

***SACCHAROMYCES CEREVISIAE*: A MODEL HOST FOR
ANTISENSE GENE CONTROL?**

A thesis presented for the
Degree of Ph.D.

By

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I hereby certify that this material, which I now submit for assessment on this programme of study leading to the award of PhD is entirely my own work, and has not been taken from the work of others, save to the extent that such work has been cited, and acknowledged within the text of my work.

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Date:

26/9/00.

***“IN TRUTH WE
KNOW NOTHING,
FOR TRUTH LIES
IN THE DEPTH”***

Democritus
(c. 460 BC – 370 BC)

DEDICATION

This thesis is dedicated to my parents, Paddy and Colette, to my sister Heidi and last but not least, to my husband Mark, for all their constant support and encouragement.

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ABSTRACT

Saccharomyces cerevisiae: A Model Host for Antisense Gene Control?

S. cerevisiae was chosen as a host for the development of a model system, which tested the effectiveness of antisense as a mechanism of controlling gene expression. The reporter gene chosen for this study is the α -amylase gene from *Bacillus licheniformis*. *S. cerevisiae* has no endogenous α -amylase activity. The sense and antisense gene copies were introduced into the yeast cell in various combinations, initially with the primary objective of obtaining an excess of antisense to sense transcripts. In several of the yeast recombinant strains used in this study the α -amylase gene (sense or antisense) was integrated into one of the yeast chromosomes. Despite achieving a copy number difference of 9:2, and a reasonably favourable mRNA ratio (4:1), together with temporal coincidence of the two transcript populations, no down-regulation of α -amylase was observed.

In these initial experiments a complete copy of the α -amylase antiscript had been used. It was considered that the lack of any observable antisense effect may have partly been due to poor interactions between the two complementary α -amylase sequences. One of the critical requirements to achieving an antisense effect is that the two complementary RNA molecules be available to base pair with one another. This requirement would not be met if the antisense RNA molecule, when synthesised, was forming stable intramolecular structures. With this in mind, several truncated forms of the gene were tested for their ability to down-regulate α -amylase expression. Northern analysis of the mRNA population from these yeast recombinant strains yielded the expected sized RNA products. These truncated anti-mRNA molecules appeared to be reasonably stable (relative to the α -amylase mRNA itself), and present in an abundance similar to that found for the complete gene antisense transcript (3-4:1). However, none of these truncated antisense molecules were found to exert an antisense effect.

In an effort to improve the spatial coincidence of the two RNA populations, the two α -amylase genes (sense and antisense), were introduced on

separate episomal plasmids. Additionally, a more favourable ratio of antisense to sense transcripts was obtained (7.5:1) by placing the antisense gene under the control of the inducible GAL1 promoter, while the sense remained under the control of the AdHI promoter. This experimental system resulted in a 12% reduction in the levels of α -amylase activity.

The final set of experiments presented involved the construction of a chimeric sense-antisense fusion (referred to as the *cis* construct), in such a way that, upon transcription, a fusion RNA product would be produced consisting of the complete α -amylase message tagged to α -amylase antisense sequences. This construct was designed to maximise the co-localisation of the two RNAs (sense and antisense) by linking their transcription and cellular distribution. Upon introduction and subsequent transcription of this gene fusion in yeast cells, a complete inhibition of α -amylase enzyme activity was achieved. Interestingly, Northern analysis revealed a shorter, but discrete transcript than that predicted. The particular location of this construct, whether episomal or chromosomal, was found not to alter the outcome.

NOMENCLATURE

Throughout this thesis this symbol (::) has been used to indicate the presence of an episomal plasmid in a particular yeast strain, for example, DBY746::pSOC-01, denotes the presence of the episomal plasmid pSOC-01 in the *S. cerevisiae* strain DBY746. *S. cerevisiae* yeast strains which have particular DNA sequences integrated into one of the chromosomes are individually named.

Abbreviations

<i>AdHI</i>	Alcohol dehydrogenase I
ARS	Autonomously Replicating Sequences
ATP	Adenine triphosphate
Bp	Base pairs
<i>CAT</i>	Chloramphenicol acetyl transferase
CEN	Centromeric
CIP	Calf Intestinal Phosphatase
CONC	Concentration
D.C.U.	Dublin City University
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DNS	Dinitrosalicylic acid
DTT	Dithithreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene Diamine Tetraacetic acid
EtBr	Ethidium bromide
<i>GAL1</i>	Galactose 1
IPTG	Isopropylthio- β -D-galactoside
Kb	Kilobase
LB	Luria Bertani
<i>Leu</i>	Leucine
<i>LEU2d</i>	Leucine deficient
LiAc	Lithium Acetate
MCS	Multiple Cloning Site
MOPS	3-[-N-Morpholino] propanesulfonic acid

OD	Optical Density
OLIGO	Oligonucleotide
ORI	Origin of Replication
PBS	Phosphate Buffered Saline
PCN	Plasmid Copy Number
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
RBS	Ribosome binding site
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	Shine-Dalgarno
SDS	Sodium Dodecyl Sulfate
SOLN	Solution
SPM	Sorbitol-Phosphate-Mercaptoethanol
TAE	Tris-Acetic acid-EDTA
TBE	Tris Borate EDTA
TE	Tris-EDTA
TK	Thymidine kinase
UAS	Upstream Activation Sequences
<i>URA</i>	Uracil
UTR	Untranslated region
X-GAL	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
YEPD	Yeast-Extract-Peptone-Dextrose
YNB	Yeast Nitrogen Base

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CHAPTER 1

Literature Review

1. LITERATURE REVIEW

1.1 INTRODUCTION

Nowadays, it is possible to isolate virtually any gene from any desired organism and to establish its DNA sequence, however there are very few techniques available that allow one to determine these genes' *in vivo* function. Antisense techniques are emerging as important tools in determining the *in vivo* function(s) of these genes, since they provide a method of preventing or reducing gene function by targeting the genetic material or its expression. These methods require only a knowledge of the DNA or RNA sequence of the target gene.

Realisation of the genetic code is a multistep process which involves the flow of information from gene to protein. This flow depends at each stage on specific base pairing interactions between complementary nucleotides which ensure the accuracy of transmission of the information. This complementary nature of the two strands of DNA in the double helix as proposed by Watson and Crick lead to the determination that both single stranded DNA and RNA can form homo and heterodimers of DNA:DNA, RNA:DNA and RNA:RNA hybrids. These complementary interactions are of basic importance to transcription, RNA processing and ultimately translation. Because of the fundamental importance of these complementary base pair interactions to the function of genes, interference with any of these interactions by using a complementary or antisense sequence offers the possibility of interfering with target gene expression in a highly selective manner. The antisense sequence may exert its' effect by preventing these complementary interactions from occurring or by occluding a process that requires a single-stranded substrate (such as the binding of ribosomes to the mRNA molecule for the initiation of translation); or by stimulating the action of nucleases specific for double-stranded regions of DNA:RNA or RNA:RNA hybrids, or finally the antisense sequence may inactivate the target directly by intercalation or cleavage. Therefore it should be obvious at this stage that antisense techniques can in principle be designed to modulate the information

flow for any gene for which sequence information is available and in any system into which the antisense sequences can be introduced.

The ultimate objective of using antisense techniques is to use a sequence complementary to the target gene, to block or to downregulate its expression, thereby creating a mutant cell line or organism in which the level of a single chosen protein or in certain cases, the replication of a virus is selectively reduced or abolished. Blocking the expression of a particular gene opens up the possibility of investigating the function of the gene in cellular or developmental processes. Antisense sequences may also be employed to reduce the expression of a gene causing an undesirable phenotype in transgenic plants or animals, or on a medical note, to interfere with oncogenic transformation or viral infection.

Despite the simplicity of the concept behind antisense RNA regulation, this technique does not always result in downregulation of gene expression and usually requires the construction of a number of different antisense genes (van der Krol et al., 1988a). In addition to the numerous successes associated with antisense RNA mediated regulation there have also been a number of failures (Salmons et al., 1986; Kerr et al., 1988; Law and Devenish, 1988; Olsson et al., 1997). Based on these observations a large research effort has been directed towards analysing the exact mechanism(s) underlying this technology with the ultimate goal of enhancing the efficiency of antisense RNA control. Now that the initial excitement and subsequent disappointment has given way to a greater pragmatism, it is clear that there is an important future for antisense approaches not only as a research tool but also in biotechnology for manipulating the characteristics of transgenic animal and plants and in pharmacology as therapeutic agents (Uhlmann and Peymann, 1990).

Within this chapter I will review natural and artificial antisense gene regulation in both prokaryotes and eukaryotes, with a particular emphasis on *Saccharomyces cerevisiae*. I will highlight the evidence for the mechanism(s) underlying antisense regulation, since one of the least satisfying aspects of antisense technology is the lack of information regarding its mode of action, which makes a rational design of antisense strategies difficult. This is partly due to the variety of different model systems (with their inherent differences) being used, but is perhaps chiefly a reflection of the large number of variables within the multistep process from gene to protein. Any one of these steps may or may

not be a target depending on mRNA structure, accessibility, transport and other factors that can differ from gene to gene, cell type to cell type and organism to organism.

1.2 NATURAL GENE REGULATION BY ANTISENSE RNA IN PROKARYOTIC SYSTEMS

1.2.1 CLASSIFICATION OF NATURALLY OCCURRING PROKARYOTIC ANTISENSE RNA SYSTEMS

Over the last decade antisense RNA has emerged as a natural component of many regulatory circuits. The targets for this newly discovered mode of regulation seems to be any process which involves single-stranded RNA, namely RNA processing, transcription and translation. In many instances this antisense RNA gene is part of a more complex regulatory system and in its natural role may not be the only or the main regulatory gene. From examples in the naturally occurring systems, it appears more likely that the antisense RNAs act as fine tuners of complex regulatory systems (Pines and Inouye, 1986). These natural prokaryotic antisense RNA systems exhibit a variety of modes of inhibiting gene expression under which they may be classified (Table 1-1). These mechanisms include interference with transcription, (transcriptional attenuation), translation (sequestering of the ribosomal binding site), indirect interference with translation by means of RNase cleavage and finally through interference with other RNA functions such as the inhibition of primer formation in plasmid replication, as in the case of ColE1.

The genomic organisation of many of the natural prokaryotic antisense genes is one in which the antisense genes and the target gene overlap such that the antisense RNA and the target mRNA are transcribed from opposite template DNA strands. These antisense RNAs can be classed as being *cis* encoded. More recently, however *trans*-encoded antisense RNAs have come to light. These RNAs are encoded by genes that are located at genetic loci other than those of the target genes. Only a few of these unlinked antisense RNA genes have been detected and they like *cis* encoded RNAs are normally small, but they exhibit only partial complementarity to their target RNAs (Delihias, 1995). Naturally

occurring antisense RNAs could be further classified as to whether they are *cis* or *trans* encoded.

Table 1-1 Classification of Naturally Occurring Antisense RNAs on the basis of Function.

Target Gene			Antisense RNA		
Name	Function	System	Name	Size (nt)	Target
Interference with transcription (transcriptional attenuation)					
<i>repC</i>	Replication	pT181	RNAI	87	LRNA*
<i>traJ</i>	Transfer	F	FinP	105	L-RNA
<i>crp</i>	cAMP receptor	<i>E.coli</i>	<i>tic</i>	92	L-RNA
<i>repR</i>	Replication	pIP501	RNAIII		L-RNA
Direct interference with translation (sequestering of ribosome binding site)					
IS10	Transposition	Tn10	RNA _{OUT}	69	RNA _{IN}
<i>gvpABC</i> operon	Formation of gas vesicles	<i>Calothrix</i> 7601 Cyanobacterium	<i>gvp</i>	420	coding region of <i>gvpA</i> , AB, ABC
<i>repB</i>	Replication	pLS1	RNAII		L-RNA
<i>repA</i>	Replication	IncB	RNAI	70	L-RNA
Q	Antitermination	λ	P _{aQ} RNA	220	L-RNA
<i>sulA</i>	Division	<i>E.coli</i>	<i>isf</i>	353	L-RNA
	Inhibition				
<i>ompF</i>	Outer membrane	<i>E.coli</i>	<i>micF</i>	174	L-RNA
Indirect interference with translation (RNase cleavage)					
cII/O inter-genic region	Replication	λ	cII/O	77	cII/O
<i>repA</i>	Replication	R1	CopA	89	CopT
Interference with other RNA functions					
RNA II	Replication	ColE1	RNAI	108	RNAII5' end
Activation of translation					
<i>hla</i>	Activation of alpha toxin	<i>S.aureus</i>	RNAIII	500	L-RNA

* L-RNA, leader RNA

Finally, prokaryotic antisense RNAs can be classified according to function for example many are involved in plasmid replication and maintenance, others regulate either bacterial or phage gene expression while a further group of these antisense RNAs are involved with transposition and conjugal DNA transfer. In the following section the antisense RNAs listed in Table 1-1 will be discussed individually, features that will be highlighted include their mechanism of action, structure and function.

1.2.2 PLASMID REPLICATION AND MAINTENANCE

Many prokaryotic antisense RNAs are involved in the regulation of plasmid copy number and different mechanisms of regulation are exhibited

between different organisms. In the ColE1 plasmid family for example, the antisense RNA, RNAI inhibits primer maturation, whereas in plasmids of the IncFII-like and IncI α /IncB families, antisense RNAs inhibit translation of a Rep (replication) protein either by inhibiting Rep protein synthesis through prevention of leader peptide translation or by the inhibition of formation of an activator RNA pseudoknot, required for Rep protein synthesis respectively. On the other hand, antisense RNAs of plasmids, like pT181 and pIP501, found in Gram-positive hosts, function by inducing transcriptional attenuation of the Rep mRNA. In all these systems antisense RNAs act as efficient inhibitors of the functions of their target RNAs. The inhibition is mainly post-transcriptional and involves antisense-target RNA base pairing (Hjalt and Wagner, 1995).

1.2.2.1 ColE1

The negative control of replication of the plasmid ColE1 (which is a 6.4 kb plasmid that encodes the colicin E1 protein as well as immunity to the lethal effect of this protein) is accomplished by an antisense RNA, RNAI which is transcribed in the opposite direction from the same DNA that encodes the primer RNA, RNAII, for DNA replication at the origin. Replication of ColE1 initiates when the primer precursor, RNAII hybridises to its DNA template and is cleaved by ribonucleaseH at the origin. Using the cleaved RNA as a primer, DNA synthesis by DNA polymerase I can ensue. The antisense RNA (RNAI) has a short sequence of 108 nucleotides, which functions by blocking processing of the primer precursor by hybridising with it, thus causing a conformational change, which then prevents formation of a suitable ribonuclease H substrate (i.e. a DNA:RNA hybrid; Green et al., 1986; Simons, 1988).

Maximal negative control of plasmid replication by RNA I is further enhanced by a plasmid encoded protein consisting of 63 amino acid residues referred to as rop (repressor of protein) or rom (RNA one modulator). This protein is capable of accelerating the association of RNA I with RNAII. The protein itself is a small rigid dimer of exact two-fold symmetry. This lead to the proposal that each subunit binds to one RNA molecule and that if two molecules have complementary loops, these are then positioned correctly for the “kissing” interaction, (Cesarini and Banner, 1985).

The actual pairing between RNA I and RNA II can be broken down to three steps. In the first step termed “kissing” RNA I and RNA II form reversible contacts between the free 5' end of RNA I and essentially the 3 equivalent loops in RNA II. Next, stable complex formation begins between the free 5' end of RNA I and its complementary in RNA II, as the kissing intermediate gives way. Mutations that delete the 5' end of RNA I prevent stable complex formation. Finally pairing is propagated from the 5' end of RNA I to the full extent of complementarity (Figure 1.1).

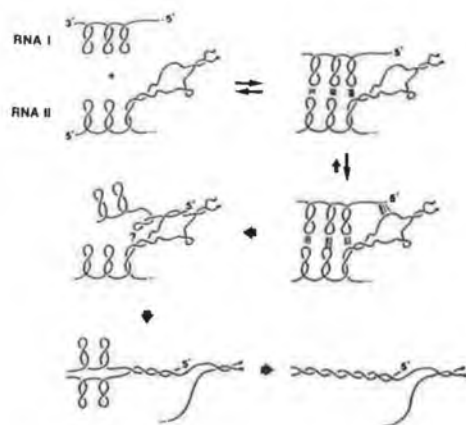


Figure 1.1 Schematic representation of the pairing reaction between RNA I and RNA II (Simons and Kleckner, 1988).

1.2.2.2 IncF

Another example of this type of organisation where the antisense and target genes overlap is involved with the control of the conjugative transmission of the IncF plasmids. Conjugative transmission in IncFI, IncFII and related plasmids involves a large number of transfer genes (*tra*) whose transcription requires the product of a single gene *traJ*. Upon transfer to a new cell, the *traJ* gene is expressed and additional rounds of conjugation occur. However, after residing in the cell for several generations *traJ* gene expression is inhibited and further transmission becomes rare (Simons, 1988; Simons and Kleckner, 1988).

The FinOP system mediates this plasmid DNA transfer between enterobacterial cells. This system consists of an antisense RNA, *finP*, and a 21.2 kDa protein FinO which together inhibit *traJ* expression. *finP* is a plasmid

specific antisense RNA, whereas FinO encodes a proteic co-repressor which is not plasmid specific but exchangeable among F-like plasmids. The *finP* gene product of plasmid R1 is encoded from the strand complementary to the *traJ* mRNA which results in a 72-base untranslated RNA transcript (*finP*) that is fully complementary to the 5' non-translated sequence, the ribosome binding site and the first two codons of the *traJ* mRNA. Hence it appears that the *finP* product is the principal player in regulatory events due to its ability to bind the 5' end of the *traJ* mRNA, including the RBS. This pairing presumably prevents the access of ribosomes and as a result inhibits *traJ* mRNA translation (Koraimann et al., 1996).

In addition *finP* exhibits a highly ordered secondary structure consisting of two stem-loop domains with a short single-stranded 5' tail. The 5' untranslated region of *traJ* mRNA adopts a conformation complementary to that of *finP*. Results presented by Koraimann et al. (1996) demonstrated that the specificity of the *finP* antisense RNA resides in loop II of the predicted *finP* RNA molecule (Figure 1.2), however further analysis of an additionally created loop I mutant showed a reduced function of *finP* which strongly suggested that the central nucleotides in both loops of *finP* play a role in the initial interaction between an antisense RNA and its target, the *traJ* mRNA. A mutant located at the periphery of loop I in *finP* did not lead to a loss of function phenotype suggesting that not all of the loop nucleotides are involved in the creation of the kissing complex. Hence, one could postulate that the primary interaction via 'kissing of the loops' is sufficient for occlusion of the *traJ* RBS and the shutting-down of *traJ* expression. *TraJ* sequence complementarity to the RBS lies partially in loop I of *finP*, a feature that is not found in other well-characterised antisense RNA systems (Koraimann et al., 1996).

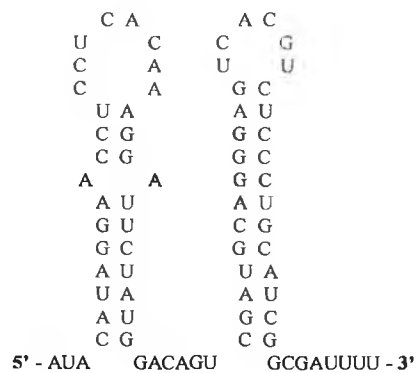


Figure 1.2 Structure of the prokaryotic antisense RNA, finP (Simons, 1988).

1.2.2.3 R1162 and pT181

Other examples of natural antisense RNAs, which are produced by the transcription of their individual target regions in the opposite orientation to the target RNA transcription, include the control of plasmid replication of the broad host range plasmid R1162 and also of the gram positive *Staphylococcus aureus* plasmid pT181. In contrast to ColE1, the antisense RNAs in these plasmids inhibit the expression of an essential plasmid encoded replication initiation protein.

In the case of pT181, two overlapping antisense RNA species are made which are complementary to the mRNAs coding for the plasmid specific replication protein, RepC. The two leftward transcripts, RNA III and RNA IV are utilised as mRNAs for RepC and contain long, untranslated 5' leaders of 190 and 260 bases respectively. The translation of the RepC mRNAs can be inhibited by two countertranscripts, RNA I (80 bases) and RNA II (150 bases) which are complementary to the 5' regions of RNA III and RNA IV. (Takayama and Inouye, 1990). The original understanding was that these species function by repressing the translation of the *rep* gene mRNA, however Highlander and Novick (1990), came to the conclusion that it appears more likely that they cause attenuation (i.e. premature termination of transcription). The attenuation is proposed to occur as a result of the antisense RNA:mRNA duplex influencing the

choice of possible secondary structures and favouring the adoption of the one that includes a transcriptional terminator, (Thomas, 1992).

The copy number of the broad host range plasmid R1162 is regulated by a 75 nucleotide antisense RNA complementary to a region of the *rep1A* mRNA, including the RBS and first two codons of the *rep1A* coding sequence. It is proposed that this antisense RNA competes with the ribosomes for binding at the ribosome binding site of the *rep1A* mRNA (Takayama and Inouye, 1990).

1.2.2.4 pIP501

Antisense RNA mediated transcriptional attenuation is also the mechanism of control for the streptococcal plasmid pIP501. This particular mechanism implies that the replication (*repR*) mRNAs must be capable of folding into two mutually exclusive structures. Formation of one of these structures is induced by the binding of the antisense RNA, RNA III to the nascent *repR* mRNA, RNA II, and this induces transcriptional termination at an inverted repeat structure located upstream of the *RepR* reading frame. As the fate of the nascent RNA transcript is dependent on the binding rate of this antisense RNA to its target, the control is kinetic. To simplify, despite the mechanism employed, all replication control circuits involve a branched pathway in relation to the fate of the target RNA; binding of the antisense RNA results in inhibition of translation, escape allows replication. As the relative rates of the competing mutually exclusive branches of the pathway determine replication frequency, it becomes clear that the control is kinetic.

The synthesis of the replication rate-limiting protein, RepR is controlled transcriptionally by CopR and at the post-transcriptional level by the antisense RNA, RNA III (Figure 1.3). The secondary structure of this RNA molecule, RNA III, possesses two minor stem-loops (I + II), a relatively unstructured middle-region and two major stem-loops towards the 3' end terminus (III + IV).

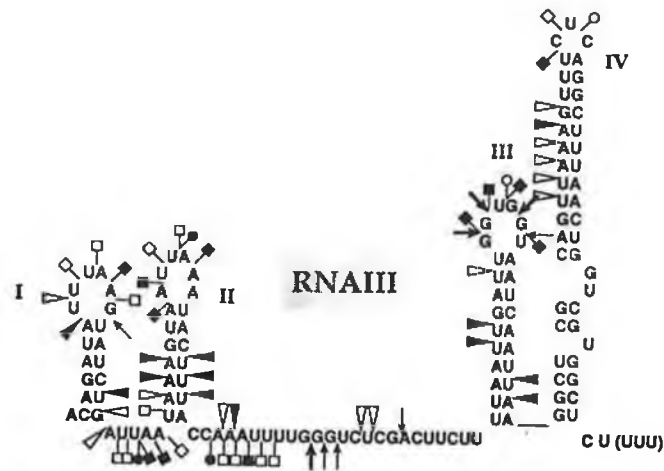


Figure 1.3 Structure of the antisense RNA, RNA III (Brantl and Wagner, 1994).

1.2.2.5 pLS1

The control of synthesis of the RepB initiator protein of the streptococcal plasmid pLS1 was proposed to be carried out by two plasmid-encoded gene products; the CopG protein and an antisense RNA, RNA II. CopG represses transcription from the single promoter for the *copG-repB* operon, whereas it was predicted that the antisense RNA would act at the translational level, perhaps by blocking the accessibility of the ribosomes to the RepB translation's initiation signals. The pLS1 region which is involved in the control of this initiator protein RepB, encompasses less than 300 base pairs and includes the genes *CopG* and *rnalI*, that read in opposite directions. The use of plasmid mutants affected in the *rnalI* gene characterised a plasmid DNA region involved in both transcription of *rnalI* and translation of RepB (del Solar et al., 1997).

1.2.2.6 IncB

The replication of IncB's mini-plasmid, pMU720, is dependent upon the expression of the *repA* gene. This gene encodes the replication initiator protein RepA. The activation of *repA* expression is negatively regulated by a small highly structured antisense RNA designated RNAI. This small antisense RNA binds to its complementary target, the stem-loop I (SLI) of the *repA* mRNA and this occludes the participation of SLI in the formation of a pseudoknot that is an enhancer of the translation of the *repA* mRNA. Therefore RNAI regulates the frequency of replication of pMU720 by controlling the efficiency of translation of the *repA* mRNA. Interestingly, despite the fact that SLI and RNAI show complete complementarity the structures of the two molecules are different (Figure 1.4). The *nal* gene resides in the leader region of *repA* and is transcribed in the direction opposite to that of SLI, so that RNAI is fully complementary to a 70 base region of SLI (Wilson et al., 1997).

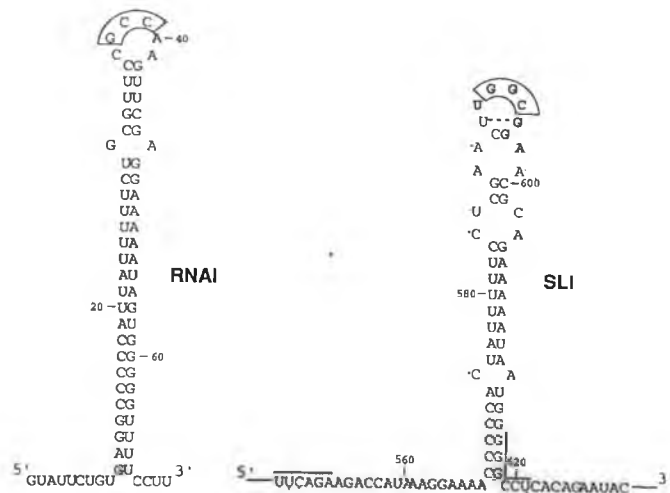


Figure 1.4 Secondary structures of RNAI and SLI (Wilson et al., 1997).

1.2.3 TRANSLATIONAL REGULATION (BACTERIAL GENE EXPRESSION)

1.2.3.1 *micF*

An example of a *trans*-encoded RNA involves control of the genes *OmpF* and *OmpC*, which encode outer membrane proteins in *E.coli*. Regulation of these proteins is due partially to the *micF* (mRNA inhibitory complementary RNA) antisense RNA encoded by the *micF* gene. The *micF* locus is transcribed divergently from the *OmpC* promoter region (47 minutes on the *E.coli* chromosome) but acts on the distant *OmpF* locus (21 minutes on the *E.coli* chromosome) showing that inhibitor-target interactions do not require the antisense RNA to be transcribed from the complementary strand of the target gene itself (Thomas, 1992).

Specifically, *micF* is involved in the post-transcriptional negative regulation of *OmpF*. Two tandem promoters of *micF* give rise to two species of RNA; an abundant 93 nucleotide 4.5S RNA and a less abundant 174 nucleotide 6S RNA. Both RNAs contain sequences complementary to the Shine-Dalgarno and translational initiation sequences of *OmpF* mRNA. These sequences inhibit *OmpF* translation and they may also be involved with destabilising the mRNA as well (Pratt et al., 1996).

1.2.3.2 *gvpABC* operon

A higher level of genomic complexity is exhibited by the naturally evolved RNA system described for the *gvpABC* operon of the cyanobacterium *Calothrix* 7601 (Csiszar et al., 1987). This operon encodes genes involved in the formation of gas vesicles, which provide buoyancy to its' cells. Four different RNA species were found to be transcribed from the *gvpABC* operon in differentiated cells. Three of the transcripts (*gvpA*, *gvpAB* and *gvpABC*) initiate from the same nucleotide, 56 bases upstream from the *gvpA* AUG start codon and extend for various distances in the 5' - 3' direction. The resulting transcripts are 326 bases (covering only the *gvpA* gene), 773 bases (covering the *gvpA* and *gvpB* genes) and 1408 bases (covering all three genes, *gvpA*, *gvpB* and *gvpC*) in length. The fourth transcript originates from the opposite strand and is a 420 base pair long antisense RNA that is complementary to a portion of each of the three mRNAs of the *gvpABC* operon. Unlike other prokaryotic naturally occurring antisense RNAs, the 420 base pair *gvp* antisense transcript is considerably larger and can hybridise along its entire length to each of the three mRNA species in the operon.

The *gvp* antisense RNA can form duplexes with the *gvpABC* RNAs which result in inhibition of translation due to the blockage of ribosome binding and/or ribosome migration during translation of the mRNAs (Csiszar et al., 1987).

1.2.4 PHAGE GENE EXPRESSION

1.2.4.1 Phage λ

In phage λ , choice between lytic and lysogenic pathways is critically dependent on the level of cII protein. This protein activates the λ_{PRE} and P_I promoters which transcribe functions required for repression and integration. cII also delays λ late gene expression, to allow sufficient time for establishment of lysogeny. This delay is thought to be mediated by an antisense RNA which reduces expression of λQ protein, the activator of late gene expression. This antisense RNA is specified by a cII-dependent promoter P_{aQ} which is located within and antisense to the Q gene. Reduction in the level of P_{aQ} RNA reduces the frequency of lysogeny.

The expression of the cII protein itself is controlled at several levels, one of these being another antisense RNA, the oop RNA. This 77 base transcript oop RNA, does not contain any open reading frames but the 3' terminal bases are complementary to the last 17 codons of cII mRNA (Takayama and Inouye, 1990), when the oop RNA is expressed from a multicopy plasmid it inhibits cII expression. Many regulatory processes are involved in the determination of the course of λ development. The cII protein plays a role in the lysis-lysogeny decision, while the antisense RNAs act in a specific way to prevent a particular target message from functioning. The inhibition of antiterminator Q expression by P_{aQ} RNA indirectly impels the phage to enter the lysogenic cycle, whereas oop RNA which targets cII mRNA functions in favour of the lytic mode.

1.2.5 TRANSPOSITION AND CONJUGAL DNA TRANSFER

1.2.5.1 Tn10

Another example of the overlapping genomic organisation of prokaryotic antisense genes is illustrated by the bacterial transposon Tn10. Insertion sequence *IS10*, the mobile genetic element of transposon Tn10, encodes a single protein, transposase, which acts at the ends of *IS10* to bring about transposition. This sequences' rate of transposition is regulated by the action of an antisense RNA (RNA-OUT) on the transposase mRNA (RNA-IN). The ends of this

composite, tetracycline-resistant transposon are inverted repeats of the insertion sequence *IS10*. *IS10*-right contains both the weak promoter P_{IN} (that directs transcription of the transposase mRNA) and the strong promoter P_{OUT} (that initiates transcription of RNA-OUT). These two promoters overlap each other by 36 base pairs with transcription from P_{IN} proceeding toward the internal region of the Tn10 transposon and transcription from P_{OUT} proceeding in the opposite direction. P_{OUT} specifies the synthesis of an antisense RNA (RNA-OUT) that is complementary to the initial 36 nucleotides of the P_{IN} directed RNA-IN including the ribosome binding site and the translation start codon for transposase (Simons et al., 1983; Simons and Kleckner, 1983).

This small, 69 nucleotide untranslated antisense RNA, transcript is composed of a simple secondary structure in which an approximately 20 base-pair stem is topped by a loosely paired loop domain. RNA-OUT is an unusually stable RNA having a half-life of 60 minutes. More than likely the metabolic stabilities of antisense RNAs evolved to suit their individual regulatory roles. For example the plasmid ColE1 antisense RNA, RNA I, which as mentioned earlier is involved in ColE1 replication and hence copy number, is unstable and expressed from a strong promoter. This set-up allows RNA I levels to rapidly respond to the relatively fast changes in ColE1 copy number. The *IS10* antisense RNA in contrast is very stable and is expressed from a modest promoter. This set-up is ideal as the concentration of RNA-OUT need respond only slowly to the very slow changes in *IS10* copy number (Pepe et al., 1994).

Deshler et al., (1995), utilised this natural prokaryotic antisense RNA, RNA-OUT by replacing its' loop domain with a hammerhead ribozyme (1.4.1.2) as a step towards developing a widely applicable system for expressing small ribozymes in a variety of cell types utilising T7 RNA polymerase. RNA-OUT was chosen because of its unusually long half-life in *E.coli* due to the stem in its secondary structure. This should endow *in vivo* stability to small RNA sequences expressed within RNA-OUTs loop domain.

traJ (section 1.2.2.2)

1.2.6 ACTIVATION OF TRANSLATION

1.2.6.1 *hla*

All discussion of naturally occurring prokaryotic antisense RNAs thus far, have shown these antisense RNAs as negative regulators of gene expression. One exception is the activation of alpha-toxin (*hla*) translation in *Staphylococcus aureus* by the *trans*-encoded antisense RNA, RNA III. According to Morfeldt et al., (1995), this is one of the first examples of an antisense RNA that stimulates translation of the target RNA.

Staphylococcus aureus, a gram-positive bacteria is a major pathogen, causing abscess formation and severe tissue damage as well as toxin related diseases such as food poisoning and toxic shock syndrome. The virulence of this bacterium has been attributed to a large number of extracellular toxins and enzymes (i.e. alpha-toxin, hemolysins, leucocidin, proteases, lipase, nuclease and staphylokinase) and to several cell-surface proteins including protein A, coagulase and fibronectin receptor. The synthesis of these virulence factors is controlled by a regulatory 0.5 kb RNA molecule RNA III, encoded by the *agr* (accessory gene regulator) locus. RNA III seems to act primarily on the initiation of transcription of virulence genes as demonstrated by transcriptional and translational fusion experiments localising the *agr* responsiveness to the promoter region of target genes.

In the report by Morfeldt et al., (1995), they have shown that in a *S. aureus* mutant lacking the regulator RNA III, the steady-state levels of alpha-toxin mRNA was reduced 5 - 10 fold, while the differential rate of alpha-toxin production was reduced 70 fold in comparison to the wild-type strain. These results indicate that RNA III activates *hla* gene expression both at the transcriptional and translational level. They suggest that translational activation is achieved by a direct interaction between RNA III and the target mRNA and transcriptionally indirectly by means of intermediary protein factors. This complex of RNA III and *hla* mRNA was found in extracts of total RNA isolated from the wild-type strain. A ribonuclease T1 digest revealed that the RBS of the *hla* transcript is blocked by intramolecular base-pairing. Hybridisation with RNA III prevents this intramolecular base-pairing and makes the *hla* mRNA accessible for the initiation of translation. There is approximately 75% complementarity

between the 5' end of RNA III and a stretch of approximately 80 base pairs within the Shine-Dalgarno region of the *hla* mRNA, which leads to the base pairing between the two molecules. As a result, in the absence of RNA III, the *hla* mRNA would be in a non-translatable form, subsequently when binding of RNA III does occur, this results in a conformational change that is amenable to translation. Thus RNA III appears to be an example of an antisense RNA that stimulates translation of the target transcript, in contrast to the known antisense RNA species which block translation by interfering with the binding of ribosomes to the target mRNA.

A question likely to crop-up in relation to RNA III is why does alpha-toxin need to be regulated both at the level of transcription and translation? A possible hypothesis may be that *hla* mRNA has a relatively long half-life of 10 - 15 minutes, therefore in order to decrease the production of alpha-toxin quickly in response to environmental signals the bacterium may need to down-regulate translation of pre-formed mRNA although transcription has been silenced. Finally, in conclusion, this antisense RNA appears to be unique in many respects; it seems to regulate both transcription and translation by independent mechanisms; it appears to be the first regulatory RNA that activates transcription and it is also the first example of an antisense RNA where interaction with the target RNA leads to stimulation of translation rather than inhibition (Morfeldt, et al., 1995).

1.2.7 STRUCTURAL FEATURES ASSOCIATED WITH NATURALLY OCCURRING PROKARYOTIC ANTISENSE RNA SYSTEMS

1.2.7.1 Stems and Loops

The majority of natural antisense RNAs analysed display a high degree of secondary structure and most of these RNAs have the potential to form one or more stem-loop structures. In many cases these structures have been clearly established (e.g. Figure 1.5). Many of the loops are the most energetically favorable size, six to eight nucleotides. Many of the stems in these structures are extremely stable, others however contain weakly paired segments usually

adjacent to their terminal loops. The antisense RNAs of ColEI plasmids also have an important 5' tail.

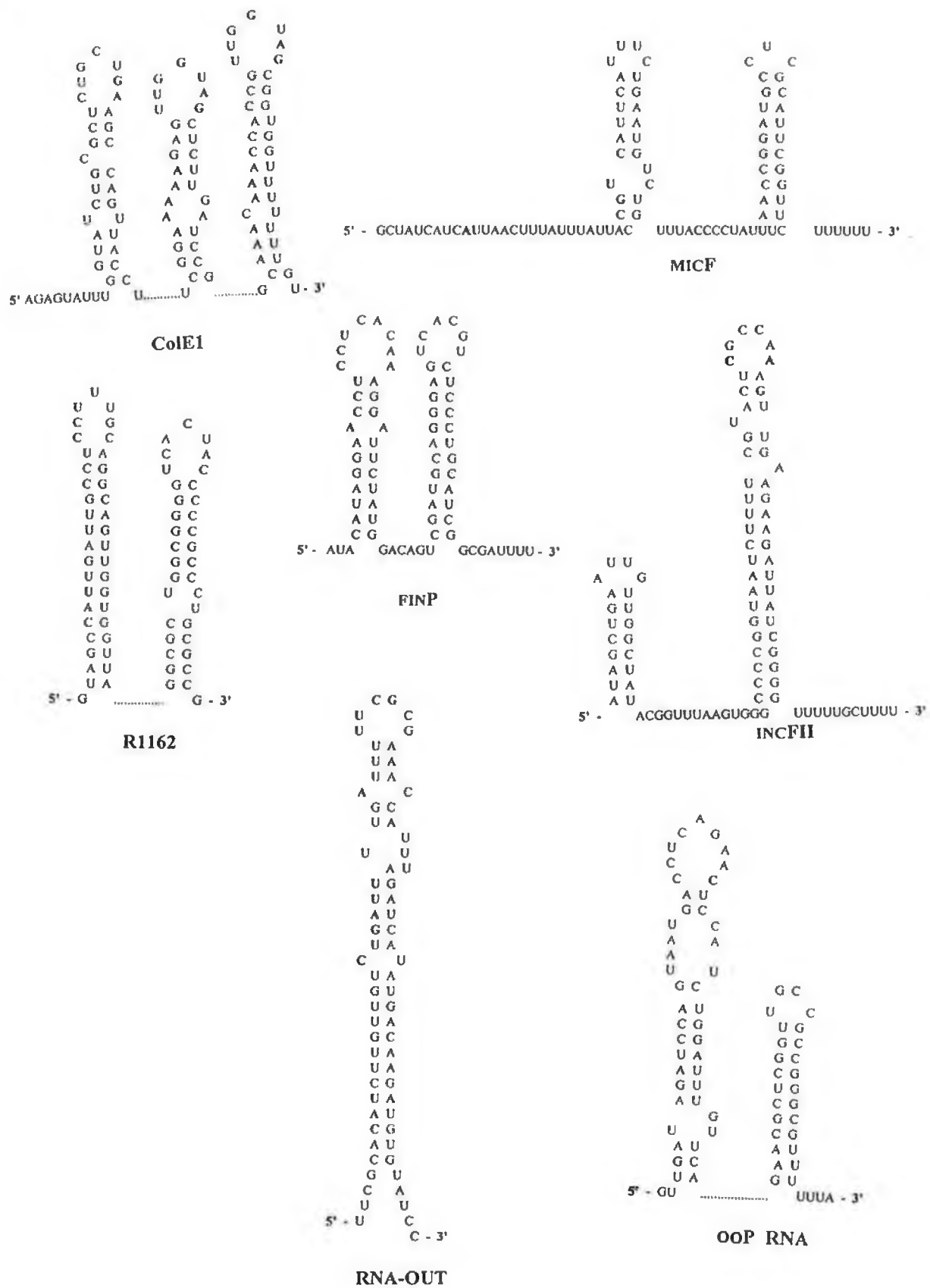


Figure 1.5 Structures of various prokaryotic antisense RNAs (Simons, 1988).

With all of these antisense reactions, critical contacts between the antisense RNA and its target occur in the loops of the antisense RNA. The loops which contain these critical sequences may represent a convenient way of ensuring that single-stranded sequences are available for pairing but are unavailable to cellular RNases. In the ColE1, Is10 and IncFII RNAs mutations in the loop often changes pairing specificity; when such a mutation is present in both the antisense and target RNAs pairing is essentially normal, but when the mutation is present in only one of the two RNAs, pairing is prevented. These effects arise in most cases from single DNA base pair changes that result in complementary changes at strategic positions in the two interacting RNA species. Furthermore the severity of these point mutations suggest that only a few Watson/Crick base pairs are involved in the initiation of pairing. Naturally occurring differences in loop sequences are thought to account for compatibility among ColE1 type plasmids and conjugational specificity among the IncF plasmids (Simons, 1988).

The stems of antisense RNAs on the other hand allow the formation of the appropriate loops and they also determine the stability/instability of the antisense molecules. Any point mutations which disrupt the pairing in the RNA-OUT stem reduced the RNA half-life *in vivo* and the appropriate second site mutations that restored the stems also restored stability. Similar mutations in the stems of the ColE1 and Is10 RNAs decrease RNA levels *in vivo*, presumably by shortening the half-life (Simons, 1988).

1.2.7.2 Bulges

Bulged out nucleotides (or internal loops within stem regions) have been found in the stem-loop structures of many naturally occurring antisense RNAs. In the paper by Hjalte and Wagner (1995) they examined the antisense/target RNA system CopA/CopT that controls the copy number of plasmid R1 to examine the possible function of these bulged out nucleotides. The CopA antisense RNA contains two regions within the major stem-loop, which contain bulged-out nucleotides. The result of their findings tentatively indicates that these bulges protect the CopA RNA from being a substrate for the double-strand specific

enzyme RNase III, as *in vitro* cleavage rates were increased when either the lower or both bulges were absent (see Figure 1.6).

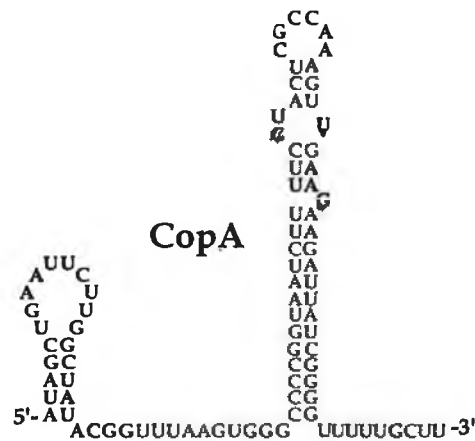


Figure 1.6 CopA RNA secondary structure (Hjalt and Wagner, 1995).

As well as playing a role in the above process, bulged-out nucleotides may also serve other functions such as protein recognition sites on RNAs. *In vitro* these bulges in CopA are required for rapid duplex formation with CopT as well as for an effective inhibition of target RNA function *in vivo* (Hjalt and Wagner, 1995).

In the majority of cases (*IS10*, *micF* and *finP*) involving prokaryotic natural antisense RNAs, the RNAs are complementary to the 5' end of their target RNAs and they usually base pair in such a way as to sequester the ribosome binding site and/or the translation initiation codon. When an antisense RNA is complementary to some other region of the target message, cleavage may play a more essential role. Indeed Krinke and Wuff (1988) have shown that inhibition of *cII* gene expression by *oop* RNA, which is complementary to the 3' portion of the *cII* coding region requires RNaseIII. Hence these antisense RNAs are also involved with destabilisation of the target message.

Despite the differences in size of the naturally occurring antisense RNAs, it is generally observed that the region of complementarity with the associated

target mRNA is equal to or shorter than the entire length of the antisense RNA. It has also been found that these naturally occurring antisense RNAs do not need to retain 100% complementarity with the target mRNA in order to exert an antisense effect (Mizuno et al., 1984). A case in point is the *micF* antisense RNA which is only 70% complementary to the 5' end of the *ompF* mRNA, in the region of the RBS, the initiation codon and the codons of the first nine amino acids of the ompF protein.

Antisense RNAs (R1162 RNA, pT181 RNA) that bind to non-coding regions of the target mRNA, such as sequences upstream from the Shine-Dalgarno sequence, it has been proposed that duplex formation in this region results in the formation of an alternative secondary structure at or near the sequence which then blocks ribosome binding (Inouye, 1988). The *tic* RNA which functions at the level of transcription is considered to bind to the extreme 5' end of the *crp* mRNA and forms an intermolecular structure that mimics the secondary structure normally recognised by RNA polymerase as a *Rho*-independent transcription termination signal. Finally, antisense RNAs also come into play at the level of events preceding DNA replication as in the case of ColE1 (Inouye, 1988).

1.3 ARTIFICIAL GENE REGULATION BY ANTISENSE RNA IN PROKARYOTIC SYSTEMS

Due to the presence of natural antisense genes in prokaryotes it was reasonable to consider that other cellular genes might be able to be specifically controlled using artificial antisense RNAs. The natural examples discussed in section 1.2.1 illustrate the many possible ways in which artificial RNAs can be designed in order to function effectively, and they provide food for thought on various parameters to be considered, before designing effective artificial antisense RNAs. Since most bacterial mRNAs are translated as soon as transcription has created a molecule long enough to be recognised by ribosomes, the degree of inhibition achieved by the antisense RNAs will depend on its rate of interaction with the target. In turn, the rate will depend on the concentration of both RNA species and their reactivity towards one another.

Another feature observed from naturally occurring antisense molecules is that they are highly structured and maximum reactivity is achieved by strict adherence to relatively rigid single-stranded loops that are involved in the initial “kissing” between antisense RNA and target. Artificial regulators should try to mimic this set-up as much as possible although this is not easy to achieve. Since most antisense RNAs are relatively unreactive, it is normally necessary for them to be made in large quantities. Furthermore, it is apparent from the many studies of these natural systems that the effectiveness of antisense RNAs is often modulated by other macromolecules in the cell, such as the Rop protein in the ColE1 system and the FinO protein involved in the conjugative transfer of plasmids. These proteins facilitate RNA-RNA interactions. Lastly the susceptibility of the double-stranded RNA molecules formed between the antisense RNA and its target to RNase activity can influence the fate of the products of the interaction and the success of the inhibition (Thomas, 1992).

1.3.1 ANTISENSE GENES

Artificial antisense RNAs can be synthesised by placing a DNA fragment of a specific target gene under the control of a promoter in reverse orientation. Several such antisense RNA genes have been constructed, and a few will be described in the following section.

1.3.1.1 Lipoprotein (*lpp*), *ompC* and *ompA* genes of *E.coli*

An artificial antisense RNA vector, pJDC402, was constructed by Coleman et al. (1984), to produce complementary RNAs against the lipoprotein (*lpp*), *ompA* and *ompC* genes of *E.coli*. This vector contains the highly expressed lipoprotein (*lpp*) promoter and the inducible lactose promoter-operator (*lac^{PO}*) in tandem and the *lpp* transcription terminator separated by a unique site into which a DNA fragment can be inserted. This arrangement ensures high-level transcription of the DNA fragment in the presence of *lac* inducers such as IPTG. The vector was designed after the natural *micF* RNA to give rise to a transcript flanked by stem-loop structures, one at the 5' end derived from the *lac^{PO}* and one at the 3' end derived from the *lpp* transcription terminator. With the anti-*lpp* gene, which includes the RBS and the start of the open reading frame there was a 16-fold reduction in lipoprotein synthesis. Interestingly, a duplication of the antisense *lpp* RNA gene resulted in a 31-fold reduction of lipoprotein synthesis upon IPTG induction, thus hinting at the presence of an antisense gene dosage effect. Northern analysis demonstrated that the *lpp* mRNA was significantly less stable when the anti-*lpp* was induced. This suggests that the effect on *lpp* was not simply due to inhibition of translation, but may also be the result of accelerated mRNA degradation due to cleavage by ribonucleases such as RNaseIII (Coleman et al., 1984).

The pJDC402 system was also used to inhibit the expression of the outer membrane protein OmpC of *E.coli*. The antisense *ompC* RNA was complementary to the first 20 bases of the leader sequence including the RBS and 100 bases of the coding sequence, induction of this antisense RNA caused an approximately fivefold reduction in OmpC synthesis. Another anti-*ompC* RNA with longer complementarity to the leader region (72 bases) was four times more effective in inhibiting the production of OmpC than the shorter anti-*ompC* RNA, thus implying that 5' leader region complementarity may be an influential factor in antisense RNA-mediated inhibition (Coleman et al, 1984).

Using the pJDC402 vector once again, several antisense genes were constructed from the *ompA* gene in order to ascertain which components contribute to the effectiveness of an antisense RNA. This gene was chosen for this study, because the leader and the coding regions of the *ompA* mRNA have

been extensively characterised. A total of five DNA fragments were individually cloned into pJDC402 in antisense orientation. The resulting antisense *ompA* fragments are shown in Figure 1.7. Each of the five constructs were capable of inhibiting *ompA* synthesis by 45%, 51%, 54%, 18% and 45% for the constructions using fragments I through V respectively. The antisense *ompA* RNA targeting fragment IV in figure 1.7 is clearly much less effective than the other four antisense *ompA* constructs. Unlike, fragments I and V, which encompass the *ompA* translation initiation site, fragment I which includes the Shine-Dalgarno sequence or fragment III which encompasses only structural gene sequences, fragment IV does not include any sequences likely to come in contact with ribosomes. This may possibly be the reason why this antisense *ompA* construct is a weaker down-regulator of *ompA* synthesis than the other four constructs. Finally, the antisense *ompA* gene spanning fragment III is complementary only to the the portion of *ompA* which spans the coding regions for amino acid residues 4 - 45 of pro-*OmpA*. This indicates that the antisense RNA does not necessarily need to hybridise to the translation initiation site and/or the 5' leader region sequence in order to exert an effect (Coleman et al., 1984).

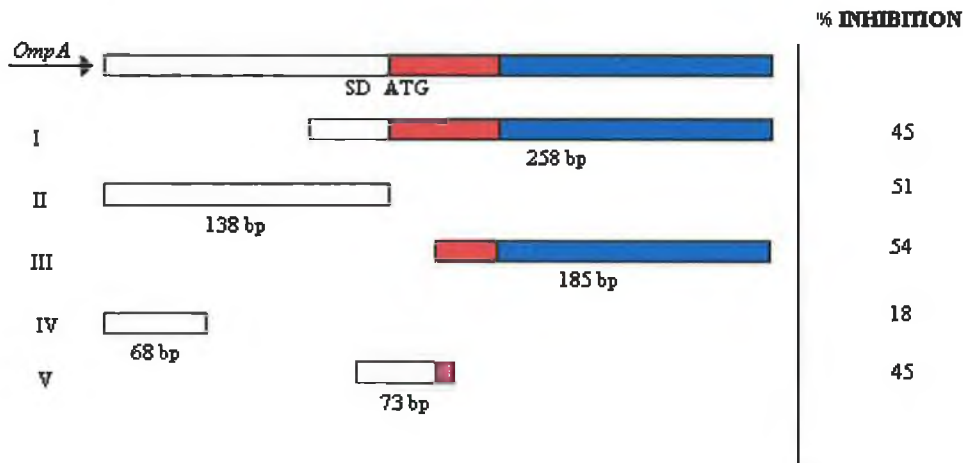


Figure 1.7 Fragments used to construct antisense *ompA* genes.

The upper line illustrates the structure of the *E. coli ompA* gene. The arrow represents the promoter and the open box represents the region encoding the 5' leader region of the *ompA* mRNA. The shaded box and the slashed box represent the regions of the *ompA* gene encoding the signal sequence and the mature OmpA protein respectively. Restriction fragments I - V were

inserted into the pJDC402 vector in the orientation opposite to that shown here (Coleman et al., 1984).

1.3.1.2 *lacZ* gene

Pestka et al., (1984), cloned an 831 base pair sequence coding for the NH₂ terminus of β -galactosidase in reverse orientation under the control of the phage λ P_L promoter. The gene coding for a temperature sensitive repression of P_L, cITs, was carried on a compatible plasmid. Thus at 30°C, no anti-mRNA would be made, but at 42°C anti-mRNA would be synthesised extensively. Under conditions in which no anti-*lacZ* mRNA is made, β -galactosidase, lactose permease and transacetylase were induced by IPTG, however when anti-*lacZ* mRNA was produced, the syntheses of all three enzymes produced from the same polycistronic mRNA were inhibited by 97%, 80% and 55% respectively.

Daugherty et al., (1989), performed many detailed experiments which examined the effects of various anti-*lacZ* RNAs of different lengths and representing the different parts of the *lacZ* coding region. Once again all the antisense genes were placed under the control of the phage λ P_L promoter. These plasmids generate anti-*lacZ* mRNA which either possessed or did not possess a synthetic ribosome binding site (RBS) adjacent to the λ P_L promoter and/or the *lacZ* RBS in reverse orientation. *LacZ* DNA from the 5' and/or 3' regions were used in these constructs. Maximal inhibition of β -galactosidase synthesis occurred when a functional RBS was present near the 5' end of the anti-mRNA, in conjunction with the anti-mRNA being complementary to the 5' region of the mRNA corresponding to the *lacZ* RBS and/or the 5' coding sequence. Furthermore when the molar ratio of anti-*lacZ* mRNA:normal *lacZ* was greater than 100:1 maximal inhibition of β -galactosidase activity was achieved. According to other reports in eukaryotic cells (Izant and Weintraub, 1984; Melton, 1985) the ratio sufficient to produce significant inhibition can vary from 1:1 to 600:1 (Daugherty et al., 1989).

1.3.1.3 Streptavidin gene

An exciting application of artificial prokaryotic antisense control is involved with the containment of microorganisms, by dual control of streptavidin

expression by an antisense RNA and the T7 transcription system, (Szafranski et al., 1997). The authors in this paper describe a potentially universal lethal system based on the derepression of the tightly regulated streptavidin gene from the actinobacterium, *Streptomyces avidinii*. The toxicity of streptavidin results from its high binding affinity for the essential prosthetic group, D-biotin. Cell death occurs as a result of the depletion of free biotin and by the direct inhibition of biotin dependent carboxylases, decarboxylases and transcarboxylases.

The system was tested on the Gram-negative soil-rod *Pseudomonas putida*, a useful microbe for the bioremediation of areas polluted with aromatic hydrocarbon-based organic solvents and petroleum. The system was coupled to regulatory elements from the *P.putida* TOL catabolic plasmid to condition survival of the bacteria only in the presence of an aromatic carboxylic acid that they can degrade. There is a problem however with cell suicide systems involving incomplete repression (leakiness) of regulatory promoters. The authors sought to overcome this problem by the combination of the bacteriophage T7 RNA polymerase-lysozyme system with the inducible synthesis of antisense RNA and the *E.coli* LacI repressor, to ensure very tight control of the derepression of cell death.

To achieve control of the induction of a lethal phenotype, a heterologous RNA polymerase and its inhibitors were generated by coupling expression of the streptavidin gene to the bacteriophage T7 transcription system (see Figure 1.8). The streptavidin gene was transcribed in this case from the T7 gene 10 promoter ($\phi 10$) by T7 RNA polymerase. The T7 gene 1, encoding the RNA polymerase was fused to the trp-lac (tac) promoter of *E.coli*, and this was negatively regulated by the LacI repressor. The leakiness of the P_{tac} promoter was compensated by an inhibitor of T7 polymerase, T7 lysozyme. The function of lysozyme is that it binds to T7 RNA polymerase and blocks its transcription activity. The *lacI* gene was transcribed from the P_m promoter, which was induced by XylS protein aromatic carboxylic acid complexes. Streptavidin should be synthesised upon inactivation of LacI with IPTG or by interception of LacI synthesis in response to depletion of a benzoic acid effector of the XylS protein. As mentioned previously, the problem with cell suicide systems is leakiness of the regulatory promoters, so in this system an additional level of regulation of the

streptavidin gene expression was achieved by placing the $\phi::stv$ fusion immediately downstream of the *lacI* gene but in the opposite orientation. This it was hoped would further decrease uninduced expression of the streptavidin gene by generating an antisense RNA complementary to the streptavidin transcript.

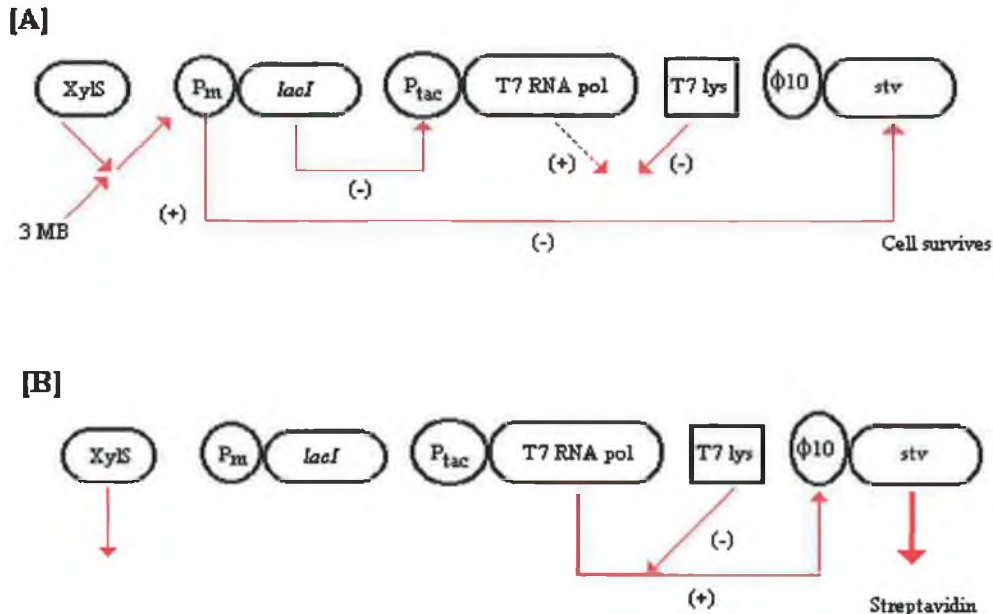


Figure 1.8 Schematic representation of the tightly regulated containment system to control survival of bacteria by availability of 3MB or other hydrocarbon effectors of the XylS protein. (A) Survival. (B) Induction of a lethal phenotype (Szafranski et al., 1997).

The rate of inactivation which is a basic determinant of the efficiency of suicide designs, in this streptavidin based system was 10^{-7} - 10^{-8} per cell per generation, this value is far lower than those reported for constructs based on single toxic functions (10^{-2} - 10^{-6}). This result reflects the very low basal level of streptavidin in cells. Although the coupled T7 transcription/LacI- O_{lac} system is less leaky than the lac system alone an additional explanation of the better protection of cells against uninduced expression of the streptavidin gene is synthesis of the antisense RNA originating from the P_m promoter, (Szafranski et al., 1997).

1.3.1.4 *galK* gene of *E.coli* and the *N* gene of coliphage lambda

On the down side of things not all attempts to use artificial antisense RNA in prokaryotic systems have been successful. Hasan et al., (1988), studied the effect of artificial antisense RNA on the expression of the *E.coli galK* and λ *N* genes. These antisense RNA target genes were either present in the *E.coli* chromosome as single copies or were cloned on plasmid vectors. The entire *galK* or *N* gene or only their N-proximal regions were cloned in antisense orientation downstream from the strong P_L , P_R or *lacZp* promoters on multicopy vectors. The authors performed many experiments with the *galK* and *N* sense and antisense genes, in both *cis* and *trans* configurations, but they did not find any significant downregulation of these genes, even though there was a 50-fold excess of antisense RNA.

1.3.2 PEPTIDE NUCLEIC ACIDS

Peptide nucleic acids (PNAs) are another means by which gene expression can be downregulated by antisense mechanisms. A PNA is a DNA mimic with attractive properties for developing improved gene-targeted antisense agents. A PNA is a molecule of synthetic origin that binds to single-stranded nucleic acids. Instead of possessing a repeating sugar-phosphate backbone a PNA has a repeating polyamide structure to which the purine and pyrimidine bases are attached in a fashion that confers the ability to bind RNA by Watson-Crick hybridisation, (Figure 1.9). The unnatural chemical structure of PNA provides stability in cells because neither peptidases nor nucleases will cleave it (Ecker and Freier, 1998).

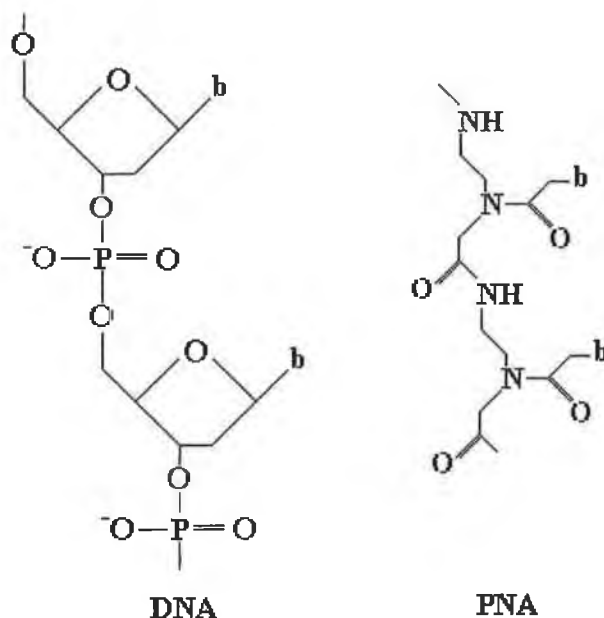


Figure 1.9 Chemical structure of a PNA and DNA oligomer (Good and Nielsen, 1998).

To study the antisense potential of PNAs in a prokaryotic system Good and Nielsen (1998) designed PNAs to target the start codon regions of the β -galactosidase and β -lactamase genes in *E.coli*. Dose-dependent and specific gene inhibition was observed *in vitro* using low nanomolar PNA concentrations and *in vivo* using low micromolar concentrations. Specificity of inhibition was demonstrated by the fact that the potency of the anti- β -lactamase PNAs was abolished by a six base substitution but was re-established using a PNA with complementary base changes. Antisense inhibition of the β -lactamase gene was sufficient to sensitise resistant cells to ampicillin. The results demonstrate gene and sequence specific antisense inhibition in *E.coli* and open up the possibilities for antisense bacterial drugs (Good and Nielsen, 1998).

Recently, it has been demonstrated that certain peptide-PNA conjugates are taken up very efficiently by at least some eukaryotic cells and that antisense downregulation of target genes in nerve cells in culture is attainable using such PNA conjugates. Perhaps, even more exciting is that antisense compatible effects have been reported using PNAs injected into the brain of rats (Nielsen, 1999).

1.4 ANTISENSE GENE REGULATION IN EUKARYOTIC SYSTEMS

1.4.1 EVIDENCE FOR NATURAL ANTISENSE GENES

An abundance of antisense transcripts have also been found to occur naturally in eukaryotes but in contrast to prokaryotes where the mechanism of action is well understood, there are relatively few examples of gene regulation mediated by antisense RNA in eukaryotic systems. Although it is possible that many of these abundant RNA species may be involved in antisense RNA regulation, their regulatory role(s), if any, remain yet to be defined. These antisense transcripts derive either from transcription of both strands of a DNA fragment (overlapping transcripts) or from transcription of complementary DNA strands located at different genomic positions (Rogers, 1988). Overlapping transcripts have been identified for many eukaryotic genes including the human *ERCC-1* encoded DNA excision repair gene (van Duin et al., 1989); the barley α -amylase gene (Rogers, 1988); mouse polyoma virus (Kumar and Carmichael, 1997) and in yeast numerous overlapping transcripts been identified including overlap within the *RHO1* and *MRP2* genes (Peterson and Myers, 1993); *SPO12* and *SPO16* genes (Malavasic and Elder, 1990) and the *PET122* locus (Ohmen et al., 1990; Marathe and McEwen, 1999) to name but a few. Once again in very few instances have actual functions been assigned to these overlaps.

1.4.1.1 snoRNAs

Further evidence for the existence of naturally occurring antisense RNAs has been the discovery of small nucleolar RNAs (snoRNAs) within vertebrates, *Drosophila* and yeasts. These snoRNAs contain long stretches of sequence complementarity to conserved sequences in mature ribosomal RNA and Bachellerie et al. (1995) propose that these antisense snoRNAs may be involved with pre-rRNA folding, base modification and ribosomal ribonucleoprotein assembly. Evidence to suggest that these snoRNAs are involved in ribosome production comes firstly from their cellular location which is nucleolar, secondly nearly all snoRNAs are associated with the nucleolar protein fibrillarin which itself is required for normal processing and methylation of rRNA and finally

some of the snoRNAs are encoded within the introns of genes coding for proteins involved in ribosome synthesis.

The complementarities of snoRNAs to rRNAs map to both single and double-stranded regions. The thermodynamic stability predicted for each snoRNA-rRNA interaction in double-stranded regions largely exceeds that of the intramolecular stem in the mature rRNA. This indicates that the snoRNAs could compete favourably in intermolecular versus intramolecular rRNA-pairing reactions. Additionally the presence of methylated nucleotides in antisense rRNA elements raise the question that possibly snoRNAs may play a role in post-transcriptional modification. Although the function behind methylation of rRNA is mainly unknown some types of methylation (particularly base methylations as opposed to ribose methylations) may affect conformation (Bachellerie et al., 1995).

1.4.1.2 Ribozymes

Another type of naturally occurring antisense molecule is a catalytically active RNA species called a ribozyme. These molecules are responsible for the cleavage and ligation of certain specific phosphodiester bonds within RNA molecules. In 1981 Cech discovered ribozyme activity in *Tetrahymena thermophila*, following this ribozyme activity has been detected in many other organisms including *E.coli*, *B.subtilis*, *Salmonella typhimurium*, plants and other eukaryotes which suggests that ribozymes occur universally (Uhlmann and Peyman, 1990). The attractive aspect of these molecules in contrast to “conventional” antisense RNAs is that they are not required in stoichiometric amounts, as a ribozyme possesses catalytic activity whereby it can cleave the target mRNA resulting in its inactivation without affecting the antisense “ribozyme” molecule which is then free to dissociate and attack other substrate molecules. The conventional antisense RNAs, in contrast must remain hybridised to block the mRNA function or to target the sense:antisense hybrid for degradation, in which case the antisense molecule is destroyed along with its substrate. Therefore the potential attraction of catalytic RNA systems for gene regulation or antiviral strategies is that a lower concentration of antisense molecules may be necessary for effective regulation.

Linked to the catalytic centre of a ribozyme molecule are RNA sequences which are complementary to, and thus serve to target the ribozyme to a unique RNA sequence (Rossi and Sarver, 1990; Figure 1.10). The cleavage reactions have a requirement for divalent metal ions and a neutral or higher pH, that results in the production of an RNA molecule with termini possessing 5' hydroxyl and 2',3' cyclic phosphate groups. The cleavage reactions presumably result from RNA conformation bringing reactive groups into close proximity. The sites of cleavage are specific and associated with domains of conserved sequence and secondary structure (Haseloff and Gerlach, 1988).

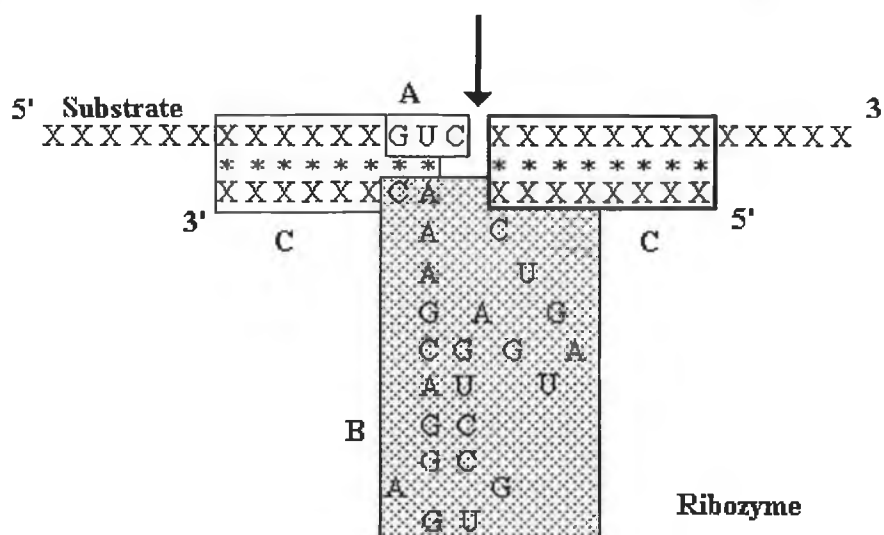


Figure 1.10 Schematic representation of a ribozyme.

Three structural domains are boxed. [A] Contains conserved sequences in the RNA substrate, immediately adjacent to the site of cleavage. [B] Comprises the highly conserved sequences maintained in the ribozyme, and the regions [C] consist of flanking helices, with base pairing between the substrate and ribozyme RNAs (Haseloff and Gerlach, 1988).

Different types of ribozymes have been reported; *Tetrahymena* Group 1, hammerhead and hairpin ribozymes. These ribozymes are classified according to their biological origin and catalytic motif (the 3D structure of the catalytic section of the molecule). The 3D structure of ribozymes is very important to their function, disruption of the catalytic motif can easily render a ribozyme inactive (Putnam, 1996).

Although ribozymes are naturally occurring they can be adapted to cleave other substrates (such as targeted mRNAs). Work has been carried out on exploiting the use of engineered *Tetrahymena* derived ribozymes after it had been shown that the reaction can also proceed when the target and catalytic sequences are present on two different RNA molecules. The minimal recognition site requirement for the wild-type ribozyme has been shown to be CUCU. However various research groups have shown that by altering the guide sequence and conditions almost any substrate can be recognised (Murray and Crockett, 1992).

1.4.1.3 Overlapping transcription units within the mouse genome

One of the first reports documenting overlapping transcription in eukaryotes was presented by Williams and Fried (1986). They identified a region in the mouse genome containing two adjacent convergent transcription units which overlap by 133 base pairs at their 3'-untranslated ends. This finding implies a complex sequence organisation in this region as the poly(A) addition signal for one transcript is contained in the exon sequence of the other and vice versa. Furthermore, for these vertebrate genes transcription does not terminate at the poly(A) addition site but continues for some distance downstream. This implies that the sequences which may be involved in transcriptional termination or in the processing of one transcript are also probably encoded in the sequence giving rise to the transcript from the opposite locus and vice versa. Therefore it may be significant that the overlap is in the 3' untranslated region of both mRNAs.

Although no function has been assigned to this overlap, the authors postulate that these overlapping transcripts may display some novel mechanism of control of mammalian gene expression. These being, if the overlapping transcripts were expressed at the same time, it might be expected that the RNA polymerase duplexes travelling in opposite directions along a DNA duplex might encounter a considerable degree of steric hindrance, especially as transcription probably continues past the poly(A) addition site. Also, although the abundance of the mRNAs is fairly low for each of the two species of mRNA, the local concentration of the two opposite strand transcripts in the vicinity of the 3' ends of the transcription units might favour RNA duplex formation with resultant

inhibition of RNA processing and/or transport from the nucleus. Perhaps either the two strands are only rarely transcribed and their mRNAs very stable or there may be some spatial and/or temporal control of expression such that their transcription occurs at different times in the cell cycle or from different alleles (Williams and Fried, 1986).

1.4.1.4 α -Amylase gene of barley aleurone cells

As a model system for the investigation of developmental and hormonal controls of plant gene expression barley aleurone cells have been used. These cells possess a tough outer layer of tissue that surrounds the starchy endosperm of the grain. During germination the embryo secretes a hormone gibberellic acid (GA_3) which in turn directs the aleurone layer to produce and secrete hydrolytic enzymes which digest the storage contents of the endosperm for use by the seedling. The most abundant hydrolases are the α -amylases and mechanisms controlling their expression have been studied extensively. Consequently this has led to the discovery of a naturally occurring antisense α -amylase RNA. This antisense α -amylase RNA may prevent unwanted expression of the α -amylase genes A and B (Rogers, 1988).

RNA complementary to the α -amylase mRNAs of either isozyme were found to be present in developing endosperm tissue and in mature aleurone tissue, but not in shoot or root tissue. The abundance of this antisense RNA was greatest (relative to the abundance of α -amylase mRNA) in mature aleurone tissue following treatment with abscisic acid (ABA). ABA produces an effect which antagonises GA_3 -mediated changes in α -amylase expression, essentially ABA prevents the GA_3 -induced increase in α -amylase mRNA abundance. It was found that the antisense RNA was not perfectly complementary to any of the 3' α -amylase genes known to be expressed, this leads to the conclusion that the antisense RNA must be derived from another uncharacterised amylase-like sequence. Northern blots of total RNA isolated from different barley tissues using single-stranded probes to detect antisense RNAs and under conditions of moderate stringency resulted in three distinct hybridising species of 1.6, 1.4 and 1.0 kb isolated from mature aleurone RNAs. It was postulated that these antisense RNAs could function to alter the stability of the α -amylase mRNA

precursors and/or to interfere with their translation even at steady-state levels that are substantially lower (relative to the mRNA target) than those required in the previously described artificial systems (Rogers, 1988).

1.4.1.5 Mouse polyoma virus

Kumar and Carmichael (1997) used the mouse polyoma virus as a model system to examine naturally occurring mammalian antisense-induced regulation of transcript levels. This is a small virus, whose double-stranded, circular, polyoma genome is divided into early and late transcription units that are expressed from opposite strands of the viral genome. The life cycle of this virus is divided into two phases; the early phase which occurs immediately after infection and before DNA replication and the late phase which begins after the onset of DNA replication. During the early phase of productive infection, early strand transcripts accumulate preferentially over late-strand transcripts, late strand transcripts are also synthesised during this period, but are degraded rapidly in the nucleus. Before DNA replication occurs, the ratio of late-strand to early-strand RNAs is less than 1:10. Subsequently, after the onset of DNA replication during the late phase of infection the late strand transcripts become more plentiful than the early strand transcripts. During this period transcription termination becomes inefficient and this allows RNA polymerase II to encircle the genome many times. The resulting multigenomic transcripts possess sequences which are complementary to the early-strand transcripts and thus have the potential to act as natural antisense regulators within the nucleus. Furthermore the region of the late strands that can form antisense hybrids with early-strand RNAs are found within intron sequences that do not appear in the late strand messages.

In their paper Kumar and Carmichael (1997) have examined the fate of these hypothetical sense-antisense hybrids in the nucleus, by asking whether the reduced levels of early-strand RNAs at late times of infection are as a result of antisense-induced RNA degradation or are early-strand transcripts as a result of base modifications merely non-complementary to the probes used in RNase protection assays? This last possibility was considered because mammalian nuclei contain an enzymatic activity capable of modifying double-stranded RNA (dsRNA) molecules. The enzyme double stranded RNA specific adenosine

deaminase (dsRAD; 1.5.4.2) was originally discovered in the nucleus of *Xenopus laevis*. Its' activity is mainly confined to the nucleus and it functions by catalysing the conversion of adenosines to inosines within dsRNA by means of hydrolytic deamination. The RNA produced contains I-U base pairs and this may lead to partial unwinding of the RNA duplex.

Using RT-PCR to specifically amplify early-strand transcripts, the products were subsequently analysed to determine whether or not they were subject to RNA editing by dsRAD. The RT-PCR amplified fragments were shown to be resistant to a particular restriction enzyme in about 50% of the early-strand RNAs, thus indicating modification. Sequence analysis of these resistant fragments showed that 40-60% of the adenosines within the early-strand RNAs had been changed to guanosines. A-G changes in the cDNAs are suggestive of A-I changes within the dsRNAs, this result is consistent with modifications induced by dsRAD. The authors therefore considered it most probable that this enzyme was responsible for the antisense-induced base modifications in the polyoma system. As the modifications caused by dsRAD can be detected only in dsRNA it was postulated that the sense-antisense transcripts almost certainly do form duplexes within the nucleus. Results using probes that specifically detect modified RNAs indicate that extensive antisense-induced base modification results in nuclear accumulation of the altered transcripts. More than likely the modified messages are trapped in the nucleus due to their base compositions, altered secondary structures or possibly because partial duplex structures resulting from the incomplete dissociation of the two RNA strands may form. On the other hand, these modified messages may be transported to the cytoplasm, where they are rapidly degraded. Either way these messages appear to be inert for gene expression (Kumar and Carmichael, 1997).

1.4.2 EVIDENCE FOR NATURAL ANTISENSE GENES IN *SACCHAROMYCES CEREVISIAE*

As with most other eukaryotic cells, several examples of complementary transcription units have been identified within the genomic DNA of yeast. These naturally occurring antisense RNAs of yeast can be categorised into examples where the two RNAs can be either in *cis* or in *trans*, with transcripts derived

from DNA templates that overlap or are distantly positioned respectively. The *cis* acting RNAs can be further subdivided as being overlapped at the 3' or 5' termini, which is the case with convergent (section 1.4.2.1 to 1.4.2.2) and divergent (section 1.4.2.3 to 1.4.2.5) genes respectively, or it could be where one transcript is fully complementary (section 1.4.2.6) when it is encoded within the complementary strand of a longer transcription template. Most of the examples described thus far for *Saccharomyces cerevisiae* have been the *cis* acting type, (Atkins et al., 1994).

1.4.2.1 *RAD10*

The homologous *Saccharomyces cerevisiae RAD10* gene and the human *ERCC-1* DNA excision repair gene, contain overlapping antisense transcription units in their 3' regions (van Duin et al., 1989). Transcription of the *RAD10* gene results in the production of three transcripts of 1.0, 1.5 and 1.8 kilobases that vary in sequence at their 3' end and an antisense transcript of 1.9 kilobases. The antisense RNA designated *ASR10* (antisense *RAD10*) overlaps with all the mRNAs with up to 600 bases of complementarity to the 1.8 kilobase transcript (Figure 1.11). Similarly, the human *ERCC1* DNA gene has a 2.6 kilobase antisense RNA that has an overlap of 170 bases with the *ERCC1* transcription unit, yielding the major 1.1 kilobase mRNA, whereas it is completely complementary to the 3.4 kilobase *ERCC1* transcript for at least 1 kilobase. The antisense function of these overlapping transcripts is still only speculation, however the fact that the transcription pattern between humans and yeast is conserved may indicate a functional relationship rather than a coincidental gene arrangement (Atkins et al., 1994).

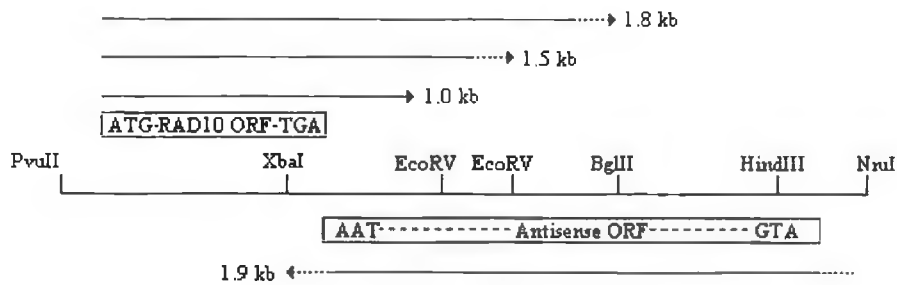


Figure 1.11 Transcript map of the *RAD10* gene and antisense gene region.

The positions of the *RAD10* and *ASR10* ORFs are indicated by the open boxes. The arrows above the *RAD10* ORF and below the antisense ORF represent the directions and approximate sizes of *RAD10* transcripts and the antisense transcript respectively (van Duin et al., 1989).

1.4.2.2 *RHO1* and *MRP2*

A study by Peterson and Myers (1993), characterised transcription of a 281 base pair region located between the convergently transcribed yeast genes, *RHO1* and *MRP2*. Their results showed that this intercoding region contains two adjacent, functionally independent 3' end formation signals, each of which contains a sequence motif implicated as a yeast polyadenylation signal. On occasion when transcription of these genes has continued through their respective termination signal the two transcripts overlap at their 3' ends by up to 111 base pairs.

The 3' ends of the *RHO1* and *MRP2* mRNAs are natural antisense RNAs complementary to each other. In order to ascertain the significance if any of this overlap, a third 3' termination signal was inserted within the intercoding region in both orientations. This extra termination signal resulted in no overlap between the two sequences and this led to no detectable effect on the expression of either gene suggesting that the complementary RNA domain was apparently dispensable. Insertion of a strong promoter into the intercoding sequence of the *RHO1* and *MRP2* genes resulted in transcription beyond the normal polyadenylation sites and this inactivated the gene located downstream. This may

suggest that the intercoding region functions not only to specify polyadenylation sites, but also to terminate transcription (Peterson and Myers, 1993).

Gene inactivation by convergent transcription could be either a *cis* or *trans* acting effect. To investigate this heterozygote diploid yeast cells were prepared in which one allele of the gene was wild type and the other possessed the artificial antisense gene. The apparent lack of an effect on the wild-type gene suggested that the antisense transcription unit was recessive and *cis*-acting. Hence the gene suppression seen in this system was due to promoter interference, rather than true antisense RNA function (Peterson and Myers, 1993).

1.4.2.3 *SPO12* and *SPO16*

Similarly, Malavasic and Elder, (1990), found that preventing overlapping transcription between the *SPO12* and *SPO16* genes did not block expression of either gene. The *SPO12* protein has an open reading frame of 173 codons and is required for meiosis I chromosome division during sporulation of the yeast *Saccharomyces cerevisiae*. *SPO16*, a second sporulation-specific gene was found adjacent to *SPO12*. These two genes are encoded on opposite DNA strands and there are only 103 base pairs between their termination codons. The *SPO12* and *SPO16* transcripts overlap by 700 bases and the 3' untranslated region of the longest *SPO16* transcript is complementary to nearly all of the *SPO12* mRNA. Removal of the regions of complementarity does not affect the efficiency of sporulation or spore viability. The complementarity therefore has either no function or only a subtle function in meiosis and sporulation (Malavasic and Elder, 1990).

1.4.2.4 *ACT1* and *ASR1*

The *Saccharomyces cerevisiae* *ACT1* intron contains the promoter and transcription start sites for an *ACT1* antisense RNA, designated *ASR1* (antisense RNA 1 in *Saccharomyces cerevisiae*). Preliminary co-culture results with an *ACT1* intron containing and an *ACT1* intronless strain where the growth rates of the two yeast strains were compared, ruled out any drastic effects of the *ASR1* transcript on the expression of *ACT1* in the vegetative stage. As *ACT1* is essential for growth a strong effect on its expression should be reflected in terms of growth rate. However, subtle effects not evident in the growth rate phenotype

cannot be ruled out. The *ASR1* transcript was found to be at very low concentration in the cell, from Northern blot analysis, whereas the *ACT1* transcripts were present in high abundance, (Thompson-Jager and Domdey, 1990).

Theories suggested for the function of this antisense transcript include RNA-RNA double strand formation between *ACT1* and *ARS1* may hinder splicing or translation of *ACT1* mRNA, or indeed it may effect transport of the mature *ACT1* mRNA to the cytoplasm. If such regulation does exist to help regulate the amount of this important protein, it must operate within a small range, because *S.cerevisiae* does not have a mechanism which allows it to survive the presence of an additional *ACT1* gene on a high copy vector (Thompson-Jager and Domdey, 1990).

1.4.2.5 *PET122*, *ORF2* and *ORF3*

Divergent overlapping transcripts have been found at the *PET122* locus in *S.cerevisiae*. *PET122* is one of three nuclear genes required for translation of the mitochondrial mRNA for cytochrome c oxidase subunit III. The nucleotide sequence of 2,862 bases encoding the *PET122* locus demonstrates very close spacing between the *PET122* gene (254 codons) and two unidentified open reading frames, designated *ORF2* and *ORF3*. *ORF2* is encoded by the same strand of DNA as *PET122* and is located 53 bases downstream of *PET122*, while *ORF3* is encoded on the opposite strand and is located 215 bases upstream of *PET122*.

From this locus five different transcripts are produced, with sizes of 2.9, 2.3, 2.1, 1.5 and 1.4 kilobases (Figure 1.12). The 2.1 and 1.4 kilobase transcripts encode *ORF3*, the 1.5 kilobase transcript encodes *ORF2* and the 2.9 and 2.3 kilobase transcripts encode *PET122*. Interestingly, the *ORF3-PET122-ORF2* transcription unit shows a 535 base overlap between the 2.3 kilobase *PET122* transcript produced from one strand and the 2.1 kilobase *ORF3* transcript produced from the opposite strand. Also the 2.9 kilobase *PET122* transcript overlaps the 2.1 kilobase transcript *ORF3* transcript by more than 900 bases and it overlaps the 1.5 kilobase *ORF2* transcript by at least 200 bases. These pairs of transcripts are antisense to each other and may have the potential to regulate the posttranscriptional expression of *ORF3* and *PET122*.

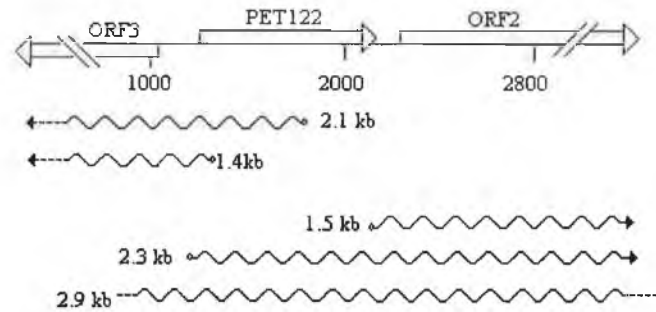


Figure 1.12 Transcription map of the ORF3-PET122-ORF2 region (Ohmen et al., 1990).

Both of the intergenic regions of *ORF3-PET122* and *PET122-ORF2* are involved in promoting divergent transcripts. *DED1-HIS3*, *MAT* and *GAL1-GAL10* are three well characterised pairs of divergently transcribed genes in yeast cells. The *MAT* and *GAL1-GAL10* promoters co-regulate expression of these divergent transcripts, while the *HIS3-DED1* intergenic regions appear to regulate the two genes independently. At this point it is not known whether the divergent transcript from *PET122* (1.4 kilobase transcript) is coregulated with the 2.3 kilobase *PET122* transcript or if the 1.5 and 2.1 kilobase transcripts are coregulated.

1.4.2.6 Overlapping transcripts in Genes coding for Glycolytic Enzymes

More recently, Boles and Zimmerman (1994), discovered open reading frames in the antisense strands of genes coding for glycolytic enzymes in *Saccharomyces cerevisiae*. The glycolytic enzymes with open reading frames in their antisense strands include phosphoglucose isomerase, phosphoglycerate mutase, pyruvate kinase and alcohol dehydrogenase I. Proteins coded in codon register by complementary DNA strands, it has been proposed, can interact with each other owing to a molecular complementarity of amino acids represented by complementary nucleic acid codons. Hence Boles and Zimmerman decided to investigate whether the open reading frames in the antisense strands of glycolytic enzymes are functional. To do this they introduced pairs of closely spaced base substitutions into the *PGII* (phosphoglucose isomerase 1) gene, which resulted in

stop codons in one strand and only silent replacements in the other by means of oligonucleotide-directed mutagenesis. The introduction of the two stop codons into the sense strand caused the same physiological defects as already observed for *pgil* deletion mutants. On the other hand no detectable effects were caused by the two stop codons in the antisense strand. Northern analysis using double-stranded probes detected only one transcript for the *PGII* gene indicating that the antisense reading frames are not functional.

In the case of these naturally occurring complementary RNAs discussed over the last few paragraphs, it is important to realise that these RNAs do not necessarily interact by hybridisation and duplex formation, but rather transcription of convergent genes may result in polymerase interference as transcription complexes on opposite DNA strands collide. Or indeed when genes have overlapping promoters in a divergent arrangement, the overlapping sequences may ensure the occlusion of RNA polymerase assembly and transit (Atkins et al., 1994).

If however the two RNAs do indeed interact by hybridisation at some point along their molecules, the functions of these partially complementary transcripts derived from overlapping genes may involve a tail-tail duplex configuration of the two mRNAs and this could result in their transport to a common location in the cytoplasm. Additionally, the stability of such a tandem transcript could be affected by the duplex state and this may serve to protect against degradation. As regards translation, this could be slowed down or inhibited by the hybridised 3' tail. Conversely, unwinding of the 3' termini could offer the possibility of induction of gene expression at the translational level. At the protein level, as regards to low expression genes, tail-tail association of the sense-antisense transcripts could provide an opportunity for complex formation between the two nascent proteins that are in close proximity to one another when the ribosomes on the opposite transcripts encounter one another, (van Duin et al., 1989).

1.5 ARTIFICIAL GENE REGULATION BY ANTISENSE TECHNOLOGY IN EUKARYOTIC SYSTEMS

1.5.1 ENGINEERED ANTISENSE GENES

One of the most usual ways of supplying antisense RNA sequences to eukaryotic cells involves the use of engineered antisense genes. This can be achieved *in vivo* by the introduction and expression of an antisense gene sequence, that is one in which part or all of the normal gene sequence is placed under the control of a promoter in reverse orientation so that the “incorrect” (complementary) DNA strand is transcribed into a non-coding antisense RNA that can then hybridise with the target mRNA and interfere with its expression. The antisense expression vector can be introduced into cells by any of the usual ways of transferring DNA such as transfection, electroporation, microinjection or in the case of viral vectors by infection. Clearly the type of transformation and choice of vector will influence whether expression is transient or stable, while the promoter chosen will decide the level, timing, inducibility or tissue specificity of the antisense inhibition (Murray and Crockett, 1992).

1.5.2 CELL CULTURE EXPERIMENTS

Many of the early studies investigating the utility of antisense genes for the ablation of specific gene expression involved the use of reporter genes such as the bacterial chloramphenicol acetyl transferase (*CAT*) gene and the *herpes simplex* virus (HSV) thymidine kinase (TK) gene. The latter gene was in fact the first gene targeted by artificial antisense RNA in eukaryotes (Izant and Weintraub, 1984). Artificial antisense genes were constructed by placing the TK structural gene in an inverted orientation relative to the HSV-TK promoter and a SV40 polyadenylation signal. The coinjection of the antisense gene constructs with a plasmid carrying the wild-type TK gene at a ratio of 100:1 respectively, resulted in a significant reduction of transient TK expression in mouse TK⁻ cells. TK activity was detected at four to five-fold lower levels when compared with cells coinjected with a control plasmid and wild-type TK.

The authors also demonstrated in this study the sequence specificity of the transcribed antisense HSV-TK RNA by showing that this antisense RNA had

no effect on the expression of a chicken TK gene present in mouse L cells. In a further experiment, designed to answer whether the activity of a chromosomal gene could be inhibited by antisense RNA the authors integrated the antisense TK plasmids into the mouse L cell genome. This was done to pre-flood the cells with antisense TK RNA before the subsequent introduction of HSV-TK gene containing plasmids. Indeed the presence of antisense HSV-TK RNA reduced the capacity of the cells to express HSV-TK.

Evidence that endogenous genes can be inhibited was provided by the author's attempts to inhibit the synthesis of cellular β -actin. If actin synthesis was reduced significantly the cells would exhibit a lethal phenotype. Co-transformation of the LTK⁻ cells with HSV-TK and an antisense β -actin construction resulted in a decrease between 10 - 20 fold of the production of TK⁺ colonies. Normally cells which took up the TK gene would form TK⁺ colonies but because of the presence of the antisense β -actin the number of TK⁺ colonies was drastically reduced, thus indicating successful inhibition of an endogenous gene.

It was the goal of Takacs and Banerjee (1997) to determine whether replication of vesicular stomatitis virus (VSV) could be inhibited by expression of a virus-specific antisense RNA in mammalian cells. VSV is a rhabdovirus, which packages within the virus an RNA-dependent RNA polymerase that transcribes the negative sense genome RNA into five mRNAs in the cytoplasm of infected cells. The active polymerase complex consists of the large RNA polymerase (L) protein tightly associated with an activator phosphoprotein P. The authors chose to target the L mRNA with an antisense RNA because it is the message transcribed in the least molar amounts and secondly because the RNA polymerase protein is essential for both virus transcription and replication.

The first 550 bases of the VSV L gene were cloned in antisense orientation into an eukaryotic expression vector. The vector chosen contained a human cytomegalovirus (HCMV) promoter for expression of the inverted sequence and a mutant dihydrofolate reductase transcription unit with an increased K_m for methotrexate. The data showed that the HeLa cell line transfected with the antisense vector did produce high levels of antisense L RNA which substantially inhibited the transcription and replication of VSV but only

for a limited time after infection. A complete inhibition of VSV infection was not obtained due to the increasing levels of virus L mRNA as the infection progressed. Because VSV replicates so fast, the concentration of target RNA will eventually become too high for inhibition by antisense RNA (Takacs and Banerjee, 1997).

1.5.2.1 PLANTS

1.5.2.2 Inhibition of the Tomato Yellow Leaf Curl Virus (TYLCV)

Engineered antisense genes have also been successful in downregulating gene expression in whole organisms in addition to the cell culture experiments just mentioned. Bendahmane and Gronenborn, (1997) designed antisense RNAs to interfere with a disease in plants caused by the Tomato Yellow Leaf Curl Virus (TYLCV). This disease is caused by a geminivirus and it is transmitted by the whitefly, *Bemisia tabaci*. Geminiviruses are single-stranded DNA plant pathogens with a wide spectrum of hosts and some can cause substantial crop losses. The authors chose the rare messenger RNA of the Rep protein encoded by the *C1* gene as the target for antisense RNA inhibition, as this gene is indispensable for the DNA replication of the geminivirus. Furthermore the Rep protein is multifunctional, hence it would serve as an attractive target.

The antisense gene cloned contained 351 nucleotides and was complementary to the full 5' leader sequence of the 1.45 kb *C1* sense transcript and the first 288 nucleotides of the *C1* gene. The sequence of the last 71 nucleotides of the antisense RNA are very conserved within all of the TYLCV genomes sequenced to date. These nucleotides encode a small protein of 11.4 kDa (C4) whose expression is required for the systemic movement of the virus in tomato. If this protein is expressed via a separate mRNA, this could then possibly represent an additional target for the antisense RNA. Finally this antisense gene was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

Transgenic *Nicotiana benthamiana* plants were derived following leaf-disc transformation using *Agrobacterium* (LBA 4404) harbouring the respective *C1* antisense or sense gene cassettes on binary plasmids. The authors found that they were unable to regenerate plants that expressed a sense copy of the complete *C1* gene, due to the deleterious effects that the constitutive synthesis of the active

Rep protein probably had on the cell. However, the transgenic plants which expressed the *CI* antisense RNA were shown to resist infection by TYLCV and this resistance was found to be effective for at least two generations of progeny.

Northern blot analysis was used to analyse the steady-state levels of RNA expression in selected TYLCV tolerant and resistant first generation lines of *Nicotiana benthamiana*. A 400 base pair single transcript lit up when using a single strand probe. This transcript was not found in non-transgenic plants. The steady-state level of this antisense transcript varied between different plant lines, however plants which exhibited an elevated transcript level consistently exhibited a higher degree of virus resistance (Bendahmane and Gronenborn, 1997).

1.5.2.3 Petunias - Inhibition of the expression of the Chalcone Synthase (CHS) gene demonstrates the variable effects of an antisense gene in eukaryotes

van der Krol and colleagues (1988b) were the first to report the inhibition of an endogenous gene in plants using antisense RNA. The gene for chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis, was placed in antisense orientation under the control of the CaMV 35S promoter. The constitutive expression of this antisense gene in transgenic petunia and tobacco plants resulted in decreased amounts of CHS protein and steady state mRNA. Phenotypically, these reduced levels were found to correlate with alterations of flower pigmentation. The differences in pigmentation pattern which were observed among the flowers in both the transgenic petunia and tobacco plants, did not appear to correspond necessarily to variances in antisense RNA steady-state levels but may be more likely to be as a result of the fact that DNA sequences adjacent to the insertion site of the antisense gene may have influenced its activity in both a quantitative and qualitative manner. Finally, Southern analysis of progeny plants demonstrated the stable inheritance and co-segregation of the antisense phenotype (van der Krol et al., 1988b).

1.5.2.4 Granule-Bound Starch Synthase (GBSS) gene

Kuipers et al., (1995) used the granule-bound starch synthase (GBSS) gene, a key enzyme in starch biosynthesis as a model system for the investigation

of antisense RNA inhibition. They constructed eleven antisense constructs derived either from the full-length GBSS cDNA, the genomic coding region (gDNA) or fragments of each of these sequences. Each construct was analysed with respect to their inhibitory effect. As regards the efficacy of antisense inhibition, very little is known about the influence of the composition of the antisense gene construct. Effective inhibition in many systems has been achieved using cDNA-derived antisense RNAs covering either the complete target mRNA or a 5' portion of varying size. In petunias, chalcone synthase gene expression was shown to be effectively inhibited by antisense genes containing the full-length cDNA or a 3' cDNA fragment (van der Krol et al., 1990). Genomic antisense genes are also suitable for effective reduction of gene expression as demonstrated by the almost complete inhibition of expression of the tomato pectin methylesterase (PME) gene by the introduction of an antisense gene based on the nearly full-length PME genomic DNA (Tieman et al., 1992).

In this study the authors found that the origin of the GBSS sequence was an important factor in determining the efficacy of antisense inhibition. The full-length GBSS cDNA and gDNA constructs were all found to be capable of complete inhibition of GBSS gene expression but it was observed that the antisense GBSS cDNA constructs resulted in complete inhibition of GBSS gene expression in a higher percentage of transgenic potato clones. The presence of intron sequences in the genomic constructs might contribute to the observed differences in antisense inhibition. The full-length GBSS gene contains 12 introns. These introns will not be processed in the antisense orientation. The duplex formed with the antisense RNA based on the genomic GBSS DNA is, therefore, expected to be most stable with the non-processed GBSS pre-mRNA, whereas the duplex formed with the GBSS cDNA-based antisense RNA will be most stable with mature GBSS mRNA. The higher inhibitory capacity of the GBSS cDNA-based antisense constructs suggests that RNA duplex formation is most effective with mature GBSS mRNA or that the cDNA-based antisense RNA interferes with RNA processing more effectively.

In this system, *A. tumefaciens* mediated introduction of the antisense genes driven by the GBSS promoter resulted in a higher percentage of transgenic clones with completely inhibited GBSS gene expression in contrast to when these constructs were driven by the CaMV promoter. This is most likely due to

the observation from promoter-GUS fusion experiments that expression from the GBSS promoter is three-tenfold higher than the expression of the 35S CaMV promoter. This implies that similar amounts of antisense RNA and GBSS mRNA are transcribed in the case of a GBSS promoter-driven antisense gene. The occurrence of antisense inhibition with comparable amounts of antisense RNA and target mRNA has been demonstrated in several other studies (van der Krol et al., 1990). For naturally occurring antisense RNAs in plants, which are thought to be involved in gene regulation, the steady-state levels of antisense RNA and mRNA do not differ greatly (Rogers, 1988). This might indicate that, in contrast to what has often been hypothesised, the formation of an excess of antisense RNA relative to sense mRNA may not be a prerequisite for successful inhibition (Kuipers et al., 1995).

1.5.2.5 Tomatoes - Inhibition of the activity of two enzymes, Polygalacturonase (PG) and Pectinmethylesterase (PME) which cause fruit softening

Growers of fresh-market tomatoes harvest their crop while the tomatoes are still firm and green, so they can be washed, sorted, packed and shipped without undergoing extensive and costly bruising. Just before the tomatoes are shipped they are bathed in ethylene gas for many days to bring about ripening. The problem with this process is that prematurely plucked and artificially ripened tomatoes do not possess the flavour that homegrown tomatoes have. Ideally, growers would like to be able to leave mass-production tomatoes on the vine for a longer period of time in order to build up the sugars and acids needed for a fresh taste and aroma, yet remaining firm enough to handle without damage (Pendick, 1992). Pectin is a polysaccharide which functions as an intercellular adhesive in the middle lamella of plant cells. When the tomato fruit softens during the course of ripening, this intercellular pectin becomes both depolymerised and solubilised which subsequently leads to a loss of cell -to-cell cohesion and fruit integrity. Polygalacturonase (PG) and pectinmethylesterase (PME) are the two enzymes involved in the degradation process of pectin. It is thought that this degradation process occurs in two steps, where pectin is first demethylated by PME and then depolymerised by PG (Pear et al., 1993).

Antisense RNA has provided a solution to these problems by the introduction of an engineered antisense polygalacturonase (PG) gene into tomatoes. This antisense gene prevents the tomato from making its normal amount of PG and as a result of this, the tomato can remain longer on the vine without getting too soft for handling. It also possesses a longer shelf-life. The commercial implications for these transgenic tomatoes are immense because in 1992 the world's first genetically engineered tomato, the "Flavr Savr" made its way onto supermarket shelves in the US after the US Food and Drug Administration cleared the way for sale of the tomato by declaring it "as safe as tomatoes bred by conventional means".

Further work carried out involved the introduction of the full-length antisense PME gene construct and a chimaeric antisense PG gene into tomato plants via *Agrobacterium*-mediated transformation. This transformation resulted in a significant reduction in both the mRNA and protein levels in ripe fruit, of these normally highly abundant cell wall hydrolases. This result indicates that antisense gene constructs in plants can be used to block multiple steps in metabolic pathways simultaneously.

However, some variability in chimeric gene expression and consequently in the degree of enzyme reduction was observed during this particular study, possibly due to the site of insertion of the T-DNA into the plant genome. Conversely, some of the transformed plants had reduced levels of PG but a much less effective reduction of PME mRNA and protein. From this, one cannot assume that effective expression of one chimeric antisense gene construct forecasts similar effectiveness of an accompanying antisense gene construct in transgenic plants. Finally, the authors are implementing this technique to transcribe multiple antisense RNA species in transgenic plants as a way of attenuating other biochemical pathways (Pear et al., 1993).

1.5.2.6 Determination of the function of a gene in tomato plants using antisense RNA

In 1990, Hamilton et al. generated transgenic plants expressing antisense RNA to a gene of unknown function, pTOM13. This clone was isolated from a ripe tomato fruit cDNA library and it was believed that this gene was involved in the synthesis of ethylene during fruit ripening and wounding of leaves. To

investigate this hypothesis, transgenic tomato plants expressing antisense genes against pTOM13 were constructed. Indeed, biochemical analysis showed that in these plants ethylene synthesis was reduced in a dose dependent manner. Plants containing one antisense gene synthesised only 13% of the normal amount of ethylene, whereas in plants homozygous for the pTOM13 antisense gene the production of ethylene was only 3% of that found in normal fruits. This particular set of experiments demonstrates that antisense RNA techniques can be used to determine the biochemical function and biological role of genes (Schuch, 1991).

1.5.3 YEAST

An examination of the field of artificial antisense RNA technology in *Saccharomyces cerevisiae* has revealed the absence of many successful antisense gene suppression experiments. This is in contrast to its very successful application in a wide range of other host systems including other yeasts, such as *Schizosaccharomyces pombe* and *Candida albicans*. However the failure of these experiments is not due to any lack of attempts but rather it may indicate particular features of *Saccharomyces cerevisiae* biology that make this organism refractory to antisense RNA technology (Atkins et al., 1994). Another interesting finding when researching this field is that many of the published findings on artificial antisense or ribozyme gene function in *Saccharomyces cerevisiae* are concerned with establishing this yeast as a model host in contrast to the few studies where the aim is to generate a yeast mutant by antisense technology.

Within *Saccharomyces cerevisiae* the silencing of genes is usually accomplished by disruption of the target gene with a selectable marker introduced by homologous recombination. The shortfall in reports of experimental attempts to inhibit the expression of genes in *Saccharomyces cerevisiae* by antisense RNA may be as a result that homologous recombination is such a powerful technique for the creation of yeast mutants. In principle, even a small amount of sequence information and a selectable gene can be used to disrupt a target gene and create a null mutation. However, a major potential advantage for using antisense RNA expression in *Saccharomyces cerevisiae* as opposed to homologous recombination is that multiple disruptions would not be required to suppress expression of a gene in a polyploid strain.

1.5.3.1 Regulation of the Sulfometuron Methyl Resistant (*SMRI*) Gene?

Xiao and Rank (1988) reported for the first time or so they thought on the regulation of gene expression in *Saccharomyces cerevisiae* by antisense RNA. Their work involved construction of chimaeric genes containing the 5' upstream and partial coding sequence of a sulfometuron methyl resistant allele (*SMRI*) of the *ILV2* locus. These fragments were placed 5' to 3' and 3' to 5' under the control of the powerful *GAL10* promoter and *CYC1* terminator in a high copy YEp plasmid. They found following galactose induction that it was only transformants containing antisense RNA exhibited biological activity against *SMRI* gene expression. Synthesis of the gene product acetolactate synthase was inhibited by the antisense RNA and this lead to the repression of cell growth which lead to a bradytrophic auxotroph revertable by addition of isoleucine and valine. However a few short years later, the results from these experiments were retracted, as a re-examination of their findings indicated that the reported YEp antisense plasmid containing transformants also harboured the corresponding sense control plasmid.

Further examination of the transformants harbouring the 5' sense *ILV2* RNA alone, revealed that these transformants produced an increased percentage of petite colonies following induction of 5' sense *ILV2* RNA transcription. The published DNA sequences for the *GAL1-GAL10* promoter, the *CYC1* transcription terminator and the *ILV2* DNA insert suggests that the 5' *ILV2* sense RNAs produced, retained the potential for translation into non-functional fusion proteins. At their 5' ends these putative proteins would contain the acetolactate synthase mitochondrial targeting signal. An overexpression of these proteins may have lead to a disruption of mitochondrial structure and/or function, hence the repression of growth observed. Finally, Arndt et al., (1994) concluded that the observed isoleucine-valine bradytrophic phenocopy was not, unfortunately the result of antisense *ILV2* RNA regulation.

1.5.3.2 Can Antisense RNA regulate the expression of the endogenous *ADE1* gene in *Saccharomyces cerevisiae*?

Also in the late eighties, Law and Devenish, (1988), examined the utility of antisense RNA as a means of regulating gene expression in *Saccharomyces*

cerevisiae. They chose to inhibit the endogenous *ADE1* gene. This gene is only one of many in the adenine biosynthesis pathway. A mutation in any one of the genes results in a red colony phenotype. This phenotype arises from the accumulation and eventual polymerisation of the substrate phosphoribosyl-5-aminoimidazole to form an insoluble red pigment. This would provide a very convenient system for screening for *ade1* cells created by antisense RNA inhibition of the *ADE1* mRNA function.

Their initial experiments involved cloning a 570 base pair fragment of the *ADE1* gene (comprising 161 base pairs of coding region and 409 base pairs of 5' flanking region) in antisense orientation into a YEp vector, between the PGK promoter and terminator. They found however that this clone was also producing sense *ADE1* even though cloned in antisense orientation. This they concluded was due to the presence of the genes own promoter. To overcome this problem they removed the promoter and this time they cloned an *ADE1* gene fragment of 179 base pairs (comprising 18 base pairs of the 5' untranslated region and the first 161 base pairs of the coding region) in antisense orientation and transformed into yeast. This clone failed to produce an *ade1* mutant phenotype.

Northern blot analysis of total cellular RNA extracted from transformed yeast cells confirmed the presence of high levels of antisense RNA to natural *ADE1* mRNA within cells. This result would appear to indicate that the failure of the antisense RNA to inhibit *ADE1* gene expression was not as a result of an insufficient amount of antisense RNA being produced. Possible reasons suggested by the authors for the failure to create phenotypic *ade1* mutants included self-annealing of the antisense RNA or formation of significant secondary structure within the 5' non-coding region of the *ADE1* gene. To investigate this hypothesis the sequences were analysed by an RNA folding computer algorithm which determines the optimal secondary configuration of an RNA molecule by minimising its conformation energy. The results indicated that the formation of significant secondary structure within the *ADE1* related sequences of the antisense transcripts or the equivalent region of the *ADE1* mRNA was unlikely, therefore hybridisation of the antisense RNA and mRNA could not be ruled out on this basis.

Other reasons for failure include the escape of a small amount of natural *ADE1* mRNA from antisense RNA inhibition, which may be sufficient to direct

synthesis of enough enzyme to maintain an *ADE1* phenotype. Lastly, may be the fragment of DNA chosen directs the synthesis of an antisense RNA which cannot efficiently block the translation of the *ADE1* mRNA even after hybridisation with the *ADE1* mRNA has occurred. This may be due to, at least in part, the presence of a tail of *PGK* coding sequences and poly(A) in the antisense RNA that arises from the presence of *PGK* terminator sequences in the expression vector, (Law and Devenish, 1988).

1.5.3.3 Does Antisense RNA in close proximity to the target gene enhance Antisense RNA control in *Saccharomyces cerevisiae*?

Arndt carried out a study in 1993 with its primary objective being to utilise the highly efficient system of homologous recombination in *Saccharomyces cerevisiae* to determine whether antisense RNA synthesis in close proximity to the target gene enhanced antisense RNA control. The *ILV2* gene, more specifically the *SMR1-410* allele, encodes the enzyme acetolactate synthase (ALS) which catalyses the first step in the biosynthetic pathway for the amino acids isoleucine and valine. Two strategies were adopted to examine the effect of localised antisense *ILV2* RNA synthesis on *SMR1-410* gene expression. The first approach involved positioning 5' and 3' antisense *ILV2* RNA expression cassettes either 5' or 3' to the *SMR1-410* gene at its endogenous chromosomal location. The second approach was designed to copy the genomic organisation of both natural prokaryotic antisense genes and overlapping transcription units in eukaryotes. To do this the *GALI-GAL10* promoter was integrated 3' to the *SMR1-410* gene such that transcription from the *GALI* start site was oriented in a direction opposite to that of the *SMR1-410* promoter. As with Xiao and Rank (1988), the criterion for a successful antisense RNA down-regulation of gene expression was a change in cellular phenotype. Induction of the antisense *ILV2-CYC1* RNA synthesis, in these integrants did not produce an isoleucine-valine bradytrophic or auxotrophic phenocopy in these cells. Northern blot analysis of the relevant integrants indicated that the 5' antisense *ILV2-CYC1* RNA was undetectable but the 3' antisense *ILV2-CYC1* RNA was expressed. The reasons for the lack of down-regulation of gene expression in these integrants is unknown (Arndt, 1993).

1.5.3.4 Is the bacterial Chloramphenicol Acetyltransferase (*CAT*) gene susceptible to antisense regulation in *Saccharomyces cerevisiae*?

In an attempt to identify a target gene susceptible to antisense regulation in *Saccharomyces cerevisiae* Arndt (1993) tested the effectiveness of an antisense RNA expressed against the bacterial chloramphenicol acetyltransferase (*CAT*) gene. To do this a chloramphenicol sensitive (Cm^{S}) yeast strain was transformed with a *CAT* gene expression cassette to produce isogenic chloramphenicol resistant (Cm^{R}) derivatives. YEp plasmids containing the antisense *CAT* RNA expression cassettes were then used to transform these cells. Following galactose induction of antisense *CAT* RNA transcription, there was no difference in this strains sensitivity to chloramphenicol. A similar result was obtained using antisense *CAT* RNA expression cassettes containing the 5' or the 3' end of the *CAT* coding region. Interestingly this gene has been shown to be susceptible to antisense control in other host systems. Using the full-length antisense *CAT* RNA, Izant and Weintraub (1985) were able to reduce *CAT* activity in mouse L cells by 5 - 20 fold. Similarly, Delauney et al., (1988) were able to reduce *CAT* activity to undetectable levels in transgenic tobacco plants.

Atkins and Gerlach (1994) have combined a number of strategies to investigate the effect of antisense gene function and hammerhead ribozymes directed once again towards the bacterial chloramphenicol acetyltransferase (*CAT*) gene, in *Saccharomyces cerevisiae*. The authors chose this gene as sensitive assays exist to detect quantitative changes in its expression and secondly this gene had been shown previously to express a functional product in this yeast. The system was designed to examine the activity of both the antisense and ribozyme genes in both a *cis* and *trans* conformation, relative to the target. They found that when the target and ribozyme or antisense genes were transcribed in the same mRNA from an expression vector, *CAT* expression was reduced by up to 90%. The steady state levels of the chimaeric transcripts were increased several fold relative to control mRNAs, this observation indicates a mechanism of suppression of *CAT* gene expression other than duplex-dependent degradation of mRNA. In contrast, when the ribozyme or antisense genes were expressed in *trans* from a plasmid-based expression vector, expression of the *CAT* gene which was chromosomally located was unaffected (Atkins and Gerlach, 1994).

1.5.3.5 Silencing of the *MIG1* gene of *Saccharomyces cerevisiae*

Olsson et al., (1997), attempted to silence the *MIG1* gene. This gene encodes a transcription factor which imposes carbon catabolite repression on invertase. In an attempt to maximise the amount of antisense transcript, the antisense sequences were driven by the constitutive *PGK* promoter. As it is difficult to predict the best region of a gene to choose as a target for antisense RNA, three regions were chosen comprising the coding region of *MIG1* alone or in combination with parts of the 5' untranslated region. The expression cassettes were introduced into a haploid *Saccharomyces cerevisiae* strain as high copy number plasmids in one configuration and in another they were integrated into the *ILV2* locus. To assess the effects of antisense gene expression on *MIG1*, the physiological characteristics of the antisense *MIG1* strains were compared with those of the wild-type strain. The invertase level in the antisense *MIG1* strains under repressive conditions was found to be 53 to 61 U/g (dry weight) regardless of the copy number or the length of the antisense fragment. As these invertase levels equal the level in the wild-type strain (61 U/g) the antisense constructs did not appear to have affected translation, even though Northern analysis showed that the antisense *MIG1* RNA was sufficiently stable to be detected. The authors conclude the failure was possibly due to the fact that the constructs were designed to produce large RNAs which may in turn have lead to an opportunity for secondary structure formation. Therefore, these RNAs may not bind to *MIG1* mRNA with a high enough efficiency to prevent translation (Olsson et al., 1997).

1.5.3.6 Control of exogenous *lacZ* gene expression in *Schizosaccharomyces pombe*

Arndt et al. (1995), designed experiments to test the suitability of the fission yeast *Schizosaccharomyces pombe* as a model for the study of antisense RNA mechanisms. A *lacZ* gene expressing yeast strain was constructed and used as a host for the expression of a series of antisense RNAs complementary to various parts of the target *lacZ* mRNA. A long *lacZ* antisense gene was designed to produce an antisense RNA complementary to 56 bases of the 5' UTR, the entire coding region and 288 bases of the 3' UTR. Short antisense genes were

designed to produce RNA complementary to either the 5' or 3' ends of the *lacZ* mRNA. The antisense genes were all placed under the control of the *Schizosaccharomyces pombe* thiamine repressible *nmt1* promoter and expressed from episomal plasmids. A corresponding sense control plasmid was constructed for each antisense plasmid. Each of the *lacZ* antisense genes were shown to express antisense RNAs of the expected size at equivalent steady state levels. In the transformed cells expressing the long, short 5' or short 3' *lacZ* antisense RNAs β -galactosidase activity was shown to be down-regulated by 45, 20 and 10% respectively, relative to the control transformants. The antisense regulation in this system was both conditional and reversible with the reduction of β -galactosidase activity being dependent on the transcription of the *lacZ* antisense RNA (Arndt et al., 1995).

To validate the use of *S. pombe* as a host for identifying antisense genes for use in human cells Clarke et al., (2000) wanted to determine if sequences identified in yeast were as equally effective in a human cell line. Their report describes the comparison of a range of *lacZ* antisense RNAs targeting a *lacZ* gene expressed in HeLa cells in a comparable manner to its expression in *S. pombe* cells in earlier studies (Arndt et al., 1995). In both cell types, the same *lacZ* gene target was expressed using the same promoter. The antisense genes were expressed episomally in both experimental systems and the levels of suppression were determined. In all cases, the relative level of suppression of the *lacZ* gene was similar in the mammalian and yeast cells. The authors postulate that this result, (at least for the *lacZ* antisense RNAs), obtained in the fission yeast may be predictive of the behaviour of antisense RNAs in mammalian cellular environment (Clarke et al., 2000).

1.5.3.7 YBR1012 gene of *Saccharomyces cerevisiae* - Determination of function using Antisense RNA

During sequencing of chromosome II of *Saccharomyces cerevisiae* the gene *YBR1012* was identified. Nasr et al., (1994), inactivated this gene using antisense RNA and found that it was essential for cellular viability. The deduced *ybr1012p* sequence was compared with the data banks and the analysis revealed the presence of a putative phosphatidylinositol kinase domain and a strong

homology to the *Schizosaccharomyces pombe rad3p*. These results indicate that *ybr1012p* may be a gene involved in signal transduction, possibly related to replication control and/or DNA damage repair. The evidence for a link with DNA damage repair was strengthened by the isolation of the *DUN1* gene as a multicopy suppressor of the *YBR1012* deletion.

To ascertain the function of the *YBR1012* gene, the authors constructed a conditional allele, using an antisense fragment of *YBR1012* DNA to regulate the expression of the gene. To do this a 235 base pair fragment from the 5' region of the gene was cloned between an inducible *GAL10-CYC1* promoter and the *PGK1* terminator. Induction of transcription by galactose should produce a small polyadenylated antisense *YBR1012* RNA. A haploid yeast strain was transformed either by control or antisense constructions, after growth on raffinose individual transformants were plated on glucose and galactose. Transformants harbouring the antisense construction were unable to grow on galactose, this result suggests that the presence of the antisense *YBR1012* RNA inhibited growth. The growth kinetics and *YBR1012* RNA levels of both the control and antisense strains were investigated following a shift to galactose medium. The control strain began to grow after a lag phase of 5 hours while the antisense strains' growth remained inhibited for a further 12 hours. Eventually the antisense strain showed slow growth indicating that suppression of the *YBR1012* gene was not complete. However, the antisense strain did stop growing at a reduced cell density when compared with the control strain.

Northern blot analysis was performed on total RNA extracted from both the control and antisense strains grown initially on raffinose, then on galactose for between 1 - 24 hours. The northern blot was hybridised with a *YBR1012* mRNA probe and an antisense *YBR1012* probe. The results showed that in the presence of galactose the *YBR1012* antisense RNA was induced and this led to a reduction in the level of *YBR1012* transcripts. After phosphoimager analysis it was observed that the *YBR1012* mRNA level in the antisense strain was 50% that found in the control.

In a continuation of this work Nasr et al., (1995), made several more antisense constructions and tested their activity. They concluded that the response of the system appeared to be very delicate as either the presence or absence of 13 nucleotides in the polylinker of the 300 nucleotide antisense

transcript could severely alter its' effectiveness. Again the authors were utilising antisense RNA to study the biological function of *YBR1012*. The results showed that some 80% of the antisense-RNA blocked cells accumulate with their DNA unreplicated, thus demonstrating that *YBR1012* has an important role in G1 and/or early S phase.

1.5.4 MICROINJECTION OF *IN VITRO* SYNTHESISED ANTISENSE RNA

In situations where there is a lack of availability of suitable vectors and promoters, microinjection of *in vitro* synthesised RNA represents an option. Indeed in some instances this mode of introduction of antisense sequences into cells is necessary due to the fact that the cells in question may be transcriptionally inactive. Another advantage with this approach is that a large excess of antisense RNA over target RNA can be injected. The drawbacks with this mode of transfer is that in multicellular organisms addition of transcripts is often difficult and in certain organisms such as *Drosophila* addition is restricted to the period of embryogenesis, before cellularisation occurs. Certainly systems like *Drosophila*, mouse embryos and *Xenopus* are more tractable to this approach than other systems because of the difficulty of accessing certain cells. Another feature of this approach which could be viewed either as an advantage or disadvantage is that the effect of the injection will be transient due to the antisense RNAs being degraded or diluted during growth. On the other hand by injecting antisense RNA at a specific time and thereby switching off gene expression it may be possible to demonstrate that the gene in question plays a role within a particular window of development.

1.5.4.1 Inhibition of exogenously microinjected β -globin mRNA in *Xenopus* oocytes

In 1985 Melton decided to test the feasibility of microinjection for blocking translation of an exogenously microinjected β -globin mRNA. Globin mRNA was chosen for these studies because it is efficiently translated in oocytes and its' protein product is easily detected. To prepare large quantities of pure antisense RNA, cDNA clones corresponding to the β -globin mRNA were cloned

into transcription vectors that contained a SP6 promoter. *Xenopus* oocytes were injected with antisense β -globin RNA and incubated for 5 hours before receiving an injection of β -globin mRNA. A 50-fold excess of antisense RNA was injected and all injections were targeted to the oocyte cytoplasm. This resulted in a complete block in translation. Further experiments indicated that this translational inhibition is due to antisense-sense hybridisation *in vivo*. Also the most effective target region in the mRNA appeared to be the RBS (Melton, 1985). Additionally the translation of an endogenous oocyte mRNA, ribosomal protein L1 mRNA has also been inhibited by the injection of antisense RNA (Wormington, 1986). With the success of microinjected antisense RNAs in oocytes Melton (1985) reasoned that this approach could be adopted to study embryonic determination in frog development.

1.5.4.2 Frog Embryos - The Discovery of Unwindase

In contrast to the success of microinjected antisense RNAs in regulating gene expression in *Xenopus* oocytes, cognate experiments in frog embryos were found to have no inhibitory effect on target gene expression (Rebagliati and Melton, 1987). Endogenous maternal mRNAs (An1, Vg1, An2, An3) could not form RNA:RNA hybrids with their respective antisense RNAs in fertilised frog eggs, even when there was a 200 - 400 fold molar excess of antisense An1 mRNA. Further studies revealed that a developmentally regulated double-stranded RNA-specific unwindase activity was present in *Xenopus* oocytes, that catalysed the unwinding of antisense:sense RNA hybrids (Rebagliati and Melton, 1987).

The enzyme double-stranded RNA adenosine deaminase (dsRAD) requires a double-stranded RNA substrate and it acts to convert adenosine (A) residues within its substrate to inosine (I) residues. Due to the fact that IU base pairs are less stable than AU base pairs the dsRNA substrate becomes increasingly single-stranded during the reaction. Since antisense techniques are dependent on the formation of a stable duplex the presence of this enzymes activity in *Xenopus* early embryos was thought to explain why antisense RNA techniques were unsuccessful in these cells. However the discovery that adenosines are modified to inosines during the reaction makes this interpretation

questionable. It is known that inosines are read as guanosines during translation therefore this modification would be expected to change the coding capacity of the DNA drastically, this would lead to the production of a non-functional protein. Thus it seems that this is more likely to contribute to the success of antisense RNA techniques (Bass, 1992).

Inhibition of target mRNA function by injected antisense transcripts is believed to occur in the cytoplasm, whereas antisense RNAs transcribed from a stably integrated gene or episomal plasmid may render its effect in either the nucleus or cytoplasm, thus with injected antisense RNAs, any potential points of regulation in the nucleus are bypassed (Murray and Crockett, 1992).

1.5.5 OLIGONUCLEOTIDES

Synthetic oligonucleotides (hereafter referred to as oligos) are widely used as another means for regulating gene expression artificially in cultured cells. An oligo is a short sequence of single-stranded DNA which is chemically synthesised and designed to be complementary to a specific intracellular target. Ideally an antisense oligo should possess a long half-life under physiological conditions, be resistant to nuclease degradation, exhibit efficient cellular uptake and affinity to its target nucleic acid and finally it is also highly desirable that the effect of the oligo is catalytic rather than stoichiometric. Most of the common oligo derivatives available today lack one or more of the aforementioned properties required for specific inhibition of gene expression (Lonnberg and Vuorio, 1996).

Oligos show great promise as therapeutic agents, however technical difficulties have slowed down their use as drugs, as structural modifications are needed to increase the stability and potency of synthetic oligos, specific delivery systems are required to facilitate their entry into target cells and more information is needed on their mechanism of action. Zamenik and Stephenson (1978) were the first to suggest the use of synthetic antisense oligos for therapeutic purposes. They were able to inhibit the growth of the Rous sarcoma virus with a 13-mer oligo targeted to a region of the viruses' RNA. From then on many papers have highlighted the great potential of antisense oligos as therapeutic agents in several human viral diseases, malignancies and dominant

hereditary diseases. In fact clinical trials are now in process to evaluate their therapeutic potential (see Table 1-2 and 1-3).

Table 1-2 Antisense Oligonucleotides - Cancer targets in Clinical Development (Ho and Parkinson, 1997).

Gene Target	Oligonucleotide	Sponsor	Backbone-Size	Disease
p53 exon 10	OL1p53	J.Armitage & Lynx*	PS-20 mer	AML-MDS/Phase I
<i>c-myc</i>	LR-3001	A.Gerwitz & Lynx*	PS-24 mer	CML-Phase I
PKC α	CGP64128A (ISIS 3521)	ISIS/Ciba	PS-20 mer	Solid tumours-Phase I
<i>c-raf</i> kinase	CGP69846A (ISIS 5132)	ISIS/Ciba	PS-20 mer	Solid tumours-Phase I
<i>bcl-2</i>	G3139	Genta & F.Cotter	PS-18 mer	Lymphoma-Phase I

Abbreviation: PS, phosphorothioate.

*Trials conducted under an investigator IND. Oligonucleotide supplied by Lynx.

Table 1-3 Antisense Oligonucleotides: Viral and Inflammatory Targets in Clinical Development (Ho and Parkinson, 1997).

Gene Target	Oligonucleotide	Sponsor	Backbone-Size	Disease
HIV gag	GEM91	Hybridon	PS-25 mer	HIV-Phase II
HPV	ISIS 2105	ISIS	PS-20 mer	Genital warts-Phase II*
CMV	ISIS 2922	ISIS	PS-21 mer	CMV retinitis-Phase III
HIV	GP0193	Chugai	PS-26 mer	HIV-Phase I
HIV integrase	AR177	Aronex	PO-17 mer!	HIV-Phase I
ICAM-I	ISIS 2302	ISIS	PS-20 mer	GVHD, RA, BD, psoriasis-Phase II
<i>c-myc</i>	LR-3280	Lynx	PS-15 mer	CAD-Phase I (+ balloon angioplasty)

Abbreviations: PS, phosphorothioate; PO, phosphodiester; GVHD, graft versus host disease; RA, rheumatoid arthritis;

IBD, inflammatory bowel disease; CAD, coronary artery disease.

! Lipid formation

* Discontinued in 1995

In theory any disease state with a gene expression component has the potential to be treated with these antisense agents. To date viral (HIV, *Herpes simplex*, Epstein-Barr, *Papillomavirus*) infections and cancer (proto-oncogenes targeted include the *myc* family (including *c-myc* and *N-myc*) *c-myc*, *c-fos*, the *ras* family (including *N-ras* and *C-H-ras*), *Bcl-2*, *c-raf-1*, *cdc-2* and *c-mos*) have

been the primary disease states under investigation for treatment with these oligos. However, other less obvious potential uses, such as restenosis, rheumatoid arthritis and allergies have also been studied (Putnam, 1996).

CHAPTER 2

Materials and Methods

2. Materials and methods

2.1 STRAINS

Strain and relevant Phenotype/Genotype	Source or Reference
<i>S. cerevisiae</i>	
DBY746	
Mat α , <i>trp1</i> -289a, <i>his</i> - Δ 1, <i>leu2</i> -3,-112, <i>ura3</i> -52.	Berkeley Stock Centre
MT235	
Mat α , <i>ade2</i> -1, <i>trp</i> -1, <i>leu2</i> -3, <i>his3</i> -11, <i>ura3</i> , <i>GAL</i> 1.	D.C.U. Stocks
I₂	
DBY746 harbouring the integrating plasmid pFAMY	D.C.U Stocks
Mat α , <i>trp1</i> -289a, <i>his</i> - Δ 1, <i>leu2</i> -3,-112, <i>URA3</i> .	
SI	
DBY746 harbouring the integrating plasmid pFAMY	D.C.U Stocks
Mat α , <i>trp1</i> -289a, <i>his</i> - Δ 1, <i>leu2</i> -3,-112, <i>URA3</i> .	
SII	
DBY746 harbouring the integrating plasmid pFAMY	D.C.U Stocks
Mat α , <i>trp1</i> -289a, <i>his</i> - Δ 1, <i>leu2</i> -3,-112, <i>URA3</i> .	
SIV	
DBY746 harbouring the integrating plasmid pFAMY	D.C.U Stocks
Mat α , <i>trp1</i> -289a, <i>his</i> - Δ 1, <i>leu2</i> -3,-112, <i>URA3</i> .	
MI	
DBY746 harbouring the ribosomal DNA integrating plasmid, pMISOC	D.C.U Stocks
Mat α , <i>trp1</i> -289a, <i>his</i> - Δ 1, <i>LEU2</i> , <i>ura3</i> -52.	
<i>E. coli</i>	
DH5α	
F'/ <i>endA</i> 1 <i>hsdR</i> 17(<i>r_k</i> - <i>m_k</i> +) SupE44 <i>thi</i> -1 <i>recA</i> 1 <i>gyrA</i> (Nal ^r) <i>relA</i> 1 Δ (<i>lacZYA</i> -argF)U169 (ϕ 80dlac Δ (<i>lacZ</i>)m15)	D.C.U Stocks

2.2 PLASMIDS

pSL5₂	pUC 8 with <i>Bacillus licheniformis</i> α -amylase gene on a <i>Hind</i> III - <i>Hind</i> III fragment.	McMahon, (1995).
pKSL5₂	pSL5 ₂ with a <i>Hind</i> III 8mer linker (CAAGCTTG), inserted in the <i>Kpn</i> I site of the α -amylase gene.	This study.
pPSL5₂	pSL5 ₂ with a <i>Hind</i> III 8mer linker (CAAGCTTG) inserted in the <i>Pst</i> I site of the α -amylase gene.	This study.
pSL5₂₁	pSL5 ₂ with the <i>Bam</i> H I site of the α -amylase gene removed.	This study.
pAAH5	Yeast shuttle vector, contains unique <i>Hind</i> III cloning site, between the constitutive <i>AdHI</i> promoter and terminator, <i>LEU</i> marker.	Ammerer, (1983).
pAAMY-01	pAAH5 containing the 1.9kb α -amylase <i>Hind</i> III fragment from pSL5 ₂ inserted in the sense orientation relative to the <i>AdHI</i> promoter and terminator.	McMahon, (1995).
pAAMY-02	pAAMY-01 with the internal <i>Bam</i> H I site of the α -amylase gene removed.	This study.
pS600	pAAH5 with the 0.6kb <i>Hind</i> III fragment of pKSL5 ₂ inserted into the <i>Hind</i> III site in its sense orientation relative to the <i>AdHI</i> promoter and <i>AdHI</i> terminator.	This study.
pS1300	pAAH5 with the 1.3kb <i>Hind</i> III fragment of pKSL5 ₂ inserted in the <i>Hind</i> III site in its sense orientation relative to the <i>AdHI</i> promoter and terminator.	This study.

pSOC-01	pAAH5 containing the 1.9kb α -amylase gene <i>Hind</i> III fragment from pAAMY-01 inserted in the antisense orientation relative to the <i>AdHI</i> promoter and terminator.	This study.
pSOC-02	pSOC-01 with the internal <i>Bam</i> H I site of the α -amylase gene removed.	This study.
pAS600	pAAH5 with the 0.6 kb <i>Hind</i> III fragment of pKSL5 ₂ inserted into the <i>Hind</i> III site in antisense orientation relative to the <i>AdHI</i> promoter and terminator.	This study.
pAS1300	pAAH5 with the 1.3 kb <i>Hind</i> III fragment of pKSL5 ₂ inserted into the <i>Hind</i> III site in antisense orientation relative to the <i>AdHI</i> promoter and terminator.	This study.
pMO2	pAAH5 with the 1.6kb <i>Hind</i> III α -amylase gene cloned in sense orientation relative to the <i>AdHI</i> promoter and terminator, this fragment lacks the genes transcriptional stop site.	Olsen, (1995).
pMO3	pAAH5 with the 1.6 kb <i>Hind</i> III α -amylase gene cloned in antisense orientation relative to the <i>AdHI</i> promoter and terminator, this fragment lacks the genes transcriptional stop site.	Olsen, (1995).
pFL34	Yeast integrating vector, <i>URA3</i> marker, unique <i>Bam</i> H I site for cloning and a unique <i>Stu</i> I site for linearisation prior to integration.	Bonneaud et al., (1991).
pFAMY	Integration vector pFL34 with <i>Bam</i> H I partial fragment from pAAMY-01 containing the entire <i>AdHI</i> promoter - α -amylase - <i>AdHI</i> terminator cassette. This vector has a <i>URA3</i> marker, unique <i>Bam</i> H I site for cloning and a unique <i>Stu</i> I site for linearisation prior to integration.	McMahon, (1995).

pFL38	ARS-CEN vector, <i>URA3</i> marker, unique <i>Bam</i> H I site for cloning.	Bonneaud et al., (1991).
pFL38-AMY	pFL38 with the 4.0kb partial <i>Bam</i> H I fragment of pAAMY-01 containing the <i>AdHI</i> promoter sense α -amylase gene - <i>AdHI</i> terminator cloned into the unique <i>Bam</i> H I site.	This study.
pMIRY	Ribosomal DNA integrating vector, <i>LEU2d</i> marker, unique <i>Bam</i> H I cloning site, and a unique <i>Sma</i> I or <i>Hpa</i> I site for linearisation of the plasmid for direction of integration into the yeasts ribosomal RNA.	Lopes et al., (1989).
pMISOC	pMIRY with the <i>Bam</i> H I fragment from pSOC-02 containing the entire antisense α -amylase gene between the <i>AdHI</i> promoter and terminator.	This study.
pYES₂	Yeast expression vector containing the <i>GALI</i> promoter for inducible expression, <i>CYCI</i> terminator, a versatile MCS, 2 μ origin of replication and a <i>URA3</i> marker.	Invitrogen.
pYES₂-AMY	pYES ₂ with the 1.9 kb <i>Hind</i> III fragment containing the α -amylase gene cloned in sense orientation with respect to the <i>GALI</i> promoter and <i>CYCI</i> terminator into the unique <i>Hind</i> III site of the MCS.	This study.
pYES₂.SOC	pYES ₂ with the 1.9 kb <i>Hind</i> III fragment containing the α -amylase gene cloned in antisense orientation with respect to the <i>GALI</i> promoter and <i>CYCI</i> terminator into the unique <i>Hind</i> III site of the MCS.	This study.

pMAS	pUC-19 which contains the 1.6 kb <i>Bam</i> H I- <i>Hind</i> III α -amylase fragment of pMO2 ligated in sense orientation to the 0.6 kb antisense <i>Hind</i> III- <i>Bam</i> H I fragment of pAS600, all ligated into the unique pUC-19 <i>Bam</i> H I site.	This study.
pMAS-H5	2.2 kb <i>Bam</i> H I fragment of pMAS blunt-ended into the unique <i>Hind</i> III site of pAAH5 in the desired orientation.	This study.
pFL34-MAS	4.2 kb <i>Bam</i> H I fragment of pMAS-H5 cloned into the unique <i>Bam</i> H I site of pFL34	This study.
pGEM-7Zf	3.0 kb vector containing T7/SP6 polymerase transcription initiation sites for use in <i>in vitro</i> transcription assays. This vector also contains a versatile multiple cloning site, an ampicillin resistance marker gene and the presence of the <i>lacZ</i> α -peptide allows recombinants to be positively selected for.	Promega.
pT7AMY	pGEM-7Zf with the 1.9 kb <i>Bacillus licheniformis</i> α -amylase gene cloned into the unique <i>Hind</i> III site. The gene is in sense orientation relative to the T7 polymerase promoter.	This study.
pSP6AMY	pGEM-7Zf with the 1.9 kb <i>Bacillus licheniformis</i> α -amylase gene cloned into the unique <i>Hind</i> III site. The gene is in sense orientation relative to the SP6 polymerase promoter.	This study.
p β ACTSP6	pGEM-7Zf with the 3.8 kb β -actin gene from pRB149 cloned into the unique <i>Eco</i> R I site. The gene is in sense orientation relative to the SP6 polymerase promoter.	This study.

pRB149 YIp 5 with a 3.8 kb yeast chromosomal β -actin gene (Ng and Abelson, 1980) cloned into a unique *EcoR* I site. YIp 5 contains a 1.1 kb *URA3* gene, an ampicillin resistance gene and a tetracycline resistance gene. Canavan, (1994).

2.3 MICROBIOLOGICAL MEDIA

All media was sterilised by autoclaving at 121°C (15 psi) for 20 minutes.

2.3.1 LB

Luria Bertani (LB) medium contains the following components:

	<i>per litre</i>
Tryptone	10.0 g
NaCl	10.0 g
Yeast extract	5.0 g

13.5 g Oxoid bacteriological agar no. 1 was added to the above for solid medium preparation.

2.3.2 SOC

(used for the growth of *E. coli* following electroporation)

Tryptone	2% (w/v)
Yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM
MgCl ₂	10 mM
MgSO ₄	10 mM
Glucose	20 mM

2.3.3 AMPICILLIN

For selective growth of *E. coli* strains, ampicillin was added after the media had been autoclaved and cooled to 55°C. The ampicillin stock solution (50 mg/ml) was made up freshly in sterile water and used at a concentration of 50 µg/ml for solid media and 40 µg/ml for broth.

2.3.4 X-GAL

A stock solution of X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was prepared by dissolving in dimethylformamide to make a 20 mg/ml solution. The tube containing the solution was wrapped in aluminium foil to prevent damage by light and stored at -20°C. It was not necessary to sterilise the X-gal solution by filtration.

2.3.5 IPTG

Isopropylthio- β -D-galactoside (IPTG) is an inducer of the β -galactosidase gene in bacteria. It was prepared by dissolving 2 g IPTG in 10 ml of water and sterilised by filtration through a 0.22 μ m disposable filter. The solution was then dispensed into 1 ml aliquots and stored at -20°C.

2.3.6 YEAST MINIMAL MEDIUM

This media is used for the selective growth of yeast auxotrophic strains carrying plasmids and it contained the following component per litre:

Yeast nitrogen base (Difco #0392-15-9)	6.7 g
--	-------

In addition the following components were added;

- (i) For a broth media, the pH was adjusted by adding 10 g of succinic acid and 6.0 g of NaOH. Solid media is buffered using 0.1 M sodium phosphate buffer, pH 6.9.
- (ii) Glucose (galactose or raffinose) was added to the media after autoclaving, from a sterilised stock solution of 40% to give a final working concentration of 2%.
- (iii) The appropriate amino acid(s) was added to the media from the sterilised stock solutions shown below, after autoclaving.

<u>Amino acid/Base</u>	<u>Supplement per litre</u>	<u>Final concentration mg/ml</u>
Uracil	8.0 ml of 0.25% soln.	20
Leucine	2.0 ml of 1.5% soln.	30
Adenine	8.0 ml of 0.25% soln.	20

(iv) 2% Lintners' starch was added, prior to autoclaving to the solid media for the routine screening of α -amylase activity.

(v) For integrative transformation it was necessary to supplement the above media with 0.75 g of the mix listed below:

Adenine	1.0 g
Uracil	1.0 g
Tryptophan	1.0 g
Histidine	1.0 g
Arginine	1.0 g
Methionine	1.0 g
Tyrosine	1.5 g
Leucine	3.0 g
Lysine	1.5 g
Phenylalanine	2.5 g
Threonine	10.0 g
Aspartic acid	5.0 g

Compounds were omitted as required, i.e. for selection on uracil - minus media uracil was omitted from this mix.

2.3.7 YEPD

YEPD is a complex medium used for routine yeast culturing and it contained the following components:

	<i>per litre</i>
Yeast extract	10.0 g
Bacteriological peptone	20.0 g
Glucose	20.0 g

20.0 g of Oxoid bacteriological agar no.1 was added to the above for solid medium preparation.

2.4 BUFFERS AND SOLUTIONS

2.4.1 TE buffer

Tris.HCl, pH 8.0	10 mM
Na ₂ -EDTA	1 mM

2.4.2 0.5 M EDTA

Disodium ethylene diamine tetraacetate (EDTA) was added to 800 ml of water and stirred vigorously. The pH was adjusted to 8.0 with NaOH and the volume made up to one litre with distilled water.

2.4.3 DNS

(3,5 - Dinitrosalicylic acid) (for measuring α -amylase activity).

	<i>per 500mls</i>
3,5 - Dinitrosalicylic acid	5.00 g
Phenol	1.00 g
Sodium sulphite	0.25 g
Sodium potassium tartrate	100.00 g

The above were dissolved in 250 ml of 2% NaOH and made up to 500 ml with distilled water. The solution was stored in the dark at 4°C.

2.4.4 PBS

(Phosphate buffered saline)

	<i>per litre</i>
KH ₂ PO ₄	1.09 g
Na ₂ HPO ₄	1.705 g
NaCl	9.0 g

2.4.5 BUFFERS AND SOLUTIONS FOR AGAROSE GEL ELECTROPHORESIS

2.4.5.1 50X TAE

	<i>per litre</i>	<i>working conc</i>
Tris-HCl,	242 g	
Glacial acetic acid	57.1 ml	0.04 M Tris-acetate
0.5 M EDTA, pH 8.0	100 ml	0.001 M EDTA

2.4.5.2 10X TBE

	<i>per litre</i>	<i>working conc.</i>
Tris-borate	108 g	0.089 M
Boric acid	55 g	0.089 M
EDTA, pH 8.0	40 ml	0.002 M

2.4.5.3 6x Bromophenol blue

Bromophenol blue	0.25% (w/v)
Sucrose in H ₂ O	40% (w/v)

DNA samples were mixed in a ratio of 5:1 with bromophenol blue prior to loading on agarose gels.

2.4.5.4 5X Formaldehyde Running buffer

(for running RNA samples).

MOPS, pH 7.0	0.1 M
Sodium acetate	40 mM
EDTA	5 mM

Made up in DEPC treated H₂O.

2.4.5.5 6x Formaldehyde Loading buffer

(RNA samples)

Na ₂ EDTA (pH 8.0)	1.0 mM
Bromophenol blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)
Glycerol	50% (v/v)

Made up in DEPC treated H₂O.

2.4.5.6 10X MOPS

(stored in the dark)

To 800 ml of DEPC (see section 2.4.10.2) treated H₂O, 41.8 g of 3-[-N-Morpholino] propanesulfonic acid was added and the pH adjusted to 7.0 with NaOH or acetic acid. 16.6 ml of 3 M sodium acetate (DEPC treated) and 20 ml of 0.5 M EDTA, pH 8.0, (DEPC treated) were then added and the solution was brought up to a volume of 1 litre with DEPC treated H₂O and sterilised through a 0.22 µm filter. This buffer oxidises with age, if autoclaved or exposed to the light. Straw coloured buffer works well but darker buffer does not.

2.4.5.7 Ethidium bromide (EtBr) (10 mg/ml)

One gram of ethidium bromide (warning, a mutagen) was added to 100 ml H₂O and stirred for several hours to ensure that the dye was dissolved. The container was wrapped in aluminium foil and stored at 4°C. Gloves were worn at all times when handling EtBr containing solutions. EtBr waste was collected, treated with activated charcoal and filtered through 3MM Whatman filter paper. The clear liquid was disposed normally and the solids contained on the filter paper were incinerated. After electrophoresis to visualise DNA the agarose gel was immersed in distilled water containing EtBr at a concentration of 1 µg/ml.

2.4.6 SOLUTIONS FOR MINI-PREPARATIONS OF PLASMID DNA

(small scale isolation of plasmid DNA from E. coli.)

2.4.6.1 Solution 1

	<i>per 10ml</i>	<i>final conc</i>
0.5 M Glucose	1.0 ml	50 mM
0.1 M EDTA	1.0 ml	10 mM
1.0 M Tris.HCl (pH 8.0)	0.25 ml	25 mM

2.4.6.2 Solution 2

(Made up freshly every fortnight and stored at room temperature)

	<i>per 10 ml</i>	<i>final conc</i>
1 N NaOH	2.0 ml	0.2 N
10 % SDS	1.0 ml	1% SDS

2.4.6.3 Solution 3

To 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H₂O were added. The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate.

2.4.6.4 STET buffer

8% (w/v) sucrose
5% (v/v) Triton-X-100
50 mM Na₂EDTA
50 mM Tris.HCl (pH 8.0)

2.4.6.5 Phenol/Chloroform mix: (Kirby mix)

100 g of AnalaR grade phenol was dissolved in 100 ml of chloroform. The phenol was then equilibrated with 100 mM Tris.HCl pH 7.5 until a pH of 7.5 was obtained (assessed by measuring the pH of the aqueous layer) then 4 ml of isoamylalcohol and 0.8 g of 8-hydroxyquinoline were added and the mixture was stored under 100 mM Tris.HCl pH 7.5 in a dark bottle at 4°C.

2.4.7 SOLUTIONS FOR MIDI-PREPARATIONS OF PLASMID DNA (QIAGEN)

(large scale isolation of plasmid DNA from *E. coli*, buffers were supplied with the kit)

2.4.7.1 Resuspension Buffer

(Buffer P1)

Tris-HCl,	50 mM
EDTA, pH 8.0	10 mM
RNase A (2.4.13.6)	100 µg/ml

Store at 4°C.

2.4.7.2 Lysis Buffer

(Buffer P2)

NaOH	200 mM
SDS	1% (w/v)

Store at room temperature.

2.4.7.3 Neutralisation Buffer

(Buffer P3)

KAc, pH 5.5	3.0 M
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Store at 4°C.

2.4.7.4 Equilibration Buffer

(Buffer QBT)

NaCl	750 mM
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MOPS, pH 7.0	50 mM
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Ethanol	15%
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Triton X-100	0.15%
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Store at room temperature.

2.4.7.5 Wash Buffer

(Buffer QC)

NaCl	1.0 M
------	-------

MOPS, pH 7.0	50 mM
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Ethanol	15%
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Store at room temperature.

2.4.7.6 Elution Buffer

(Buffer QF)

NaCl	1.25 M
------	--------

Tris-HCl, pH 8.5	50 mM
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Ethanol	15%
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Store at room temperature.

2.4.8 SOLUTIONS FOR THE ISOLATION OF TOTAL GENOMIC DNA FROM YEAST

2.4.8.1 SPM

Sorbitol	0.9 M
Sodium phosphate buffer (pH 7.5)	0.05 M
2-mercaptoethanol (freshly added)	0.14 M

2.4.8.2 Lyticase

Lyticase enzyme (Sigma) was resuspended in 0.05 M potassium phosphate buffer (pH 7.5) to a final concentration of 8,000 U/ml.

2.4.8.3 Proteinase K

10 mg/ml Proteinase K was resuspended in freshly prepared 150 mM NaCl.

2.4.9 SOLUTIONS FOR THE ISOLATION OF YEAST CHROMOSOMAL DNA FOR PULSE FIELD GEL ELECTROPHORESIS

2.4.9.1 SCE buffer

Sorbitol	1.0 M
Sodium citrate	0.1 M
EDTA, pH 7.5	10.0 mM

2.4.9.2 Solution I

SCE buffer (2.4.9.1)	10 ml
2-mercaptoethanol	0.5% (v/v)
Zymolyase 60,000	10 mg

2.4.9.3 Solution II

EDTA, pH 9.0	0.45 M
Tris-HCl, pH 8.0	10.0 mM
2-mercaptoethanol	7.5% (v/v)

2.4.9.4 Solution III

EDTA, pH 9.0	0.45 M
Tris-HCl, pH 8.0	10.0 mM
Sodium N-lauroylsarcosinate	1.0%
Proteinase K	1 mg/ml

2.4.10 SOLUTIONS FOR THE ISOLATION OF RNA FROM YEAST

2.4.10.1 Letts buffer

LiCl	0.1 M
EDTA	0.1 M
Tris.HCl (pH 7.4)	0.01 M
SDS	0.02%

2.4.10.2 DEPC treatment

Diethylpyrocarbonate (toxic) was used to inhibit RNases in the preparation of solutions for RNA manipulations. DEPC (0.1%) was added to the solution, shaken vigorously, left at room temperature for at least 20 minutes and then autoclaved to inactivate the DEPC. DEPC was not added directly to solutions containing Tris as it

is highly unstable in these solutions decomposing rapidly into ethanol and carbon dioxide (Maniatis et al., 1982). Gloves were worn at all times in the preparation of solutions for the manipulation of RNA to avoid the introduction of RNases.

2.4.11 SOLUTIONS FOR LITHIUM ACETATE YEAST TRANSFORMATION

2.4.11.1 Lithium acetate stock solution (1.0 M)

10.2 g of lithium acetate was dissolved in 100 ml of distilled water, the pH was then checked and should be between 8.4 - 8.9. The solution was then autoclaved and stored at room temperature.

2.4.11.2 PEG 3350 stock solution, 50% (w/v)

50 g of PEG was weighed into a graduated 150 ml beaker, water was added slowly until the level was just below the 100 ml mark. A stirring bar was placed in the beaker and mixed on a magnetic stirrer until the PEG had dissolved, this takes approximately 30 minutes at room temperature. The viscous solution was then decanted into a 100 ml measuring cylinder and water was added to the 100 ml mark, sealed with parafilm and mixed thoroughly by inversion. The solution was then autoclaved. An important point to note was that the solution was stored in a tightly sealed container to prevent evaporation because if the solution was stored in a loosely capped bottle, the concentration of PEG steadily increases, causing a slow but noticeable decline in transformation efficiency with time.

2.4.12 SOLUTIONS FOR ISOLATION OF DNA FROM AGAROSE

(GeneClean)

2.4.12.1 Sodium Iodide solution

90.8 g NaI was dissolved in 100 ml H₂O.
The solution was filtered through Whatman No.1 filter paper. 15 g of Na₂SO₄ was added and the solution stored at 4°C in the dark.

2.4.12.2 Ethanol Wash solution

(New Wash)

Ethanol	50%
NaCl	100 mM
Tris.HCl (pH 7.5)	100 mM
EDTA	1 mM

This solution was stored at -20°C.

2.4.13 BUFFERS AND SOLUTIONS FOR DNA MANIPULATIONS.

2.4.13.1 Restriction buffers

Buffers were supplied with the restriction enzymes and used according to the manufacturers instructions.

2.4.13.2 Ligation buffer

Supplied and used according to the manufacturer's instructions.

2.4.13.3 Klenow buffer

Supplied and used according to the manufacturer's instructions.

2.4.13.4 Dithiothreitol (1M DTT)

DTT (3.09 g) was dissolved in 0.01M sodium acetate (pH5.2) and filter sterilised. Aliquoted and stored at -20°C.

2.4.13.5 ATP (0.1M)

ATP (60 mg) was dissolved in 800 µl of sterile H₂O and the pH adjusted to 7.0 with 0.1 M NaOH. The volume was adjusted to 1 ml with sterile H₂O, aliquoted and stored at -20°C

2.4.13.6 RNase (DNase free)

10 mg/ml RNase A (Sigma) was dissolved in 10 mM Tris.HCl (pH 7.5), 15 mM NaCl; heated to 100°C for 15 minutes, allowed to cool slowly to room temperature and stored at -20°C.

2.4.14 SOLUTIONS FOR SOUTHERN HYBRIDISATION

(radioactive hybridisation)

2.4.14.1 Depurination solution

HCl	0.25 M
-----	--------

2.4.14.2 Denaturing solution

	<i>per litre</i>	<i>working conc.</i>
NaCl	87.66 g	1.5 M
NaOH	20.0 g	0.5 M

2.4.14.3 Neutralising solution

	<i>per litre</i>	<i>working conc.</i>
Tris-HCl	121.1 g	1 M
NaCl	87.66 g	1.5 M

Dissolved in 800 ml of distilled H₂O, the pH was adjusted to 8.0 and the volume to 1 litre.

2.4.14.4 Transfer buffer

(20X SSC)

NaCl	175.3 g
Trisodium acetate	88.2 g

Dissolved in 800 ml of distilled H₂O, the pH was adjusted to 7.0 and the volume brought up to 1 litre.

2.4.14.5 50X Denhardt's solution

per 500 ml

Ficoll	5.0 g
Polyvinylpyrrolidone	5.0 g
B.S.A	5.0 g
H ₂ O to	500 ml

This solution was stored at -20°C.

2.4.14.6 Prehybridisation solution

6X SSC
5X Denhardt's solution
0.5% SDS
100 µg/ml denatured salmon sperm
DNA (section 2.4.14.8).

2.4.14.7 Hybridisation solution

as for prehybridisation solution except
it contains in addition EDTA (0.1 M)
and the ³²P labelled probe.

2.4.14.8 Preparation of salmon sperm DNA (10 mg/ml)

Salmon sperm DNA was used in hybridisations with radiolabelled probes and was prepared as follows; 500 mg of salmon sperm DNA (Sigma, type IV) was dissolved in 50 ml distilled H₂O for at least 4 hours. The DNA was sheared by passing it through a syringe with a microlance at the top (21G Becton Dickinson, Dublin), aliquoted and stored at -20°C. The DNA was denatured before use, by placing it in a boiling water bath for 5 minutes and then placing immediately on ice.

2.4.15 SOLUTIONS FOR NORTHERN HYBRIDISATION

(radioactive hybridisation)

2.4.15.1 Prehybridisation

6X SSC

2X Denhardts

0.1 %SDS

100 µg/ml denatured salmon sperm

DNA (section 2.4.14.8).

2.4.15.2 Hybridisation

as for prehybridisation solution except
it contains the ^{32}P labelled probe.

2.4.16 SOLUTIONS FOR DIG - DNA

(non-radioactive hybridisation, kit supplied by Boehringer Mannheim)

2.4.16.1 Prehybridisation solution

(Southern) at 68°C

5X SSC

1% Blocking reagent (2.4.16.4)

0.1% N-Laurolysarcosine

0.02% SDS

2.4.16.2 Prehybridisation solution

(Northern) at 42°C

5X SSC

50% Formamide

2.0% Blocking reagent (2.4.16.4)

0.1% N-Lauroylsarcosine

0.02% SDS

2.4.16.3 Buffer I

Tris-HCl, pH 7.5	0.1 M
NaCl	0.15 M

2.4.16.4 Blocking stock solution (10%)

Blocking reagent was dissolved in Buffer I to a final concentration of 10% (w/v) with shaking and heating on a heating block, pH had to be exactly 7.5 for the reagent to dissolve. After autoclaving the solution turns pink and was stored at 4°C.

2.4.16.5 Buffer II (1% Blocking reagent)

Blocking stock solution diluted 1:10 in Buffer I.

2.4.16.6 Washing Buffer

Buffer I and 0.3% (w/v) Tween-20.

2.4.16.7 Buffer III (Detection buffer)

Tris-HCl, pH 9.5	0.1 M
NaCl	0.1 M
MgCl ₂	0.05 M

2.4.16.8 Buffer IV

Tris-HCl, pH 8	0.01 M
EDTA	0.001 M

All the above solutions were made up in ultrapure H₂O and autoclaved at 121°C (15psi) for 20 minutes.

2.5 GROWTH AND STORAGE OF STRAINS

All *E. coli* strains were grown overnight at 37°C in LB medium. All *S. cerevisiae* strains were grown overnight at 30°C in complex media or for 36 hours in minimal medium. Both yeast and bacterial strains were stored as glycerol stocks. An equal volume of 80% glycerol was added to a late log phase culture and stored at -70°C. Working stocks were stored on plates at 4°C.

2.6 RECOMBINANT DNA TECHNIQUES

2.6.1 TRANSFORMATION OF *E. COLI*

(based on a method by Mandel and Higa, 1970).

2.6.1.1 Preparation of competent *E. coli* DH5α cells for transformation.

100 ml of LB was inoculated with 2 ml of an overnight culture of *E. coli* and incubated in a shaking waterbath at 37°C to an OD₆₀₀ of 0.3 - 0.4. The cells were cooled on ice and subsequently centrifuged at 4°C, 3,000 rpm for 10 minutes. The media was poured off and the pellet resuspended gently in 25 ml cold 50 mM CaCl₂, then it was left on ice for 20 minutes. The cells were harvested by centrifugation as before, then resuspended in a total volume of 2.5 ml 50 mM CaCl₂. The cells were then left on ice for between 1 hour and overnight. Cells are competent at this stage.

2.6.1.2 Transformation of competent *E. coli* DH5 α cells.

200 μ l of competent cells were added to the ligation reaction/plasmid, vortexed briefly and left on ice for 30 minutes. The cells were then heat shocked at 43°C for exactly 2 minutes, 1 ml of LB without the selection antibiotic was added and the microfuge tubes incubated at 37°C for 1 hour to allow the cells to recover. The cells were collected by centrifugation in a microcentrifuge for 1 minute, the supernatant was poured off and the cell pellet was resuspended in 100 μ l of sterile H₂O. Appropriate volumes of the cells were spread on to selective media and incubated at 37°C overnight. The transformants were picked off, purified and plasmid DNA was isolated using one of the methods described in section 2.6.5. pUC 18/19 based transformations were carried out in DH5 α to exploit the *lacZ* phenotype. Selection of the transformants included inoculation onto ampicillin plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and isopropylthio- β -D-galactoside (IPTG). This was achieved by adding 40 μ l of a stock solution of X-gal (20 mg/ml in dimethylformamide, section 2.3.4) and 4 μ l of a solution of IPTG (200 mg/ml in H₂O, section 2.3.5) to a premade LB agar plate containing the appropriate antibiotic and spreading over the entire surface of the plate. The plate was incubated at 37°C until all of the fluid had disappeared, this can take up to 3 hours if the plate is freshly made due to the low volatility of dimethylformamide. Colonies with inserts in the plasmid remained white, while those without inserts remained blue.

2.6.1.3 Transformation of in-gel ligation.

Competent cells were prepared as in section 2.6.1.1. The ligation reaction mixture was heated to 68°C for 5 - 10 minutes to remelt the agarose. The ligation reaction was diluted with sterile distilled water such that 1 μ l of diluted ligation mix contained 1 - 10 ng of DNA. 1 μ l of diluted ligation was then added to 200 μ l of competent cells. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 3 minutes, then incubated at 37°C for 1 hour and plated on selective media.

2.6.2 ELECTROPORATION OF *E. COLI*

2.6.2.1 Preparation of electrocompetent *E. coli* DH5 α cells.

1 litre of LB was inoculated with 1% (v/v) of an overnight *E. coli* culture and shaken vigorously at 37°C to an OD₆₀₀ of 0.5 - 0.8. The flask was chilled on ice for 15 - 30 minutes and the cells were centrifuged at 4000 rpm for 15 minutes at 4°C. The cell pellet was resuspended by gently shaking in an ice water bath, in 1 litre of ice cold distilled H₂O. The suspension was centrifuged as before and resuspended in 0.5 litres of ice cold distilled H₂O. Following resuspension the cells were centrifuged as before and resuspended in 20 ml of 10% (v/v) ice-cold glycerol. Once again, the cells were centrifuged and resuspended finally in 2-3 mls of 10% glycerol. The cells were dispensed into 100 μ l aliquots and stored at -80°C for up to six months.

2.6.2.2 Electroporation of competent *E. coli* DH5 α cells.

The apparatus used was a BioRad Gene Pulser™, set at 25 mF capacitance, 2.49 kV and 200 ohms to deliver a single exponentially decaying pulse. The electroporation cuvettes were chilled on ice for 5 minutes, the cells thawed on ice and 1 - 5 μ l of plasmid DNA/ligation mix was added and left for 1 minute before electroporating. The DNA must be cleaned of excess protein and salts to minimise arcing. The mixture of cells and DNA was then transferred to an electroporation cuvette, the outside of which was dried, then tapped sharply off the bench to get rid of air bubbles which can also cause arcing. The cuvette was placed in the sliding holder and the pulse applied by pressing the two red buttons simultaneously. Immediately after a high pitched beep is heard, (indicating that the pulse has been delivered) 1 ml of SOC or LB medium was added to the cuvette and the cell suspension placed at 37°C for 1 hour. Dilutions of the cells were plated onto selective medium and incubated at 37°C overnight.

2.6.3 LITHIUM ACETATE YEAST TRANSFORMATION

2.6.3.1 Preparation of yeast competent cells.

100 mls of YEPD was inoculated with 20 μ l of an overnight culture of *S. cerevisiae* and grown at 30°C with agitation to an OD₆₀₀ of 0.7. The cells were harvested at room temperature by centrifugation at 5000 rpm. After two washes in half the original volume of TE pH 7.5, the cells were resuspended in one tenth the original culture volume of 100 mM LiAc and incubated at 30°C with agitation for 60 minutes. The cells were harvested as before and resuspended in the same volume of 100 mM LiAc. Routinely 10 ml samples of culture were used yielding 1.0 ml of competent cells.

2.6.3.2 Transformation of yeast competent cells.

Plasmid DNA (0.1 μ g - 10 μ g) was added to 300 μ l of competent cells. 0.7 ml of 40% PEG₃₃₅₀/ 0.1 M LiAc was added, mixed gently by inversion and incubated at 30°C for 60 minutes. The cells were then placed at 42°C for 5 minutes and plated directly onto selective media.

2.6.4 TRANSFORMATION OF *S. CEREVISIAE* BY ELECTROPORATION

2.6.4.1 Preparation of cells.

The yeast strain was grown in YEPD to late log phase or early stationary phase. The cells were harvested by centrifugation at 5,000 rpm for 5 minutes then washed three times with sterile distilled water. The cells were finally resuspended in water to 10% of the original volume and cooled to 0°C. The electroporation cuvettes (0.2 cm electrode gap) were also cooled to 0°C. To 100 μ l of cells approximately 1 μ g of DNA and 50 μ l of 40% PEG 3000 were added.

2.6.4.2 Electroporation of electrocompetent *S. cerevisiae* cells.

Electroporation of cells was accomplished using a BioRad Gene Pulser™, set at a resistance of a 1,000 ohms, a capacitance of 25 µF and a voltage of 0.8 kV, to deliver a single exponentially decaying pulse. The cells were pulsed, then 0.5 ml of 20% sucrose was added, mixed and the mixture transferred to a microcentrifuge tube, a range of concentrations were then plated onto selective media plates.

2.6.5 SMALLSCALE ISOLATION OF PLASMID DNA FROM *E. COLI*

2.6.5.1 STET prep

(Described by Holmes and Quigley, 1981).

An overnight culture (2 ml) or a toothpick colony was resuspended in 300 µl of STET (section 2.4.6.4) by vigorous vortexing then 20 µl of 10 mg/ml lysozyme was added to the microcentrifuge tubes, the tubes were then left at room temperature for 10 minutes. Following this, the samples were denatured in a boiling water bath for exactly 60 seconds, then spun immediately at 13,000 rpm for 10 minutes. The supernatant was removed and precipitated with an equal volume of isopropanol. After incubation at -20°C for 30 minutes, the samples were spun at 13,000 rpm for 5 minutes. The resulting plasmid DNA pellet was washed with 500 µl of ether, dried at room temperature for 5 minutes and resuspended in 50 µl of TE containing DNase free RNase (50 µg/ml). This was incubated for 10 minutes at 37°C to degrade RNA that may mask small fragments of DNA in an agarose gel.

2.6.5.2 Solution 1,2,3 prep

(Described by Birnboim and Doly, 1979).

1 ml of an overnight broth culture was pelleted at 13,000 rpm and resuspended by vortexing in 100 µl of Solution 1 (section 2.4.6.1) and incubated at room temperature for 5 minutes. 200 µl of Solution 2 (section 2.4.6.2) was added and the microfuge tubes were left on ice for a further 5 minutes. To this, 150 µl of Solution 3 (section 2.4.6.3) was added, the samples were left on ice for 10 minutes and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and 400 µl

of phenol-chloroform mix (section 2.4.6.5) added. After thorough mixing, the samples were spun at 13,000 rpm, the aqueous layer removed and precipitated with 0.8 ml ice-cold ethanol. Following 10 minutes at room temperature, the plasmid DNA was pelleted by centrifugation at 13,000 rpm. The pellet was washed twice in 70% (v/v) ethanol, dessicated and resuspended in 50 μ l TE.

2.6.6 LARGE SCALE ISOLATION OF PLASMID DNA FROM *E. COLI* (QIAGEN COLUMN-PLASMID MIDI-KIT)

For large-scale isolation of plasmid DNA from *E. coli*, the Qiagen method was employed, (Qiagen Inc., USA), in which an affinity resin column and the appropriate buffers are supplied. A single colony was used to inoculate 5 ml of LB (containing the appropriate antibiotic) which was grown overnight, then used to inoculate a further 100 ml of the same medium. This was again grown overnight, and harvested by centrifugation in a Beckman J2-21 (JA-14 rotor) at 6,000 rpm for 15 minutes at 4°C. The pellet was resuspended in 4 ml of resuspension buffer (section 2.4.7.1), and transferred to a glass "corex" tube. Lysis buffer (4 ml, section 2.4.7.2) was added, mixed, and the tube incubated at room temperature for 5 minutes. Then 4 ml of neutralisation buffer (chilled, section 2.4.7.3) was added, mixed gently, and further incubated, on ice for 15 minutes. This was then spun (JA-20 rotor) at 12,000 rpm for 30 minutes at 4°C, but if the resulting supernatant was not fully clear, the step was repeated for a further 15 minutes, and the clear supernatant then promptly removed. A Qiagen-tip 100 column was equilibrated with 4 ml equilibration buffer (section 2.4.7.4) just before use, then the supernatant applied and allowed to flow through. The column was then washed with 2 x 10 ml of wash buffer, (section 2.4.7.5) and eluted with 5 ml elution buffer (section 2.4.7.6). The eluent was collected into a "corex" glass tube, 0.7 volumes isopropanol added, and immediately centrifuged at 12,000 rpm and 4°C for 30 minutes. This served to precipitate the DNA, which was visible as a faint glassy pellet. The pellet was then washed with 70% ethanol, spun again, and air dried, before being resuspended in 100 - 300 μ l TE, and stored at -20°C.

2.6.7 ISOLATION OF PLASMID DNA FROM *S. CEREVISIAE*

5 ml of cells from a late exponential phase culture were harvested by centrifugation, 5,000 rpm for 5 minutes, and then resuspended in 0.5 ml 1M sorbitol/0.1 M EDTA. 50 µl of lyticase (8,000 units/ml) was then added and the cells were incubated for 60 minutes at 37°C. The cells were then spun at 1,000 rpm for 1 minute and resuspended in 0.5 ml 50 mM Tris-HCl, pH 7.4/ 20 mM EDTA. 50 µl of 10% SDS was then added and the mixture was incubated for 30 minutes at 65°C. 200 µl of 5 M potassium acetate was added and the mix was left on ice for 60 minutes, spun for 5 minutes at 5000 rpm after which the supernatant was removed and precipitated with an equal volume of isopropanol. After 5 minutes at room temperature the DNA was pelleted by spinning for 10 seconds. The pellet was then dried at 55°C and resuspended in 300 µl of TE pH 7.4. 15 µl of RNase (1 mg/ml) was added to the resuspended DNA and incubated for 30 minutes at 37°C after which the DNA was precipitated by the addition of 30 µl of 3 M sodium acetate and 300 µl cold isopropanol. The DNA was pelleted by centrifugation (5 minutes/5,000 rpm) and resuspended in 50 µl TE. This volume of DNA was then used for transformation of *E. coli*.

2.6.8 ISOLATION OF GENOMIC DNA FROM *S. CEREVISIAE*

2.6.8.1 Long Method

(This method is a modification of that of Cryer et al., 1975).

5 ml of a YEPD culture was inoculated with the appropriate strain and the culture was grown to stationary phase. If the cells were grown in minimal media a 25 ml culture volume was inoculated and the cells were once again grown to stationary phase. The cells were harvested by centrifugation. The pellet was washed once with 10 ml distilled water and once with 2 ml SPM (section 2.4.8.1). The cells were resuspended in 0.5 ml SPM. A 20 µl sample was taken and diluted in 5 ml distilled H₂O and the OD₆₀₀ measured. This value represented time zero OD value for evaluation of protoplasting efficiency. 100 µl of lyticase enzyme (section 2.4.8.2) was added and mixed. The sample was incubated at 30°C for 1 - 2 hours, or until a reduction of OD₆₀₀ to 10 - 30% of the original OD₆₀₀, when a 20 µl sample was

diluted in 5 ml distilled H₂O. 100 µl Proteinase K (section 2.4.8.3), 50 µl of 1 mM EDTA:0.25 M sodium phosphate (pH 8.0) and 70 µl of 25% SDS were added to the yeast spheroplasts. This lysis mixture was incubated for 30 minutes at 37°C. On completion of incubation, 500 µl of phenol/chloroform was added and mixed by inversion. The sample was left on ice for 10 minutes, before centrifugation at 12,000 rpm for 5 minutes. The upper aqueous phase was carefully removed using a wide bore pipette (cut-off 1 ml tip) to a fresh tube. The lower phase (including the interphase) was back extracted with 200 µl of TE buffer, centrifuged as before and the supernatant pooled with the aqueous phase of the previous extraction. Two further phenol/chloroform extractions were performed as above except with no back extraction of the interphase to ensure complete deproteinisation and extraction of lipids. The aqueous phase was extracted once with 700 µl chloroform at room temperature. 1 ml of ethanol (-20°C) was added and the sample mixed by inversion and incubated at -20°C for at least 2 hours. The total DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes. The DNA pellet was dried at 50°C washed twice with 70% ethanol and carefully dissolved overnight in 300 µl TE buffer. 15 µl of RNase (10 mg/ml) was added to the dissolved DNA, and incubated for 30 minutes at 37°C. The DNA solution was again deproteinised, precipitated with ethanol and washed in 70% ethanol as before. Finally, the DNA pellet was carefully dissolved in 100 µl TE buffer.

2.6.8.2 Quick Method for Preparation of *S. cerevisiae* genomic DNA.

The yeast was inoculated into 10 ml of YEPD medium in a 250 ml flask. The cultures were grown overnight at 30°C with shaking. The cells were harvested at 4,000 rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 0.5 ml of 1 M sorbitol solution, this suspension was transferred to a microcentrifuge tube and 25 µl of lyticase solution (5 mg/ml) was added. The tube was incubated at 37°C for 30 minutes. After this incubation, the microcentrifuge tube was spun for 1 minute at 13,000 rpm and the supernatant was discarded. The cells were resuspended in 0.5 ml of TE buffer by pipetting up and down. 25 µl of 20% SDS was then added and the tube was inverted 10 times to mix. The tube was then incubated at 65°C for 20 minutes. Following this, 0.4 ml of 5 M KAc was

added, the tube was inverted 10 times to mix, then it was left on ice for half an hour. The microcentrifuge tube was then spun at 13,000 rpm for 5 minutes. The supernatant was transferred to a fresh microcentrifuge tube and 0.75 ml of isopropanol was added at room temperature. The tube was inverted 10 times to mix, then allowed to sit at room temperature for less than 5 minutes. The tube was then spun for 5 minutes at 13,000 rpm, the supernatant was poured off, then the tube was respun. This time the remainder of the supernatant was removed by pipette and the pellet was air-dried for 5 minutes. The DNA was resuspended in 300 µl of TE by finger-flicking. 30 µl of 3M NaAc and 200 µl of isopropanol were then added, the tube was inverted 5 times to mix. The tube was then spun for 5 minutes, the supernatant was discarded, the tube was respun and once again the remainder of the supernatant was removed by pipette. The DNA was air-dried for 5 minutes then finally resuspended in 150 µl of TE buffer.

2.6.9 *S. CEREVISIAE* CHROMOSOMAL DNA ISOLATION FOR PULSE-FIELD GEL ELECTROPHORESIS

(Carle and Olson, 1985).

2.6.9.1 Standard DNA plug preparation for Pulsed Field Gel Electrophoresis.

Cells were grown to late logarithmic phase in 100 ml of YEPD medium and then harvested and washed twice with 50 mM EDTA, pH 7.5 at 0°C; the final cell pellet was suspended in 3.25 ml of 50 mM EDTA, pH 7.5; 3 ml of the cell suspension was mixed at 37°C with 5 ml of 1% low melting point agarose (which was prepared in 0.125 M EDTA, pH 7.5, and cooled to 42°C) and 1 ml of solution I (section 2.4.9.2); the mixture of agarose, cells and cell-wall-removing enzyme was then quickly pipetted into the wells of a plastic mould suited for running on a CHEF Mapper and placed in a refrigerator to accelerate gelling; after setting, the plugs were removed from the mould (with a narrow piece of x-ray film) into a petri dish (6mm diameter) containing 5 ml of solution II (section 2.4.9.3), and incubated overnight at 37°C in a plastic bag with gentle shaking; solution II was then replaced by 5 ml of solution III (section 2.4.9.4), the plastic bag was resealed, and the plates were then incubated overnight at 50°C. Plugs should look clear after proteinase K

treatment. Finally the plugs were washed twice with 0.5 M EDTA pH 9.0. Plugs were now ready for use or they could be stored in 0.5 M EDTA pH 9.0 at 4°C. For electrophoresis conditions see section 2.10.3.

2.6.10 ISOLATION OF RNA FROM *S. CEREVISIAE*

Total RNA was isolated from yeast using a method adapted from Sherman et al., (1986). In order to minimize RNase activity during the isolation of RNA the following precautions were taken. All solutions (section 2.4.10) used in this procedure were made up in DEPC treated H₂O and autoclaved at 15 psi for 20 minutes. Glassware and glass beads were washed in 3 M HCl and baked at 180°C for 8 hours before use. Plasticware was rinsed in chloroform and autoclaved before use.

Cells were grown in appropriate media to an OD_{600nm} of approximately 0.8. 100 ml of cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C and resuspended in 2 mls Letts buffer. The cell suspension was transferred to a 15 ml corex tube containing 8 g glass beads (40 mesh for GLC, BDH) and 2 ml phenol/chloroform that had been equilibrated with Letts buffer. The cells were lysed by vortexing for 3 minutes (6 x 30 second bursts with 30 seconds intervals on ice between each burst). 2 ml Letts buffer was added, the sample mixed by vortexing and spun for 5 minutes at 8,000 rpm at 4°C. The aqueous layer was removed and the sample extracted 3 times with phenol/chloroform. The RNA was precipitated in 0.1% 5 M LiCl and 2 volumes ice cold absolute ethanol and incubated at -20°C for at least 3 hours. Total RNA was harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was washed in 80% ethanol, air dried and resuspended in 1.0 - 1.5 ml DEPC treated H₂O.

2.6.11 GENERAL DNA MANIPULATIONS

2.6.11.1 Restriction digestion.

Restriction buffers were supplied by the manufacturers and used under the recommended conditions. When restricted DNA was required for further manipulations it was cleaned up either by extraction with phenol/chloroform and ethanol precipitations (section 2.6.11.7), or by Spin Column (section 2.6.11.8) or

finally by GeneClean (section 2.6.11.9). For restriction of 20 μ l of total yeast DNA for a Southern blot, the DNA was restricted in a volume of 200 μ l, using 4 μ l of restriction enzyme(1 unit/ μ g) and incubated at 37°C for 12 hours.

2.6.11.2 Klenow reaction.

After restriction digestion and prior to recircularisation or the addition of linkers to plasmids, the Klenow reaction was used to fill-in cohesive termini generated by certain restriction enzymes. Following restriction digestion, the DNA was cleaned using the GeneClean (section 2.6.11.9) procedure and the Klenow fill-in reaction was carried out as follows. To a 1.5ml microcentrifuge tube on ice the following were added:

10X React 2 buffer	3 μ l
0.5 mM dATP	1 μ l
0.5 mM dTTP	1 μ l
0.5 mM dGTP	1 μ l
0.5 mM dCTP	1 μ l
DNA	0.5-1 μ g
Large fragment of DNA polymerase I	1 unit
Sterile distilled water	to 30 μ l.

The above reaction was mixed gently, then centrifuged briefly to bring the contents to the bottom of the tube. Incubation was at room temperature for 30 minutes, and the reaction was terminated by phenol extraction. Alternatively, for restriction digestions which left 3'overhangs the exonuclease activity of Klenow was exploited and the above reaction was carried out minus the nucleotides.

2.6.11.3 Dephosphorylation of digested DNA.

0.5 μ l of Calf-Intestinal Phosphatase (CIP, 1 unit/ μ l) and 1.5 μ l of CIP buffer (5X) were added to 13 μ l (0.25 μ g - 1.0 μ g) of linearised plasmid DNA and incubated at

37°C for 20 minutes. The reaction was stopped by heat inactivation of the enzyme at 65°C for 10 minutes, followed by phenol extraction and ethanol precipitation.

2.6.11.4 DNA ligations.

Ligations were carried out in the presence of 0.1 unit of T₄ DNA ligase for cohesive end ligations and 1.0 unit of T₄ DNA ligase for blunt end ligations. Vector (CIP treated, section 2.6.11.3) and insert fragments were routinely ligated in a ratio of 1:10. Ligations were incubated at 4°C overnight.

2.6.11.5 In-gel ligation.

The DNA to be used as insert was digested with restriction enzyme, the DNA was then electrophoresed in the appropriate concentration of NuSieve GTG agarose (see Table 2-1 below). The gel was stained with ethidium bromide, destained and the gel band containing the insert was excised and placed in a sterile preweighed tube. Exposure of DNA to UV irradiation was minimised to less than 1 minute. The excised band could be stored at 4°C until ready for use.

Table 2-1.Determination of the optimal agarose concentration.

Size range in bp	% Agarose conc. (TAE)
500 - 1000	2.5
150 - 700	3.0
100 - 450	3.5
70 - 300	4.0
10 - 100	4.5
8 - 50	5.0

2.6.11.6 Ligation of vector and insert in remelted agarose.

The ligation reaction mixture was set up such that the final ligation reaction volume was approximately 50 µl, and the amount of agarose in the final reaction mixture did not exceed 1.5%. The agarose slice containing the insert was remelted by heating to 68°C for 10 minutes, the sample was then maintained at 37°C to prevent gelling. A volume of melted agarose containing the required quantity of insert was transferred to a preheated (37°C) microcentrifuge tube and the following were added to a 50 µl final volume:

T ₄ ligase buffer (10X)	5 µl
Vector	0.1 µg
Deionised water	
T ₄ ligase enzyme (1 unit/µl)	5 µl

The above were mixed gently, transferred to a ligation temperature of 14°C for a minimum of two hours or overnight.

2.6.11.7 Ethanol precipitations.

This procedure was derived from Bethesda Research Laboratories protocol **Focus** (1985) 7(4): 1 - 2.

Precipitation of DNA was achieved by the addition of 1/10 th the volume of 3.3 M sodium acetate and two volumes of ice-cold (-20°C) 95% ethanol. The contents of the tube was mixed and stored at -20°C for at least 30 minutes or overnight. The mixture was then centrifuged for 30 minutes at 13,000 rpm, the supernatant was carefully poured off and the DNA pellet washed with 2 volumes of 80% ethanol (-20°C). The contents of the tube were mixed by inverting the tube several times, the mixture was stored at -70°C for 15 minutes, then centrifuged once again for 30 minutes at 13,000 rpm. The supernatant was poured off and the pellet dried under vacuum. The DNA was reconstituted in TE buffer.

2.6.11.8 Spin Columns

(Hunter, 1991).

Spin columns were used for removing impurities from DNA preparations and for changing buffer conditions. Fifty μ l of glass beads (40 mesh for GLC, BDH) were placed at the bottom of a 0.5 ml microfuge tube which had been pierced with a hot lance. To this 0.5 ml of 70% sepharose CL6B (cross-linked 6% beaded agarose, Sigma) was added and the column was equilibrated 3 times with sterile distilled water. To do this, a volume of water equal to the sample volume was added to the column and centrifuged at 1,500 rpm for exactly 2 minutes into an empty microfuge tube. Once the column was equilibrated and eluting the exact sample volume, the sample was added and the column spun at 1,500 for 2 minutes into a clean sterile microfuge tube.

2.6.11.9 Isolation of DNA fragments from agarose

(GeneClean).

This method for the recovery of DNA fragments from agarose gels was based on Vogelstein and Gillespie, (1979). This method was also used to clean up DNA preparations, change buffer conditions and clean ligations prior to electroporation. The DNA was separated by agarose gel (TAE) electrophoresis. The fragment of interest was cut from the gel using a clean blade. The gel slice was chopped into small pieces, weighed and sodium iodide solution was added (2 ml/g agarose). The sample was mixed and incubated at 55°C for 5 minutes or until the agarose had dissolved. 1 - 2 μ l of glass bead slurry (prepared as in section 2.6.11.10) was added, the sample was mixed by pipetting up and down and left at room temperature for 5 minutes. The sample was spun at high speed for 5 seconds the supernatant was removed and the pellet was washed 3 times with 100 μ l of New Wash (section 2.4.12.2), spinning for 5 seconds between each wash. The pellet was resuspended in 10 - 20 μ l of TE, pH 8.0, and the DNA allowed to elute from the beads for 3 minutes at 55°C. The beads were then spun and the eluate (DNA) removed into a sterile microfuge tube. This elution step was repeated to ensure maximum recovery of the DNA.

2.6.11.10 Preparation of Silica 325 Mesh Glass Beads for GeneClean Procedure.

250 ml of silica powder was suspended in distilled water and the volume made up to 500 ml in a 600 ml beaker. The suspension was stirred for 1 hour and then allowed to settle for a further hour. The supernatant was then removed to two centrifuge tubes and spun at 5,000 rpm for 5 minutes. The pellet was resuspended in 100 ml of distilled water and transferred to a 600 ml beaker and the volume adjusted to approximately 140 ml. 350 ml of 70% HNO₃ was then added to give a final concentration of 50% HNO₃. The suspension was then heated to near boiling on a hot plate with continuous stirring. The suspension was then transferred to 2 centrifuge tubes and spun at 5,000 rpm for 5 minutes. Each pellet was then resuspended in 100 ml of distilled water and spun down at 5,000 rpm for 5 minutes. This process was repeated 3 times. The volume of the final pellet was then estimated and resuspended in an equal volume of distilled water and the suspension stored at 4°C.

2.6.11.11 Determination of DNA concentration.

Method I:

Serial 1:1 dilutions were prepared by mixing 5 µl of DNA sample with 5 µl of TE buffer. After addition of 1 µl of bromophenol blue dye, 5 µl of each dilution was loaded onto a 0.7% agarose gel. In the same way dilutions of DNA (of known concentration and similar size) were prepared and loaded onto the same gel. Electrophoresis was performed at 100 V for 20 minutes. The gel was then stained with ethidium bromide and destained in H₂O. The concentration of the sample DNA was estimated by comparing the intensity of fluorescence of the samples with that of the standard DNA samples.

Method II:

Alternatively, the concentration of DNA in any given preparation was measured by absorbance spectrophotometry. A 5 µl aliquot was taken diluted in 1 ml of TE, decanted into a quartz cuvette and the absorbance read on a UV spectrophotometer (Unicam 8625, UV\Vis Spectrometer). The absorbance of a DNA solution was

determined at 260 and 280 nm using an absorption coefficient of $50 \mu\text{g/ml} = 1 \text{ OD}_{260}$ unit for double-stranded DNA and $40 \mu\text{g/ml}$ for single-stranded DNA. The method is subject to interference by RNA and protein and an assessment of the purity of the sample can be gauged from the $\text{OD}_{260} / \text{OD}_{280}$ ratios.

2.6.12 SOUTHERN BLOTTING - TRANSFER OF DNA TO NITROCELLULOSE FILTERS

This procedure described by Southern (1975) results in the transfer of electrophoresed DNA from an agarose gel to a nitrocellulose filter, thereby allowing the long term storage of the DNA and its subsequent analysis using hybridisation to a labelled probe. After electrophoresis, the gel was visualised and photographed. When the fragments of interest were larger than 10 kb, the gel was placed in depurination solution (0.25 M HCl) and gently agitated at room temperature for 10 minutes or until the bromophenol blue turned yellow. The gel was then rinsed well with distilled H_2O and incubated in several volumes of denaturing solution (section 2.4.14.2) with agitation for 1 hour. The denaturing solution was decanted and the gel was soaked for 1 hour in neutralising solution (section 2.4.14.3), with constant shaking. At this stage the pH of the gel was below 8.5. The transfer took place using either the standard procedure or a modified bidirectional method involving two nitrocellulose filters.

For the standard Southern blot, a piece of nitrocellulose (Schliecher and Schnell) slightly larger than the gel was floated on the surface of 2X SSC for 5 minutes and then submerged for a further five minutes. This ensures even wetting of the nitrocellulose, eliminating air bubbles. The gel was placed on a 3MM Whatman filter paper wick, the ends of which were in contact with 20X SSC (section 2.4.14.4). The wet nitrocellulose was laid carefully on the gel and the air bubbles removed by rolling a glass test tube over the surface. Two pieces of filter paper soaked in 2X SSC were placed on top of the nitrocellulose and the stack (5-8 cm high) built up with paper towels. A weight was placed on top to aid the transfer. Parafilm was used to prevent the towels coming in contact with the gel or 20X SSC, thereby preventing the liquid bypassing the nitrocellulose.

The bidirectional blotting procedure was essentially the same except the gel was sandwiched between two nitrocellulose filters and four 3MM Whatman filter papers

soaked in 20X SSC with towels on either side. There was no need for a wick or reservoir of 20X SSC in this method. During the first 2 hours of transfer, the gel stack was inverted every 30 minutes to ensure even transfer. The wet towels were also removed and replaced with dry ones.

After the overnight transfer of DNA to nitrocellulose with either procedure, the towels and filter paper were removed and the position of the wells were marked on the nitrocellulose. The nitrocellulose filters were then washed in 6X SSC to remove any fragments of agarose, air dried for 1 hour and baked between two pieces of filter paper for 2 hours at 80°C. Routinely the filters were stored at room temperature sealed in plastic.

2.6.13 SOUTHERN TRANSFER OF PULSE-FIELD GEL ELECTROPHORESIS GELS

2.6.13.1 Alkaline transfer protocol.

Alkaline transfer was not recommended for use with Schleicher & Schnell nitrocellulose membrane as this membrane is not suitable. Instead BioTrace PVDF (GelmanSciences) was used. This is a hydrophobic microporous polyvinylidene fluoride membrane that binds biomolecules with high affinity. This membrane has high resistance to discoloration upon exposure to basic solutions and it possesses high mechanical strength. Due to the hydrophobic nature of PVDF, the membrane does not wet out in aqueous solutions, therefore the membrane was wet out by lowering into a container of 80 - 100% ethanol. The membrane should change from white to translucent almost immediately. The membrane was agitated in the alcohol solution for a few minutes to ensure complete wetting. The alcohol was rinsed from the membrane with several changes of distilled water prior to equilibrating in transfer buffer.

The pulse-field gel was stained with 1 µg/ml EtBr for 20 minutes and destained in distilled water for a further 20 minutes. While photographing the gel, minimising the exposure to UV light was essential. The gel was then immersed in 0.25 M HCl at 30°C for 5 minutes. Neutralisation of the gel took place in 0.5 M Tris, pH 7.0 for

30 minutes at room temperature, with gentle shaking. Transfer of DNA onto the PVDF membrane took place using 0.4 N NaOH, 1.5 M NaCl as transfer buffer. The blot was set up as for a Southern, and transfer was allowed to take place for 24 hours. After transfer the membrane was neutralised in 0.5 M Tris, pH 7.0 for 5 minutes, followed by rinsing briefly in 2 X SSC. The membrane was blotted dry and baked at 80°C for 1 hour.

2.6.14 NORTHERN BLOTTING - TRANSFER OF RNA TO NITROCELLULOSE FILTERS

This procedure differs slightly to the Southern blot procedure for DNA transfer. Electrophoresis was carried out as described (section 2.10.2) Electrophoresis and transfer to nitrocellulose were carried out sequentially, as it is not recommended to leave a time interval between carrying out these procedures. Being single-stranded, native RNA molecules are capable of binding to nitrocellulose filters. However, in order to transfer efficiently from agarose, a denaturing step is recommended. Following electrophoresis the gel was washed several times in DEPC treated H₂O (section 2.4.10.2) then soaked in 0.05 N NaOH to partially hydrolyse the RNA for 20 minutes. The gel was then rinsed in DEPC treated H₂O and soaked in 20X SSC (section 2.4.14.4) for 45 minutes. The nitrocellulose was placed in DEPC treated H₂O for 1 minute and then in 20X SSC for 5-10 minutes. The gel was blotted either one way or bidirectionally as described for Southern blotting. The following day, the wells were marked and the filters were baked at 80°C for 2 hours. The blots were stored dry at room temperature.

2.6.15 RNA TRANSCRIPTION *IN VITRO*; PREPARATION OF RIBOPROBES

(Promegas' Riboprobe^R in vitro transcription system)

2.6.15.1 DNA template preparation.

The vector (pGEM-7Zf with insert) was linearised with a suitable restriction endonuclease (i.e. any restriction endonuclease which does not generate a 3' overhang, if restriction endonuclease does leave a 3' overhang it must be converted

to a blunt end using the 3' - 5' exonuclease activity of DNA polymerase I Large (Klenow fragment, see section 2.6.11.2). Following restriction digestion, the linearised plasmid was extracted with phenol:chloroform before using the DNA for *in vitro* transcription reactions.

2.6.15.2 Synthesis of high specific activity radiolabelled RNA probes.

NOTE: Throughout these procedures precautions should be taken to protect against ribonuclease contamination.

Standard transcription protocol

Transcription Optimised 5X Buffer

(contains 10 mM spermidine, supplied with kit) 4 µl

DTT (100 mM) 2 µl

Recombinant RNasin Ribonuclease inhibitor (supplied in kit) 20 units

ATP, GTP and UTP (2.5 mM each)

(prepared by mixing 1 volume deionised water with 1 volume of each of the 10 mM ATP, GTP and UTP stocks supplied) 4 µl

100 µM CTP (diluted from stock) 2.4 µl

linearised template DNA

(0.2 - 20 mg/ml in water or TE buffer) 1 µl

[α -³²P]CTP (50 µCi at 10 µCi/µl) 5 µl

SP6, T7 or T3 polymerase 15-20 units

20 µl

Note: The mixture was kept at room temperature during the addition of each successive component. Since DNA can precipitate in the presence of spermidine if kept at 4°C.

The above reaction was then incubated at 37 - 40°C for 1 hour.

2.6.15.3 Removal of the DNA template following transcription.

RNase free DNase (supplied with *Promegas' Riboprobe^R in vitro transcription system* kit) was added to give a final concentration of 1 unit /µg. The reaction was allowed to proceed for 15 minutes at 37°C. After this the reaction was extracted

with 1 volume of TE saturated phenol:chloroform (pH 4.5), then vortexed for 1 minute and centrifuged at 12,000 rpm for 2 minutes. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamylalcohol (24:1) was added. The contents of the tube were once again vortexed for 1 minute followed by centrifugation at 12,000 rpm for 2 minutes. The upper aqueous phase was transferred to a fresh tube and 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol were added. The contents of the tube were mixed and placed at -70°C for 30 minutes or at -20°C for a minimum of 2 hours. Following centrifugation (20 minutes), the supernatant was carefully removed and the pellet washed with 70% ethanol. The pellet was dried and resuspended in 10 - 20 µl of TE buffer or water.

2.6.16 PREPARATION OF RADIOACTIVE LABELLED DNA PROBE

Random primer labelling of probe DNA was performed using the Promega "Prime a gene" kit, based on the method developed by Feinberg and Vogelstein (1983). 25 ng of DNA was linearised and denatured by heating to 100°C for 5 minutes, then immediately transferred to ice. The reaction (50 µl, total volume) included the following:

5X Klenow buffer		10 µl
Mixture of 3 unlabelled dNTPs	(500 µM)	2 µl
Denatured linear DNA template		25 ng
Acetylated BSA (1 mg/ml)		2 µl
[³² P] dATP (3,000 Ci/mM)		5 µl
Klenow polymerase		5 units

The components were mixed gently and the reaction tube incubated at room temperature for 1 hour. The reaction was terminated by heating to 100°C for 2 minutes, then chilled on ice. 2 µl of 0.5 M EDTA was added and the labelled, denatured probe DNA was used directly for hybridisation to nitrocellulose filters.

2.6.17 PREPARATION OF NON-RADIOACTIVE LABELLED DNA PROBES

DIG DNA labelling

(Boehringer Mannheim Biochemica).

This procedure involved the labelling of DNA by random primed incorporation of digoxigenin labelled deoxyuridine triphosphate. The steroid hapten digoxigenin is linked to the dUTP molecule via a spacer arm. Following hybridisation, the target DNA hybrids were detected with colourimetric or chemiluminescent alkaline phosphatase substrates by an enzyme linked immunoassay. If a colourimetric substrate was used, the signal develops directly on the membrane. The colour precipitate starts to form within a few minutes and the reaction is usually complete after 12 hours. The signal was detected on X-ray film (as with ^{32}P) when a chemiluminescent substrate was used. Exposure times in this case are typically in the range of 15-30 minutes. This procedure allows the efficient labelling of small (10 ng) and large (up to 3 μg) amounts of DNA. Linearised DNA labels more efficiently than circular. The DNA must be thoroughly heat denatured prior to labelling.

100 ng of DNA was routinely linearised and denatured and the following components were added to a sterile microcentrifuge tube;

freshly denatured probe DNA	100 ng
hexanucleotide mixture	2 μl
dNTP labelling mix (containing dATP, dCTP, dGTP, (1 mMol/L); dTTP (0.65 mMol/L); DIG-dUTP (0.35 mMol/L)	2 μl
Klenow polymerase (2 units/ μl)	1 μl

The components were mixed gently and incubated at 37°C for at least 1 hour. The reaction was quenched by addition of 2 μl EDTA (0.2 mol/l) and the labelled DNA precipitated by addition of 2.5 μl LiCl (4 mol/l) and 75 μl ethanol (-20°C). The mixture was left to stand at -20°C for 2 hours or 30 minutes at -70°C. The DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes, washed with 70% ethanol,

dried under vacuum and dissolved in 50 µl TE buffer. Labelled DNA could be stored at -20°C or used directly for hybridisation to nitrocellulose filters.

2.6.18 HYBRIDISATION USING THE HYBAID HYBRIDISATION OVEN

The Hybaid roller bottles (200 ml) were placed in an Hybaid hybridisation oven and allowed to warm up (approximately 1 hour). A piece of support mesh appropriate for the size of the membrane was then selected. The mesh and nitrocellulose filter were pre-wetted in a suitable tray containing 2 X SSC. After ensuring that the hybridisation membrane exactly overlaid the mesh, both were rolled up into a tight roll. The roll was inserted into a hybridisation bottle in a way such that the leading edge (inside the roll) and the trailing edge are positioned relative to each other. The bottle was placed on a flat surface and the mesh and membrane were slowly unwound around the inside of the bottle by rocking and gently rolling the bottle along the surface of the bench. No air bubbles should be visible between the membrane and the bottle. If bubbles were found to be present, the membrane was removed and re-rolled. The mesh ensured that the probe had access to all parts of the hybridisation membrane, including those parts of the membrane which overlap.

2.6.18.1 Pre-hybridisation.

Once the membrane was placed in the bottle the 2X SSC was discarded and replaced with pre-hybridisation fluid (0.2 ml/cm² of nitrocellulose filter) or 10 - 20 ml of solution was recommended for the 200 ml bottle size used. Higher volumes were required when there was more than one membrane in the bottle. The bottle was placed in the hybridisation oven, the rotisserie switched on and prehybridisation was allowed to proceed for between 1 - 4 hours at 68°C (or at 42°C for Northern blots when the hybridisation solution contained formamide).

2.6.18.2 Hybridisation and washing conditions when using a radioactively labelled probe.

After pre-hybridisation, the fluid was replaced with hybridisation solution (50 $\mu\text{l}/\text{cm}^2$ filter) containing the ^{32}P -labelled denatured probe. The bottle was again incubated at 68°C with constant rolling for 16 - 20 hours. Following hybridisation, the radiolabelled probe was poured into a radioactive waste container and the blot was then washed according to the blot type.

For a Southern blot probed with homologous DNA the washes were as follows;

2X SSC/0.5 % SDS at room temperature for 5 minutes,

2X SSC/0.1% SDS at room temperature for 15 minutes,

0.1X SSC/0.5% SDS at 68°C for 2 hours,

0.1X SSC/0.5% SDS at 68°C for 30 minutes

For a Northern blot probed with a radioactively labelled probe, the washing conditions were;

1X SSC/0.1% SDS, twice at room temperature for 15 minutes each,

0.2X SSC/0.1% SDS, three times at 68°C for 15 minutes each time.

The wash solutions were pre-warmed and the bottles were half-filled (approximately 100 mls) with these solutions. Note that washing with solutions which are not pre-warmed may result in background problems. The blots were air dried after washing and those probed with a radioactively labelled probe were placed into heat sealable bags. Exposure to autoradiography film was carried out in light proof cassettes for between 24 hours and one month, depending on the strength of the signal, after which the film was developed as described in section 2.12.

2.6.18.3 Hybridisation and washing conditions using a non-radioactively labelled probe.

3.5 ml of hybridisation solution was required for a blot of 10 x 10 cm containing a probe concentration of 5 - 25 ng/ml which had to be denatured prior to addition.

Hybridisation was allowed to proceed for 16 - 20 hours at 68°C or at 42°C for hybridisation solutions containing formamide. Following hybridisation the solution was poured off and washed. For either a Southern and Northern blot probed with a DIGoxigenin -labelled probe, the washing conditions were as follows;

2X SSC/0.1% SDS, twice at room temperature for 5 minutes each,
0.5X SSC/0.1% SDS twice at 68°C, for 15 minutes each

2.6.18.4 Detection of non-radioactively labelled probe.

The filters were washed briefly in buffer I (section 2.4.16.3) before incubating for 30 minutes in buffer II (blocking solution, section 2.4.16.5). The filter was incubated for a further 30 minutes in buffer II. The filter was then incubated for a further 30 minutes in 20 ml of diluted antibody i.e. 1/5000 dilution or 150 mU/ml of anti-digoxigenin AP conjugate. Unbound antibody conjugate was removed by washing for 2 x 15 minutes with 100 ml buffer I. The filter was equilibrated for 2 minutes in 20 ml of buffer III (section 2.4.16.7). For colour development, the filter was incubated in 10 ml colour solution in the dark until band formation one hour to twenty-four hours later. The reaction was stopped by washing the filter in buffer IV (section 2.4.16.8) for 5 minutes.

2.6.18.5 Chemiluminescent detection of non-radioactively labelled probe.

After hybridisation and post-hybridisation washes, the membrane was equilibrated in washing buffer (section 2.4.16.6) for 1 minute. The chemiluminescent substrate was allowed to come to room temperature. Using a freshly washed dish the membrane was blocked by gently agitating it in blocking solution (section 2.4.16.4) for 30 - 60 minutes. The antibody conjugate was then diluted 1:10,000 in blocking solution. The blocking solution was poured off and the membrane was incubated for 30 minutes in the antibody containing solution. After this, the membrane was washed gently twice, 15 minutes per wash in washing buffer (section 2.4.16.6). The washing buffer was then poured off and the membrane was equilibrated in detection buffer (Buffer III, section 2.4.16.7) for 2 minutes. The chemiluminescent substrate

CSPDTM was diluted 1:100 in detection buffer. It is important that the filter is kept wet before the chemiluminescent substrate is added to avoid high backgrounds. The membrane was placed between two sheets of acetate (plastic page protectors). The top sheet of plastic was lifted gently, and with a sterile pipette, approximately 0.5 ml (per 100 cm²) of the chemiluminescent substrate was added to the top of the membrane by scattering drops all over the surface of the membrane. The top sheet of plastic was then lowered and with a damp lab tissue the top sheet was wiped to remove any bubbles present under the sheet and to create a liquid seal around the membrane. The blot was then incubated at 37°C for 15 minutes before exposure to X-ray film. (Note - it is advisable to use a nylon membrane when using a chemiluminescent substrate).

2.6.19 POLYMERASE CHAIN REACTION (PCR)

A Hybaid Omnigene Thermal cycler, fitted with a 48 well heating block taking 0.5 ml tubes was used for all PCR reactions. The reactions were set up in the laminar air flow, under sterile conditions, and the target DNA was added last to each microcentrifuge tube to prevent cross contamination. The oligos and dNTPs were kept on ice at all times. A typical PCR reaction was set up as follows:

Primers (50 µM stock)	1 µl X 2
dNTPs (500 µM stock)	2 µl
10X buffer	5 µl
MgCl ₂ (25 mM stock)	3 µl
Target DNA	1 µl
H ₂ O	36 µl
Taq Polymerase	5 units

The tube contents were mixed, centrifuged briefly and overlaid with 60 µl of sterile mineral oil. Following the PCR reaction a 5 µl sample was examined on an agarose gel (usually 0.7%) and the tubes stored at 4°C.

2.7 CURING *S. CEREVISIAE* OF PLASMID DNA

In order to cure yeast cells of plasmid DNA the strains were grown up in YEPD for 2 days and then replica plated onto YEPD and selective media. The colonies which grew on YEPD and not on selective media were considered cured.

2.8 CELL ENUMERATION

Total yeast cell counts were performed using a Neubauer haemocytometer. All counts were performed in duplicate. Standard curves of cell number versus OD₆₀₀ were constructed for cells in YEPD and selective medium. These standard curves were used to estimate the number of cells per ml in a culture by simply reading the OD₆₀₀.

2.9 PLASMID STABILITY STUDIES

Strains were inoculated into 5 ml selective media lacking the amino acid used for selection and grown to stationary phase. The cell number was determined by direct cell counting and 10⁶ cells were inoculated into 100 ml YEPD. After overnight incubation at 30°C the cell counting and inoculation were repeated, this procedure was followed for up to 7 days. The number of generations per day was between 9 and 11. Each day after the cell count had been determined suitable dilutions were plated onto YEPD plates in duplicate. After 2 days at 30°C 100 colonies from the YEPD plates were replica plated onto YEPD and selective media. The percentage of colonies retaining plasmid (percentage plasmid stability) was determined from the ratio of the number of colonies growing on the minimal media plate, compared to the YEPD plate.

2.10 ELECTROPHORESIS

2.10.1 ELECTROPHORESIS AND VISUALISATION OF DNA

Agarose (0.7% w/v) in TAE was routinely used for the analysis of plasmid and chromosomal DNA. Mini-gels, used for analysis of plasmid DNA preparations, were run at 100V in 1X TAE buffer on a gel box system for approximately 1 hour, while total (genomic) DNA digests were run at 40V on a gel box for 14-16 hours. A concentration of 6X bromophenol blue dye (see section 2.4.5.3) was added to samples before loading. Gels were stained in EtBr (5 µg/ml) for 20 minutes, destained in H₂O for 5 minutes and the DNA was visualised using UV transillumination with the aid of a UVP UV transilluminator.

2.10.2 FORMALDEHYDE GEL ELECTROPHORESIS AND VISUALISATION OF RNA

RNA molecules were separated on formaldehyde gels before blotting onto nitrocellulose. All manipulations of agarose gels containing formaldehyde were carried out in the fume cupboard.

2.10.2.1 Preparation of Formaldehyde gel.

For a 200 ml gel (1.2%), 2.4g of agarose was boiled in 144 ml DEPC treated H₂O and allowed to cool to 60°C. 40 ml 5X formaldehyde buffer (section 2.4.5.4) and 36 ml 37% formaldehyde were added and the gel allowed to set at room temperature.

2.10.2.2 Preparation of RNA samples for loading.

For a total sample volume of 20 µl, 4 µl 5X formaldehyde buffer, 5 µl deionised formamide, 2 µl 37% formaldehyde and 1 µl ethidium bromide (1 mg/ml) were mixed in a microcentrifuge tube. Up to 8 µg of RNA was added. A separate sample containing 4 µl 5X bromophenol blue and 4 µl H₂O and no RNA was also prepared as a marker for running the gel. The samples were heated to 65°C for 10 minutes

before loading. Before running the gel, the electrophoresis tank was thoroughly rinsed with DEPC treated H₂O, dried with ethanol and then filled with 1X Formaldehyde running buffer. The gel was run at 3V/cm² in 1X formaldehyde buffer for 4-6 hours until the dye had reached halfway down the gel. The gel was rinsed several times in DEPC treated H₂O to remove the formaldehyde and the remainder of the procedure was carried out on the bench.

2.10.3 GEL PREPARATION AND ELECTROPHORESIS CONDITIONS FOR PULSE-FIELD

The 1% gel was prepared using BioRad Ultra-pure DNA Grade certified agarose (catalogue no. 162-0133, Molecular Biology certified agarose) dissolved in 0.5X TBE. The running conditions consisted of a 60 second pulse for 15 hours followed by a 90 second pulse for 9 hours at 6V/cm, a temperature of 14°C and a pulse angle of 120°

2.11 PHOTOGRAPHY

Photographs were taken using an Olympus OM-20 camera and T-Max 100 black and white film (Kodak). An A003 red filter (Corkin, France) was used to block UV light when necessary, and also close-up lenses (Vivitar) were fitted when required. Negatives were developed using a 35 mm Film Tank (Photax), the reel of film being loaded onto the spool and placed in the tank in darkness, 250 ml of D76 (Kodak) developer was added. Air bubbles were removed by tapping a few times off the bench. After the required development time (temperature dependent), the developer was poured out, the tank rinsed with tap water and 250 ml Unifix (1/3 in H₂O, Kodak) were added. When completely fixed, usually after 10 minutes, the negatives were rinsed for at least one hour in tap water and then air dried. Prints were later made onto Kodak Kodabrome II RC F4 high contrast paper using an enlarger (darkroom), developed with Polymax (Kodak) and fixed also with Unifix fixer. After rinsing thoroughly in tap water, the prints were dried.

Photographs were also taken using an image analyser, UVP ImageStore 7,500. The photographs were then printed on a Sony Video Graphic printer UP-860CE.

2.12 X-RAY FILM DEVELOPMENT

In the darkroom with a red filter (GBX-2 safelight, Kodak), the film was marked by inserting a few holes in the blots and film to aid orientation. The film was placed in Kodak developer LX24 (1:4) for 5 minutes, washed in water briefly and fixed in Kodak Fixer LX20 (1:4) for 5 minutes until clearing occurred. The film was washed in tap water and dried. Kodak Min-RE film (blue, 18x24cm) was used routinely for radioactive blots.

2.13 ASSAYS FOR MEASUREMENT OF α -AMYLASE ACTIVITY

2.13.1 PLATE ASSAYS FOR DETECTING α -AMYLASE ACTIVITY

In order to visualise α -amylase activity on plates, amylase secreting strains of *E. coli* and *S. cerevisiae* were grown up on either LB, YEPD or minimal medium, whichever was appropriate, supplemented with 2% Lintners starch. The amylase activity was visualised by incubating the plates at 4°C for 24 hours, after which the undegraded starch had precipitated leaving clear haloes corresponding to areas of amylase activity.

Alternatively, the plates were inverted over iodine crystals for 60 seconds. Areas where the starch had been hydrolysed appeared as clear haloes on a dark blue background.

2.13.2 DNS ASSAY FOR MEASURING α -AMYLASE ACTIVITY

In order to ensure accurate quantitation of enzyme activity in cell supernatants, it is necessary to remove all remaining glucose in the growth media, prior to assaying. This was achieved by dialysis. Dialysis removes the sugars effectively without loss of enzyme. Samples of supernatant (2.0-5.0 ml volumes) were placed in the prepared dialysis tubing (section 2.13.2.1) and sealed with dialysis tubing clips, to ensure minimal volume increase during dialysis, the samples were pressurised into the smallest amount of tubing then dialysed against 1X PBS at 4°C for 12 - 16 hours. The buffer was changed at least 3 times during that period.

The following reaction mix was set up in a test tube:

0.1 M Sodium phosphate buffer, pH 6.9	1.0 ml
1% Lintners starch	1.0 ml
0.5 M NaCl	0.2 ml

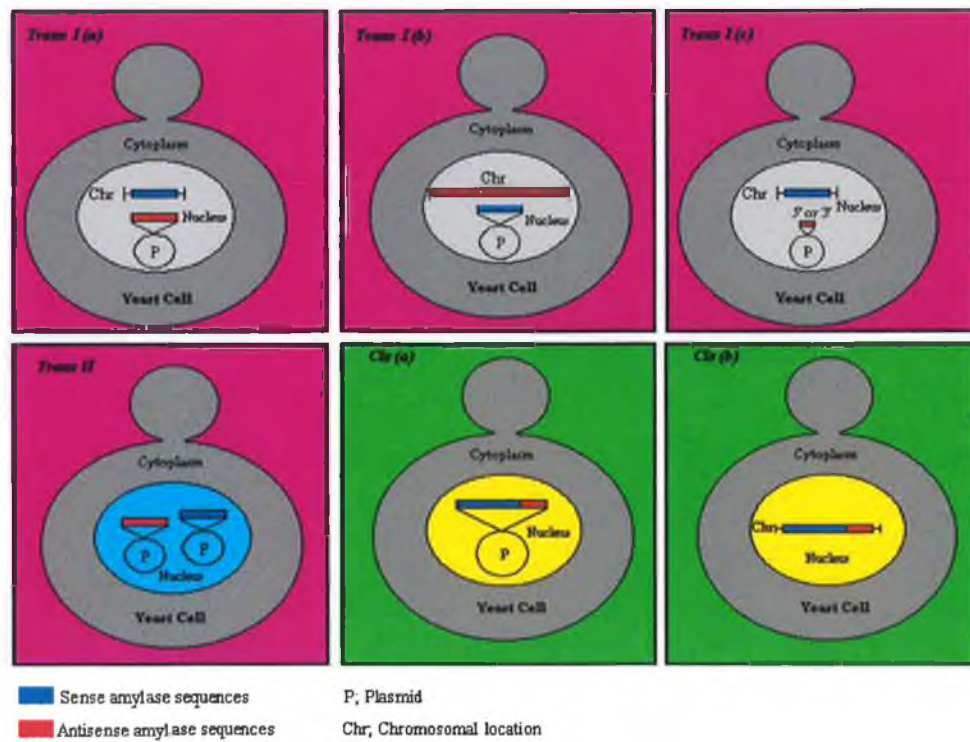
The reaction mix was preincubated at 93°C for 10 minutes. 0.5 ml of culture supernatant (diluted as necessary) was then added to the reaction mix and mixed thoroughly by vortexing. The reaction was incubated for 30 minutes at 93°C. 1 ml of DNS solution (section 2.4.3) was then added to the tube and the reaction was boiled for 15 minutes, allowed to cool, and the OD measured at 640 nm. One unit of enzyme activity was defined as that amount of enzyme producing 0.15 μ mol of reducing sugar in 30 minutes in the standard assay.

2.13.2.1 Preparation of dialysis tubing.

The dialysis tubing was boiled in 10 mM EDTA for 10 minutes, to regulate the pore size, then boiled in distilled water for a further 10 minutes. The tubing can be stored in distilled water at 4°C. Gloves must be worn when handling.

CHAPTER 3

Introduction to Results



3. Introduction to results

Since 1953, much effort has gone into unravelling the complexities of how genetic information is expressed - in particular, how gene expression is controlled. Initially the focus was on simpler organisms, with the long term goal of being able to understand all the complexities of the human body. Ultimately, equipped with such knowledge, together with the aid of current molecular genetic techniques it should be within our reach to control or correct things when they go wrong. Although it might be a long way off from understanding the complexities of an organ like the human brain, this simple single-celled eukaryote, *Saccharomyces cerevisiae*, has been invaluable in the understanding of many complex pathways, such as cell cycle control. With the development of a method for transforming *S. cerevisiae* cells in 1979, a range of techniques were developed which have enabled us to dissect many of the complexities of this simple organism. By the early eighties, this had opened a whole new approach to regulating individual genes - namely antisense technology, and more specific to this project, "antisense RNA technology".

The basis of this technology is the production of an RNA strand with a nucleotide sequence that is complementary to a particular mRNA. The regulation of gene expression by this nucleic acid sequence is thought to depend on the formation of a hybrid duplex between the sense mRNA sequence - the one that bears the genetic information - and the complementary one, the so-called antisense sequence. The formation of this sense-antisense duplex is thought to interfere with protein synthesis at the ribosome. This type of inhibition of gene expression seems to be highly specific. Therefore this mode of regulation may be considered for any gene whose sequence is known. The simplicity, specificity and elegance of this technique make it an extremely attractive one.

The work presented in the following chapters describes the development and use of a reporter system to explore the possibility of using antisense technology as a general mechanism for controlling gene expression in the yeast *S. cerevisiae*. It was hoped that the results of such a study might be useful if one was to consider applying such an approach to control the growth of pathogenic yeasts such as *Candida*. *S. cerevisiae* serves as an attractive host for the development of this model system as it is well characterised genetically and is

amenable to sophisticated molecular genetic manipulations such as precise gene replacement/exchange within individual specified chromosomes (Botstein and Fink, 1988). In addition to having the advantage associated with being unicellular and having a rapid cell division cycle, it is a eukaryote and has been clearly shown to regulate cellular processes (such as cell cycle, signal recognition from the environment and intracellular protein targeting) in a manner similar to that of higher eukaryotes. Lastly, with the complete sequence data now available for *S. cerevisiae*, this can only serve to shed more light both on the variety of mechanisms this organism uses naturally to regulate its' own genetic information, and in turn on what possibilities are likely to be realistic approaches to regulating gene expression from without.

The experiments described in this thesis were designed to test whether antisense could be used to regulate gene expression in *S. cerevisiae*, as has been shown to operate in a range of plant and animal cells. The α -amylase gene was chosen as the reporter gene. Although *S. cerevisiae* has no endogenous α -amylase activity, this gene has been successfully cloned from *Bacillus licheniformis* (Yukki et al., 1985) into *S. cerevisiae* and all detectable α -amylase activity is found in the supernatant; a feature which simplifies the analysis of the product produced. Several convenient assays are available for monitoring α -amylase activity, including a simple plate assay which can be used for screening a large number of samples.

When this work began there were surprisingly very few reported cases of this type of control in *S. cerevisiae* compared with other cell systems (Atkins et al., 1994). Since *S. cerevisiae* was fast being developed as the model eukaryote expected to shed light on a great variety of other cellular processes, it seemed important and worthwhile to investigate this further. When starting this work, my initial goal was to create hybrid yeast cells which contained the α -amylase gene on an expression cassette together with this gene (or part of) in an antisense construct, in such a way so that when the two cassettes were being expressed simultaneously, a much higher proportion of antisense transcripts would be produced.

The work presented can be divided into two sections; the first describes what I have chosen to call *trans* expression systems, the second, *cis* expression

systems. A *trans* experimental system is one in which the sense and antisense genes are transcribed from different transcriptional units. In this thesis the *trans* expression systems are divided into *Trans*-Experiment I and II, *trans*-Experiment I being further subdivided into (a), (b) and (c). In contrast to the *trans* expression systems, in the *cis* experimental systems, both genes are transcribed from the same transcriptional unit, resulting in a single fusion transcript. These expression cassettes are located either episomally (*cis*-Experiment (a)) or chromosomally (*cis*-Experiment (b)).

Trans-Experiment I (a) involved the construction of a yeast strain in which the α -amylase gene, under the transcriptional control of the *AdHI* promoter, was integrated into the yeast genome in low copy number. The dihybrid strain was obtained by the introduction of a full-length episomal antisense gene in higher copy number than the integrated α -amylase gene (Figure 3.1). This antisense fragment was required to work in *trans* to reduce α -amylase gene expression. Although both RNAs were being made by the cell, and there was an excess of anti-mRNA to mRNA, no downregulation of α -amylase gene expression was observed.

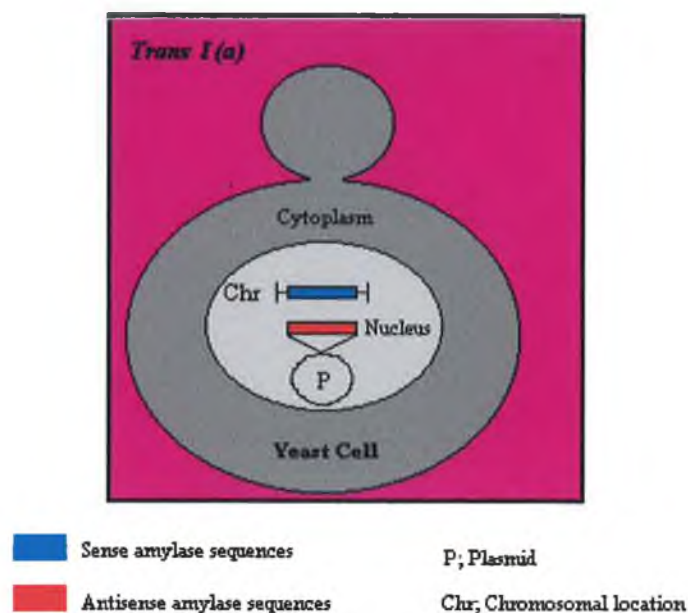


Figure 3.1 Schematic representation of *Trans*-Experiment I (a) with the sense α -amylase gene being chromosomally transcribed and the antisense gene being episomally transcribed.

The result obtained from *trans* I (a), lead to the design of *trans*-Experiment I (b), (Figure 3.2), where the aim was to improve the ratio of antisense to sense mRNA. This design sought to integrate the entire antisense α -amylase gene in multiple copies into the yeasts' ribosomal RNA (rRNA). This region of the genome was chosen as it contains 140 tandem repeats of a 9.1 kb rDNA unit. According to Lopes et al., (1989) who designed a vector for integrating DNA fragments into the rDNA locus of *S. cerevisiae*, it is possible to integrate up to 140 copies of the vector which are stably maintained over long periods of growth under non-selective conditions. It was hoped therefore, that by integrating the entire antisense α -amylase gene under the control of the constitutive *AdHI* promoter, that the copy number and hence the amount of anti-message being made, would be in far greater excess than the mRNA being produced from the sense α -amylase gene, which was cloned into an ARS-CEN vector. ARS-CEN vectors are present in only 1 - 2 copies per cell and once again the sense α -amylase gene was under the control of the *AdHI* promoter to ensure

temporal coincidence of the two RNAs. Activity assays however, demonstrated no downregulation or ablation of α -amylase gene expression. Northern blot analysis revealed that no anti-mRNA was being produced in these constructs; only sense α -amylase mRNA was detected. In some systems where antisense RNA is successful in downregulating or ablating the expression of a particular gene it has sometimes been the case that no anti-mRNA could be detected in the cell.

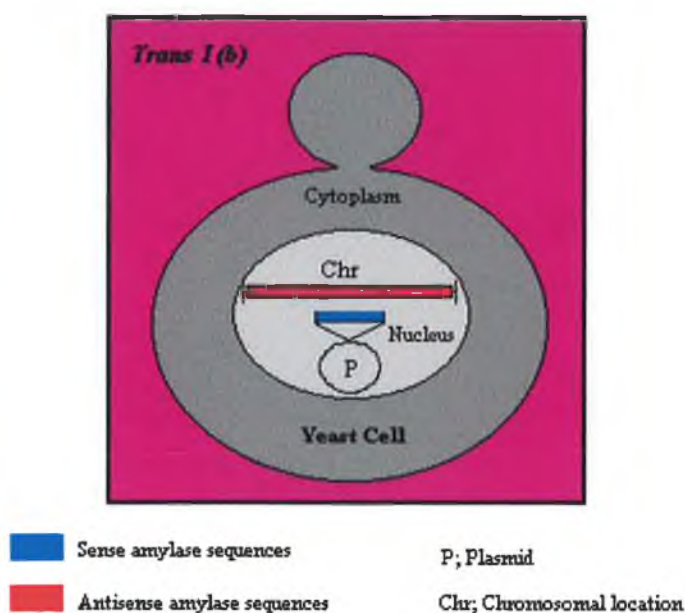


Figure 3.2 Schematic representation of *Trans*-Experiment I (b) with integration of the antisense gene into the rDNA of chromosome XII, the sense gene being expressed episomally.

Perhaps the absence of antisense activity in *trans* I (a) and (b) was caused by a poor interaction of the antisense RNA with the sense RNA. This poor interaction may be due to either different locations of the antisense and sense transcripts within the cell, and/or intramolecular secondary structures, present within one or both of the interacting RNAs, which may have precluded efficient interactions. According to Nellen and Lichtenstein (1997), the difficulty with intramolecular secondary structures can sometimes be overcome by using a

different part of the target gene for the antisense construct. This idea lead to the design of *trans* I (c) (Figure 3.3), where antisense genes targeting certain regions of the α -amylase gene were constructed. These antisense α -amylase sequences were directed to the 5' binding site of the α -amylase gene, to the coding regions, and the 3' end of the gene. Each of these constructs was introduced on a higher copy number plasmid, into an integrated sense α -amylase yeast strain (which was constructed in *trans* I (a)). Similarly, to *trans* I (a), although both sense and antisense transcripts were being made by the cell, and there was a slight excess of antisense to sense RNA, no effect on α -amylase activity was noted.

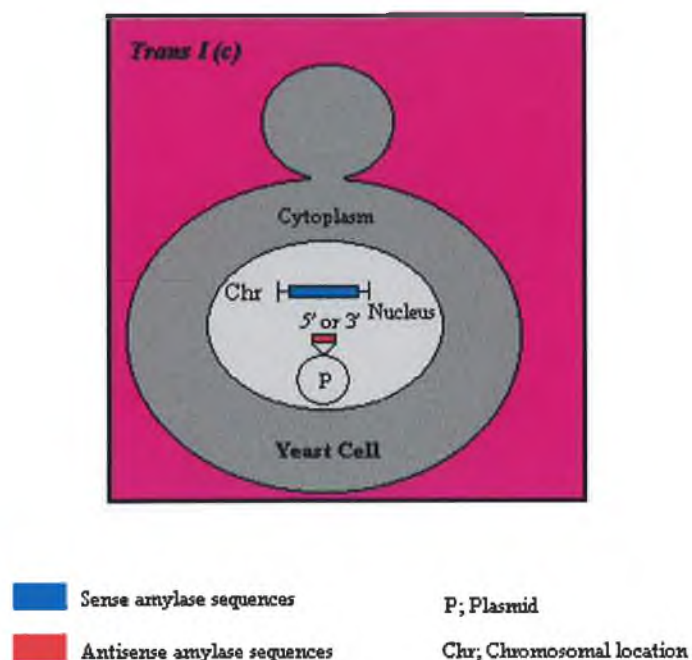


Figure 3.3 Schematic representation of *Trans*-Experiment I (c) with the sense α -amylase gene being chromosomally transcribed and the antisense genes targeting different regions of the sense α -amylase gene being episomally transcribed.

Trans-Experimental system II, designed and described in chapter 5 (Figure 3.4) sought to once again improve the ratios of anti-mRNA to mRNA, in addition to improving the spatial coincidence of the two RNAs. For two RNAs to interact they must be both temporally and spatially coincident. In *trans*

experimental systems I, the sense and antisense genes were under the control of the same promoter which should ensure temporal coincidence, as indeed it did in *trans* experimental system I (a) and (c) as both sense and antisense RNAs were visualised on Northern blots. Spatial coincidence however may have been a different matter as the sense and antisense genes were transcribed from different locations within the cell i.e. from either a chromosomal or episomal location.

Work carried out by Xing et al., (1993) has postulated that the spatial fate of a mRNA can be correlated with gene location, hence in *trans*-experiment II it was decided to introduce both the sense and antisense genes into the yeast cell on episomes, thereby hoping to ensure spatial coincidence. Once again generation of excess antisense RNA to mRNA was an issue. The full-length antisense α -amylase gene was cloned into a high copy episomal vector under the control of the very powerful and inducible *GALI* promoter. The sense gene was introduced on a 2 μ based episomal plasmid under the control of the constitutive *AdHI* promoter. A prerequisite for the introduction of both plasmids into the cell was that each carry a different selection marker. Temporal coincidence could be ensured in this experimental system by the fact that the sense α -amylase gene is being constitutively expressed. Following the addition of galactose to the media the *GALI* promoter is switched on with subsequent expression of the antisense α -amylase gene. An excess of anti-mRNA to mRNA was achieved in this experimental system and activity assays showed a small but significant reduction in α -amylase activity.

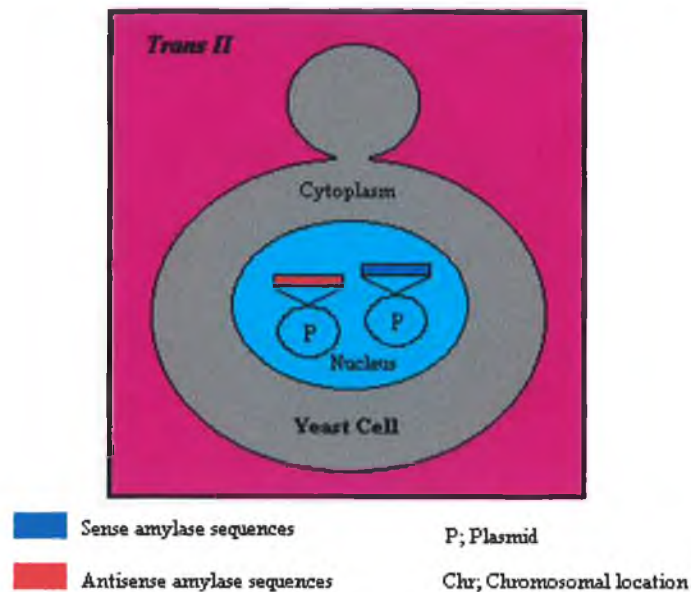


Figure 3.4 Schematic representation of *Trans*-Experiment II where both sense and antisense genes are episomally transcribed.

Finally, temporal and spatial coincidence were the major consideration in the design of the *cis*-Experimental systems (a) and (b) (Figure 3.5). The approach adopted in these systems involved the cloning into a single expression unit part of the α -amylase gene - in antisense orientation - fused to the 3' end of the complete gene. This sense-antisense α -amylase gene sequence was placed under the control of the *AdHI* promoter. This expression cassette was cloned into both a yeast episomal (*cis*-Experiment (a)) and yeast integrating vector (*cis*-Experiment (b)) to examine whether the location of the transcriptional unit would alter the outcome. In contrast to all of the *trans* systems described, where little or no effect on α -amylase activity was observed, using this *cis* configuration 100% inhibition was achieved - irrespective of the location of the transcriptional unit.

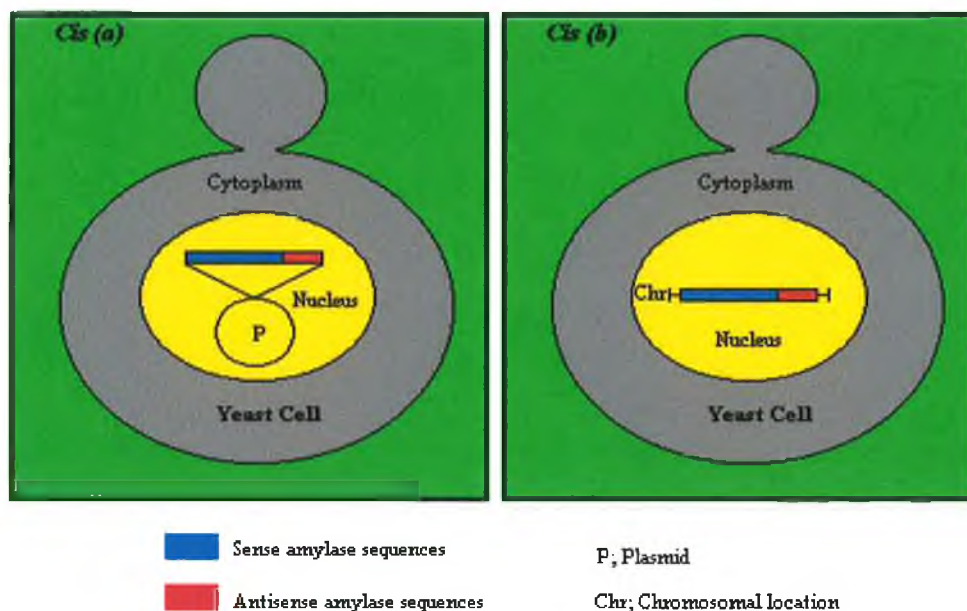
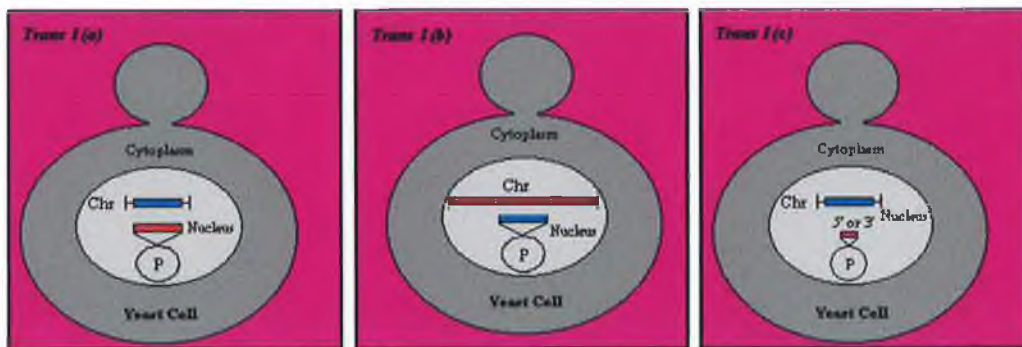


Figure 3.5 Schematic representation of the *cis*-Experimental systems (a) and (b) where the sense and antisense gene were transcribed from either the same episomal (*cis* (a)) or chromosomal (*cis* (b)) location.

The reasoning behind each of these experimental systems and their outcomes will now be discussed in more detail in chapters 4 to 6. An overall discussion and conclusion will be provided in chapter 7.

CHAPTER 4

Trans-Experiment I (a), (b) and (c)



■ Sense amylase sequences
■ Antisense amylase sequences

P; Plasmid
 Chr, Chromosomal location

4. Introduction

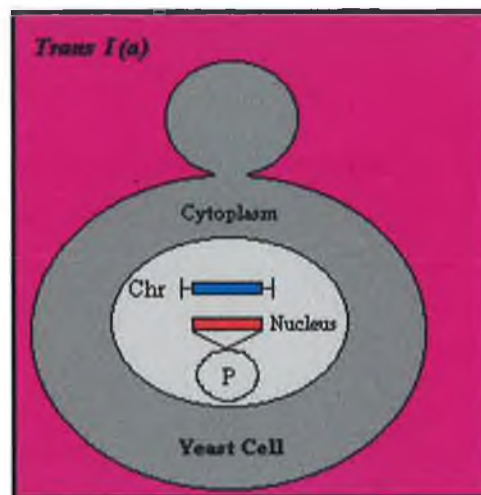
In each of the *trans* experiments discussed in this thesis the envisaged mechanism of action is the interaction of sense and antisense α -amylase RNA strands, resulting in the formation of a double-stranded structure. Although the sequences of the sense and antisense RNAs are complementary, the interaction of these two molecules is dependent upon the accessibility of these two molecules to each other. Taking into consideration that each of the respective molecules has its' own inherent intramolecular structure and also, each RNA may have its' own collection of bound proteins. Both of these factors will affect the strength of the interaction between the sense and antisense RNAs. Therefore, for an interaction to take place, the respective intramolecular structures of the sense and antisense RNAs need to be disrupted to allow formation of this double-stranded bimolecular complex, from this, it follows that for conversion of the target RNA molecules into this complexed form, the antisense RNA must be present in excess molar concentration (Riesner, 1997). *Trans*-Experiments I (a) and (b) were designed in such a way so as to provide this excess of antisense:sense RNA.

A further consideration concerning the intramolecular structures is that it is possible that only certain regions of the sense RNA will be sufficiently free of secondary structure so as to be amenable to hybridization with the antisense RNA. Possibly, the lack of an effect on α -amylase activity in *trans* I (a) was caused by poor interaction of the full-length antisense α -amylase RNA with the sense RNA. If indeed intramolecular secondary structures prevented an efficient interaction of the sense and antisense RNAs, this problem, it has been documented, can sometimes be solved by using a different region of the target gene for the antisense construct (Nellen and Lichtenstein, 1997). *Trans*-Experiment I (c) examined this hypothesis by constructing antisense genes to target different regions of the α -amylase gene.

Trans-Experiments I (a), (b) and (c), were grouped together in this chapter because conceptually, all 3 experimental systems possess a similar type of *trans* configuration i.e. if the antisense genes were transcribed from episomes, then the sense genes were transcribed from a chromosomal location, or vice versa. Furthermore, temporal coincidence was ensured the same way in all of these *trans* experimental systems, by utilizing the same promoter to control

expression of both the antisense and sense genes. Finally, underlying each of these experimental systems was the desire to create a favourable ratio of antisense:sense RNA.

Trans-Experiment I (a)



Sense amylose sequences
 Antisense amylose sequences

P; Plasmid
Chr; Chromosomal location

4.1 TRANS-EXPERIMENT I (a) - INTRODUCTION

Sometimes it is desirable to achieve a very high plasmid copy number for optimal expression of the cloned gene. In other cases it is important that the plasmid copy number is kept low, because either the gene product is deleterious to the host organism at elevated intracellular concentrations or as in my case, to achieve an optimal antisense:sense mRNA ratio. Ultimately in *trans*-Experiment I (a) the aim was to construct a *Saccharomyces cerevisiae* strain containing both sense and antisense α -amylase sequences under the control of the same promoter, *AdHI*, but on separate transcriptional units. Therefore, assuming a similar level of transcription from each copy of these α -amylase sequences, one might expect an optimum suppression of translation of the sense transcripts when the antisense:sense ratio is high. To achieve this objective the sense α -amylase gene was integrated in low copy number into the *ura3-52* locus of the yeast strain DBY746. The experiment was completed by the introduction of a full-length antisense α -amylase gene on a 2 μ based yeast episomal vector which has a copy number of at least 10 plasmids per cell.

The first half of *trans* I (a) describes how the gene was integrated into the yeast chromosome, followed by the characterisation of this integration event. The integrant most appropriate for the introduction of the antisense genes had to fulfil the following criteria: (I) It must have a reasonably low copy number relative to the antisense genes to be introduced into the integrated strain, but (II) it must not possess too low a copy number so as to result in an insensitive assay. The remainder of *trans* I (a) focuses on the construction and analysis of an antisense gene complementary to the entire *Bacillus licheniformis* α -amylase gene.

4.1.1 CONSTRUCTION OF THE *SACCHAROMYCES CEREVISIAE* STRAIN WITH THE SENSE α -AMYLASE GENE LOCATED ON CHROMOSOME V

The yeast chosen for this study was *Saccharomyces cerevisiae* strain DBY746. A number of features associated with this strains' genetic background were important for the successful expression of antisense α -amylase RNA and

sense α -amylase RNA. The *leu 2-3*, *leu 2-112* mutations in these cells could be complemented by the *LEU2* selectable marker located on the YEp-antisense plasmids. The *ura3-52* mutation served as a region for targeting homologous integration of a *URA3* gene from an integrating plasmid thus converting the strain from uracil auxotrophy to uracil prototrophy. Finally, strain DBY746 is [*cir*⁺], which means that this strain contains endogenous 2 μ plasmid DNA which is essential as this plasmid supplies *trans*-acting factors required for the maintenance and amplification of YEp shuttle vectors in yeast cells and it is important for maintaining their episomal state.

Ultimately, I wanted to create a yeast cell expressing both sense and antisense α -amylase genes, ideally with the sense gene in lower copy number. Therefore I set about to integrate the sense copy of the gene into one of the yeasts' chromosomes. This was achieved by transformation of *S. cerevisiae* with a plasmid, containing the α -amylase gene on an expression vector, that lacks an origin of replication. Integration occurs by recombination between homologous regions on the plasmid and the *S. cerevisiae* genome, producing an integrated plasmid. When yeast cells are transformed with a plasmid containing a double-strand break within sequences homologous to the *S. cerevisiae* genome, the following three consequences have been observed: (1) The plasmid transforms at higher frequency, (2) the double-strand ends target the plasmid to integrate at the homologous locus in the genome and (3) the gap is repaired from chromosomal information during plasmid integration (Orr-Weaver and Szostak, 1983).

It was decided to target integration of the sense α -amylase gene into the *ura3-52* locus of *S. cerevisiae* yeast strain DBY746 using a low-copy integrative *S. cerevisiae*/*E. coli* shuttle vector. The vector chosen, pFL34 (Appendix I) is based on the pUC-19 plasmid, its properties include an origin of replication for *E. coli*, the ampicillin resistance gene and a large polycloning site, this vector is also amenable to β -galactosidase α -complementation which allowed for the fast screening of plasmids with α -amylase gene inserts, and most importantly this vector contains the yeast selectable marker *URA3*. See Figure 4.1 for a schematic representation of the probable integration event.

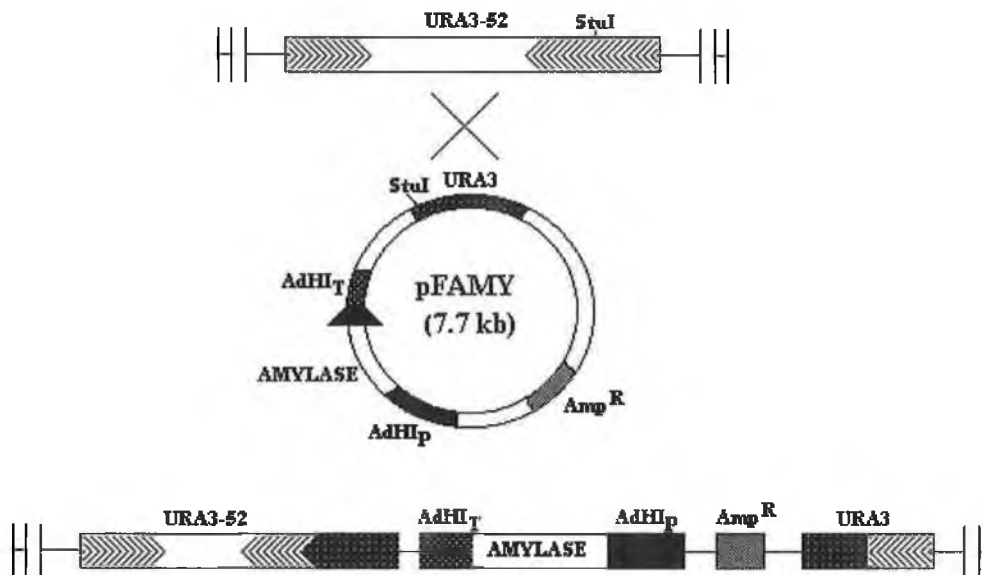


Figure 4.1 Schematic diagram of the probable integration event.

A partial *BamH* I fragment (3.9 kb), containing the *AdHI* promoter, the entire α -amylase gene and the *AdHI* terminator, was cloned into the integrating vector pFL34 to yield the plasmid pFAMY (McMahon, 1995; Figure 4.1). The *URA3* marker on this vector contained a unique *Stu* I site for linearisation of the plasmid and for directing integration of the plasmid to the *ura3-52* locus of DBY746. This *ura3-52* mutation is a non-reverting *URA3* mutation which resulted from a Ty insertion mutation within the coding region of the *URA3* gene, (Rose and Winston, 1984). Integration leads to a duplication of the *URA3* gene and conversion of the strain to uracil prototrophy and it also ensures an identical target copy number of α -amylase genes within all test cells.

Before integration of the plasmid pFAMY (McMahon, 1995) into the *ura3-52* locus of DBY746, this plasmids' construction was confirmed by restriction enzyme analysis (Figure 4.2). The diagnostic restriction enzymes chosen were *Hind* III (chosen to prove the presence of the 1.9 kb α -amylase gene) and *BamH* I (chosen to release the parental vector, pFL34 fragment of 3.8 kb, a band of 2.35 kb which contains the α -amylase gene and the *AdHI* terminator and a band of 1.55 kb containing the *AdHI* promoter). The restriction

enzymes *Stu* I and *Eco*R I were chosen to linearise the plasmid to yield a band size of 7.7 kb.

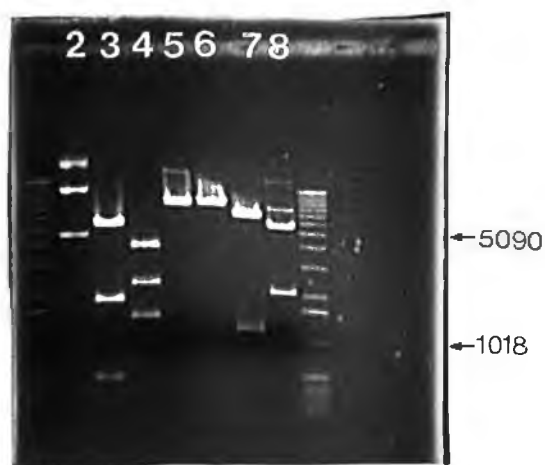


Figure 4.2 Restriction digests of the integrating plasmid, pFAMY.

The sizes of the fragments are shown in base pairs (bp) below.

Lane 1: 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)

Lane 2: pFAMY undigested

Lane 3: pFAMY digested with *Hind* III (5,350; 1,900; 450 bp)

Lane 4: pFAMY digested with *Bam*H I (3,800; 2,350; 1,550 bp)

Lane 5: pFAMY digested with *Stu* I (7,700 bp)

Lane 6: pFAMY digested with *Eco*R I (7,700 bp)

Lane 7: pFAMY digested with *Sal* I (6,480; 1,220 bp)

Lane 8: pFAMY digested with *Kpn* I (5,560; 2,140 bp)

Lane 9: 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)

Figure 4.2 does indeed confirm the construction of the integrating plasmid pFAMY. The *Hind* III digest (lane 3, Figure 4.2) produced the expected fragment sizes of 5.35, 1.9 and 0.45 kb. The *Bam*H I digest (lane 4, Figure 4.2) also produced fragments of the expected size, these being 3.8, 2.35 and 1.55 kb respectively. The *Stu* I and *Eco*R I restriction digests (lanes 5 and 6, Figure 4.2) linearised the plasmid to produce a 7.7 kb fragment. The *Sal* I and *Kpn* I digests

(lanes 7 and 8, Figure 4.2) also produced the expected restriction fragments and are a further confirmation of this plasmids construction.

Following transformation of *S. cerevisiae* DBY746 cells with pFAMY DNA, integrants were selected for, by plating on selective media supplemented with all the amino acids (see section 2.3.6) minus uracil.

4.1.1.1 Characterisation of the integration event.

4.1.1.1.1 Growth on selective plates

Only the transformants which received the *URA3* gene from the plasmid pFAMY would be capable of growth on uracil minus minimal media. As the plasmid, pFAMY does not contain an origin of replication, growth of transformants on media lacking uracil would strongly suggest the presence of the integrated plasmid. As DBY746 is auxotrophic for both uracil and leucine, the transformants were plated on minimal media containing only leucine. All *ura*⁺ transformants were tested for α -amylase activity by plating on selective media containing 2% starch. According to the predicted mechanism of integration, if the *ura* gene is integrated, one would expect the α -amylase gene also to be inserted into the chromosome. As expected all the transformants tested exhibited α -amylase activity, as demonstrated by formation of a clear halo around the colonies as a result of starch degradation (Figure 4.3).

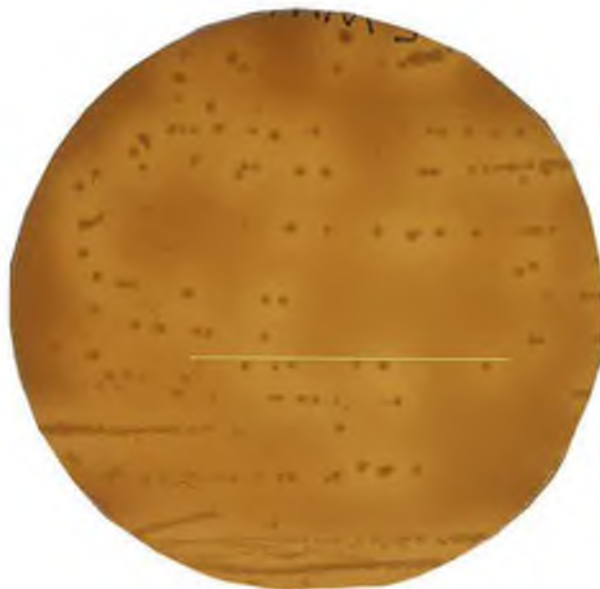


Figure 4.3 Minimal media plate containing 2% starch showing haloes surrounding α -amylase positive clones after flooding the plate with iodine vapour.

4.1.1.1.2 *Stability of the Integrated Strains*

To examine the mitotic stability of the *ura3-52* integrated exogenous DNA sequences, the yeast transformants were grown initially in minimal medium. Following this, they were transferred to YEPD media where they were kept growing for over 200 generations by repeatedly transferring them into fresh YEPD medium. The percentage of cells with the *ura*⁺ marker was then determined by plating single colonies on YEPD and checking for the loss of the plasmid associated marker by replica plating onto selective medium. The episomal plasmid pAAMY-01 was used as a control. The number of *leu*⁺ cells harbouring the YE_p-type plasmid pAAMY-01 which contains the α -amylase gene, showed a decrease over the first few generations and dropped to approximately 10.0% after 50 generations. In contrast, no decrease in the number of *ura*⁺ cells was observed among the *ura3-52* integrants even after 150 generations. These results indicated that the *ura3-52* integrated sequences were

maintained very stably during mitotic growth in rich medium. The high stability of the *ura*⁺ phenotype was also another indication that integration into the yeast genome had occurred. A number of these stable integrants that will appear in subsequent experiments have been given the following names; I₂, SI, SII and SIV.

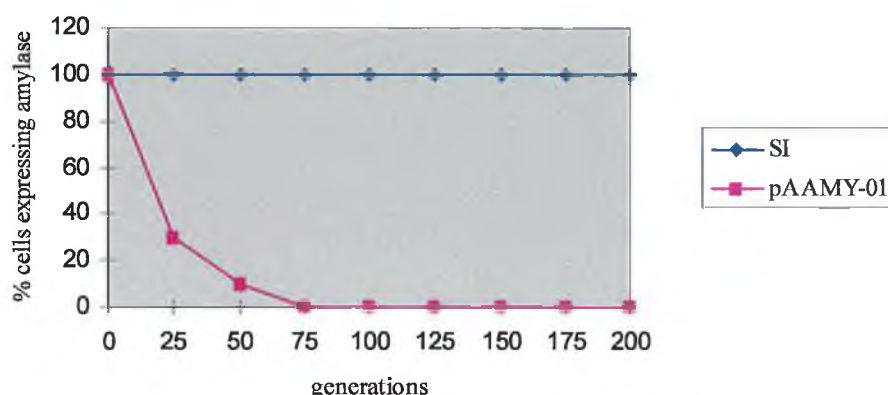


Figure 4.4 Graph of the stability of one of the pFAMY transformants, SI, together with that of the episomal plasmid pAAMY-01.

4.1.1.1.3 *Development of a semi-quantitative well assay for measuring α -amylase activity.*

The α -amylase plate assay was developed for detecting α -amylase activity in the supernatants of transformants. This involved making up plates consisting of 2% starch and 2% agar made up to volume in 0.1 M sodium phosphate buffer, pH 6.9. Wells were then removed from the firm agar using sterile pipette tips (Sarstedt 70.760/002, 10-100 μ l). Following growth, aliquots of growth medium (supernatant) were then loaded into the wells and the plates were incubated at 37°C for 16 hours. To estimate the amount of α -amylase produced the halo size was compared to a standard curve produced for α -amylase over the concentration range 0.00625 units to 100 units of amylase activity (Figure 4.5). The amylase used was from a commercial source (Sigma *B. licheniformis*

amylase Type X11A). One unit of amylase activity is defined as the amount necessary to liberate 1mg of maltose from starch in 3 minutes at pH 6.9 and 20°C. Figures 4.6 and 4.7 show the well assays used for the construction of the calibration curve which is shown in figure 4.5.

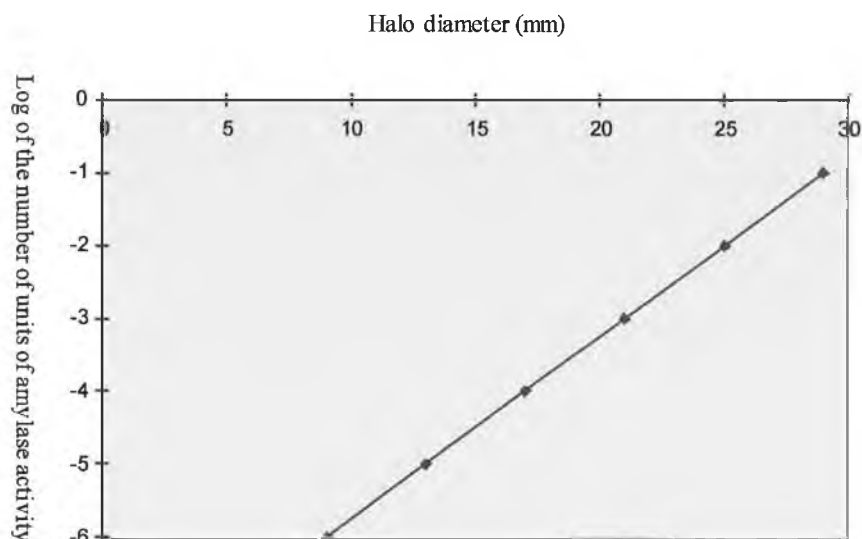


Figure 4.5 Standard Curve for Well Assay.

The plate assay was used as a semi-quantitative tool for the measurement of α -amylase activity. The haloes were visualised by either iodine vapour (Figure 4.6) or starch precipitation (Figure 4.7). In the former the plates were exposed to iodine vapour for 30 - 60 seconds and with the latter the plates were incubated at 4°C overnight in order to precipitate any undegraded starch, the α -amylase producing supernatants were surrounded by distinct clear haloes, while non-amylolytic supernatants stained dark blue with the iodine vapour. Both assay procedures had similar sensitivity, but the definition of the haloes was superior with the iodine assay.



Figure 4.6 Well Assay plates after flooding with iodine vapour.



Figure 4.7 Well Assay plates after overnight incubation at 4°C.

Another advantage of this plate assay is that unlike the DNS method (section 2.13.2) for measuring α -amylase activity, the samples do not need to be dialysed prior to analysis.

4.1.1.1.4 Southern Blot Analysis I - Proof of integration

This particular experiment was designed to demonstrate that integration into the yeasts' chromosomal DNA had taken place. Genomic DNA extracted from several independently isolated integrants I₂, SI, SII and SIV was digested with a restriction endonuclease which cuts outside the plasmid DNA. This should yield a single band of high molecular weight on the gel when Southern blotted and probed with ³²P-labelled *EcoR* I linearised pFAMY if integration has been successful. If, although unlikely, integration into the genome had not occurred one would expect to see bands identical in size to undigested pFAMY plasmid.

To ascertain what restriction enzymes do not cut within the plasmid pFAMY, firstly the sequence of the parental plasmid pFL34 was obtained (accession no. X70479) from GenBank Then this sequence and the sequence of the *B. licheniformis* α -amylase gene were analysed using SeqAid which provided information on restriction endonucleases which do not cut within these sequences. From the data retrieved, the restriction endonucleases *Mlu* I and *Xho* I neither of which cut within the plasmid were chosen.

The *Mlu* I data indicated integration had occurred by the appearance of a single band which was of high molecular weight for each of the four integrants digested with *Mlu* I (lanes 10 to 13, Figure 4.9). Lane 8 of the gel shown in Figure 4.8 clearly shows that the restriction enzyme chosen did not cut within the plasmid pFAMY, the banding pattern is identical to lane 2 which contains undigested pFAMY plasmid DNA. A further point which confirms the *Mlu* I data is the observation that in the undigested lanes, (lanes 4 to 7, Figure 4.9), where no restriction enzyme was used, there appeared a high molecular weight band comigrating with the genomic DNA. If integration had not occurred one would expect a banding pattern as for pFAMY undigested DNA (lane 2, Figure 4.8).

Similar analysis was carried out using the enzyme *Xho* I which confirmed the above results (data not shown).

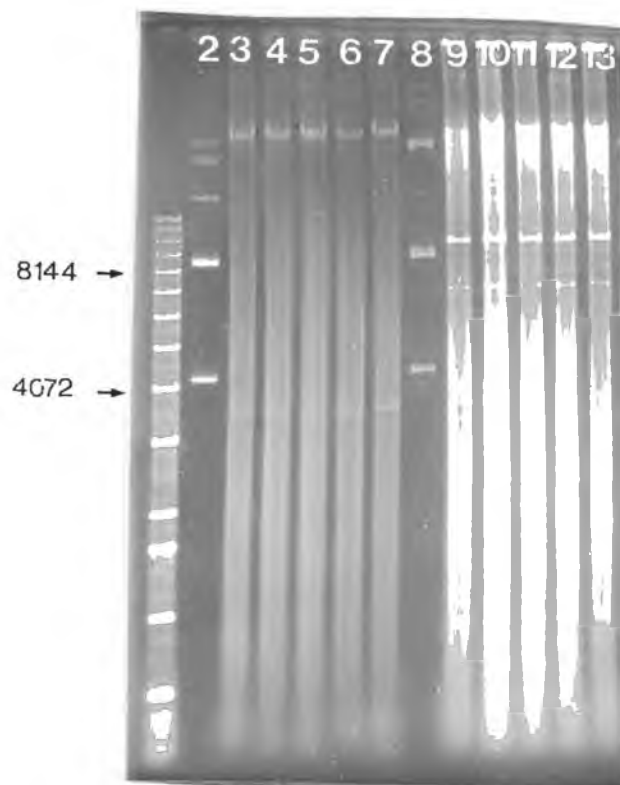


Figure 4.8 Agarose gel of genomic DNA from DBY746, the integrants I₂, SI, SII and SIV and the plasmid pFAMY unrestricted and restricted with *Mlu* I.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** DBY746 undigested
- Lane 4:** I₂ undigested
- Lane 5:** SI undigested
- Lane 6:** SII undigested
- Lane 7:** SIV undigested
- Lane 8:** pFAMY digested with *Mlu* I
- Lane 9:** DBY746 digested with *Mlu* I
- Lane 10:** I₂ digested with *Mlu* I
- Lane 11:** SI digested with *Mlu* I
- Lane 12:** SII digested with *Mlu* I
- Lane 13:** SIV digested with *Mlu* I

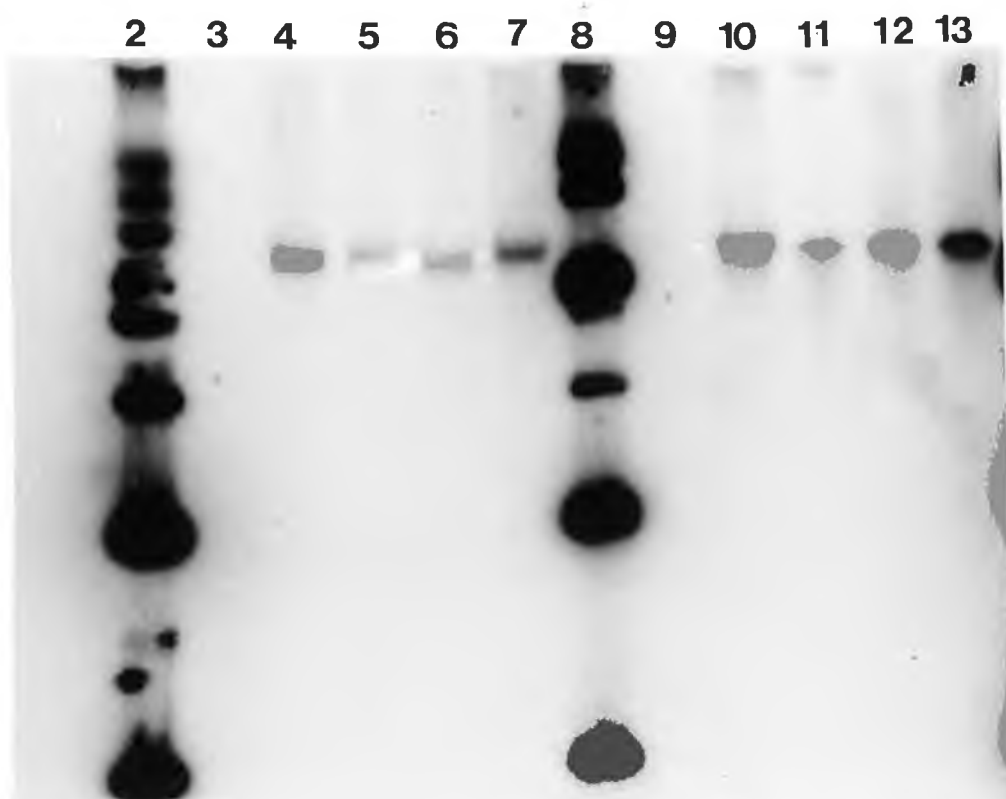


Figure 4.9 Southern Blot of genomic DNA from DBY746, the integrants I₂, SI, SII and SIV and the plasmid pFAMY unrestricted and restricted with *Mlu* I and probed with ³²P-labelled *Eco*R 1 linearised pFAMY.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** DBY746 undigested
- Lane 4:** I₂ undigested
- Lane 5:** SI undigested
- Lane 6:** SII undigested
- Lane 7:** SIV undigested
- Lane 8:** pFAMY digested with *Mlu* I
- Lane 9:** DBY746 digested with *Mlu* I
- Lane 10:** I₂ digested with *Mlu* I
- Lane 11:** SI digested with *Mlu* I
- Lane 12:** SII digested with *Mlu* I
- Lane 13:** SIV digested with *Mlu* I

4.1.1.1.5 Southern blot analysis II - A single or tandem integration?

To examine further the fate of pFAMY in the *S. cerevisiae* transformants, with respect to whether integration had occurred as a single or multiple integration event, genomic DNA was extracted, digested with various enzymes, electrophoresed, Southern blotted and probed with *EcoR* I linearised ³²P-labelled pFAMY. Initially the restriction enzymes chosen were *Hind* III, *EcoR* I and *Sal* I as these enzymes cut within both the plasmid and Ty element. Since some sequence data for the 5' end of the Ty element was available at the time that these experiments were being designed it was possible to predict the expected sizes of the *Hind* III, *Sal* I and *EcoR* I 5' flanking fragments. *Kpn* I was chosen as it cuts within the plasmid but not within the Ty element. The presence of junction fragments of the predicted size would provide proof of the integration of the plasmid at the *ura3-52* locus. If integration had occurred by a single cross-over event as outlined in Figure 4.10 the following fragment sizes (in base pairs) would be observed (Table 4-1 below). Some of the junction fragments are of unknown size as the sequence 3' to the integration site had not been determined when these experiments were being carried out.

Table 4-1 Expected fragment sizes (in base pairs, bp) following restriction if integration had occurred by a single cross-over event.

Restriction enzyme	Plasmid fragments (bp)	Junction fragments (bp)
<i>Hind</i> III	1,900; 450	2,750; ND*
<i>EcoR</i> I	*****	6,600; ND*
<i>Sal</i> I	1,220	2,740; ND*
<i>Kpn</i> I	2,150	ND*

*ND: not determined.

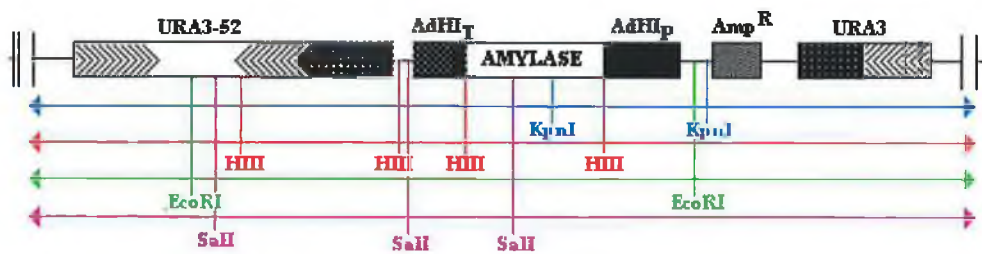


Figure 4.10 Schematic representation of a single integration event with restriction fragments highlighted. (Restriction site *Hind* III is indicated as HIII.)

Furthermore, due to the fact that about 50% of the resulting transformants in integration experiments can result in double or multiple tandem integrations (Orr-Weaver and Szostak, 1983) the expected band sizes for a tandem integration event shown diagrammatically in Figure 4.11 were also calculated. The following fragment sizes (in base pairs, Table 4-2) would be expected:

Table 4-2 Expected fragment sizes (in base pairs, bp) following restriction if a tandem integration event had occurred.

Restriction enzyme	Plasmid fragments (bp)	Junction fragments (bp)
<i>Hind</i> III	5,300; 1,900; 450	2,750; ND*
<i>EcoR</i> I	7,700	6,600; ND*
<i>Sal</i> I	6,480; 1,220	2,740; ND*
<i>Kpn</i> I	5,560; 2,140	ND*

*ND: not determined.

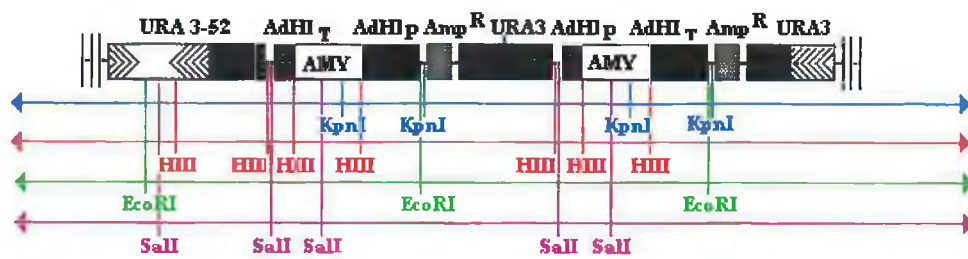


Figure 4.11 Schematic representation of a multiple (tandem) integration event with restriction fragments highlighted. (Restriction site *Hind* III is indicated as HIII.)

Finally, the occurrence of the integration event alone, irrespective of single or tandem integration events could once again be confirmed in this experiment by the presence of a single high molecular weight band comigrating with the genomic DNA on a Southern blot of undigested DNA probed with ³²P-labelled *Eco*R I linearised pFAMY. In contrast, if the α -amylase gene had remained episomally, bands characteristic of pFAMY should be detected.

The results of the Southern blotting experiment can be seen in Figure 4.13. Possibly the best choice of enzyme to demonstrate the type of integration event was *Eco*R I. It appears that a multiple integration event was the most likely event for SI, SII and SIV, as both plasmid and junction fragments were seen on the Southern blot (lane 13, 16 and 19, Figure 4.13) corresponding to band sizes of 7.7 kb, (a plasmid band indicating multiple integration), 6.6 kb (a junction fragment) and a much larger band of indeterminate size (which may be the second junction fragment).

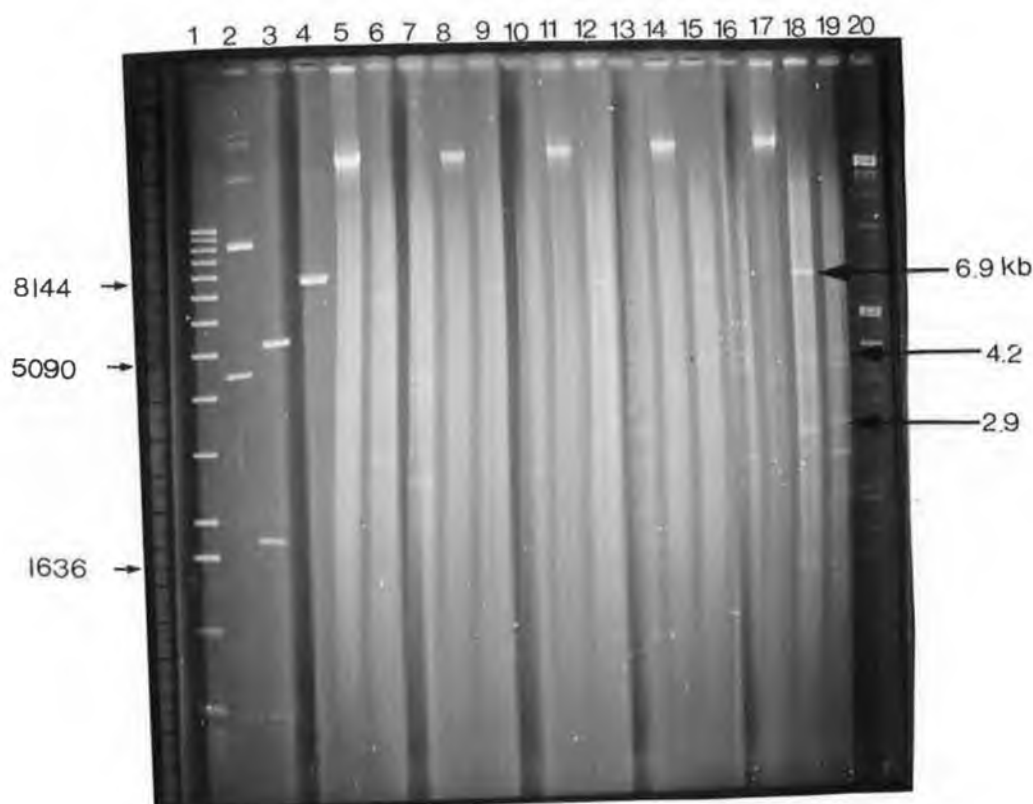


Figure 4.12 Agarose gel of genomic DNA from DBY746 and the integrants I_2 , SI, SII and SIV, electrophoresed unrestricted and restricted with *Hind* III and *EcoR* I.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** pFAMY digested with *Hind* III (5,350; 1,900; 450 bp)
- Lane 4:** pFAMY digested with *EcoR* I (7,700 bp)
- Lane 5:** DBY746 undigested
- Lane 6:** DBY746 digested with *Hind* III
- Lane 7:** DBY746 digested with *EcoR* I
- Lane 8:** I_2 undigested
- Lane 9:** I_2 digested with *Hind* III
- Lane 10:** I_2 digested with *EcoR* I
- Lane 11:** SI undigested
- Lane 12:** SI digested with *Hind* III
- Lane 13:** SI digested with *EcoR* I
- Lane 14:** SII undigested
- Lane 15:** SII digested with *Hind* III

Lane 16: SII digested with *EcoR* I
Lane 17: SIV undigested
Lane 18: SIV digested with *Hind* III
Lane 19: SIV digested with *EcoR* I
Lane 20: λ DNA *Hind* III/*EcoR* I digested (21,226; 5,148; 4,973; 4,277; 2,027; 1904;
1,584; 1,330; 983; 831; 564 bp)

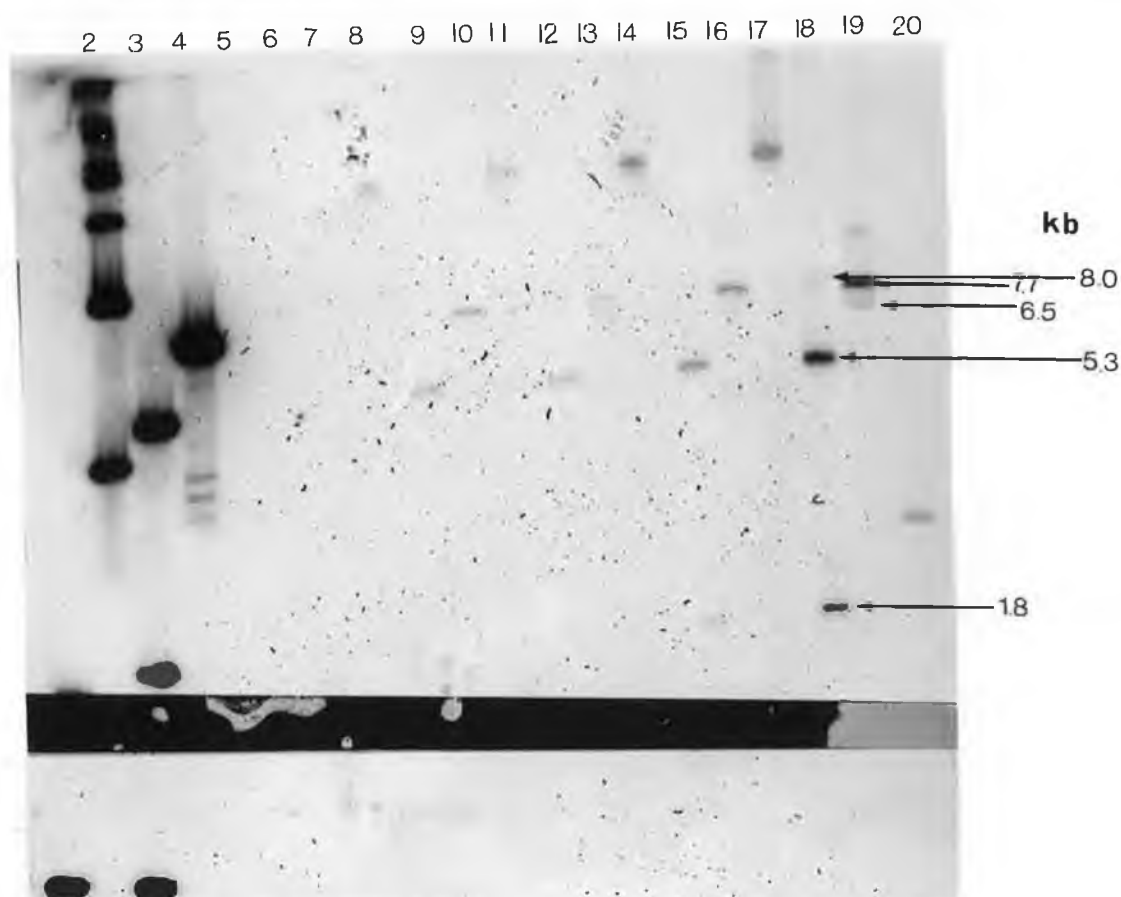


Figure 4.13 Southern blot of genomic DNA from DBY746 and the integrants I₂, SI, SII and SIV, electrophoresed unrestricted and restricted with *Hind* III and *EcoR* I, and probed with ³²P-labelled *EcoR* I linearised pFAMY.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** pFAMY digested with *Hind* III (5,350; 1,900; 450 bp)
- Lane 4:** pFAMY digested with *EcoR* I (7,700 bp)
- Lane 5:** DBY746 undigested
- Lane 6:** DBY746 digested with *Hind* III
- Lane 7:** DBY746 digested with *EcoR* I
- Lane 8:** I₂ undigested
- Lane 9:** I₂ digested with *Hind* III
- Lane 10:** I₂ digested with *EcoR* I
- Lane 11:** SI undigested
- Lane 12:** SI digested with *Hind* III
- Lane 13:** SI digested with *EcoR* I
- Lane 14:** SII undigested
- Lane 15:** SII digested with *Hind* III
- Lane 16:** SII digested with *EcoR* I
- Lane 17:** SIV undigested

Lane 18: SIV digested with *Hind* III
Lane 19: SIV digested with *Eco*R I
Lane 20: λ DNA *Hind* III/*Eco*R I digested (21,226; 5,148; 4,973; 4,277; 2,027; 1904; 1,584; 1,330; 983; 831; 564 bp)

The lane containing DBY746 DNA restricted with *Eco*R I (lane 7, Figure 4.13) highlighted two faint bands of 10.0 kb and 8.0 kb respectively, (seen on the autoradiograph but not on the photograph, Figure 4.13). The presence of these bands was most likely due to the uracil portion of the plasmid hybridising with the uracil gene in the yeast genomic DNA. This result was in accordance with the findings of Canavan (1994), when the same DNA was restricted with *Eco*R I but probed with a 32 P-labelled uracil probe. Also because integration was directed to the *ura3-52* locus of DBY746 and because the band of 10 kb appears to have shifted in the integrated strains one could take this to be a further proof of integration.

The *Hind* III digest data of the four integrants were somewhat incomplete. If multiple integration had occurred one would expect to see all of the plasmid bands (5.3 kb, 1.9 kb and 0.48 kb) lighting up plus two junction fragments. What was observed however was the 5.3 kb plasmid fragment lighting up for all four integrants (lanes 9, 12, 15 and 18, Figure 4.13). This band should only light up if a tandem integration event had occurred (see Figure 4.11). With SI, SII and SIV (lanes 12, 15 and 18, Figure 4.13) one can also see the 1.9 kb plasmid fragment and in the case of SIV (lane 18, Figure 4.13) can also make out the 0.45 kb plasmid fragment although it was very faint and it did not photograph well. The fact that this small band of 0.45 kb was not visualised for all the integrants may be due to the fact that not enough genomic DNA had been loaded on the gel. A band at 8.0 kb was seen clearly for SIV (lane 18, Figure 4.13) and this possibly represents one of the junction fragments and the same band but of lower intensity was also seen in the case of SI (lane 12, Figure 4.13) as expected. Also the junction fragment of 2.7 kb could be seen for SIV (lane 18, Figure 4.13). Once again the fact that this fragment (which would be present only once) was not visualised for all of the integrants could be due to not enough genomic DNA being loaded. However conclusive proof of multiple (tandem)

integration for all 4 integrants from this *Hind* III data was the presence of the 5.3 kb band which would only light up in the case of a multiple integration event.

The *Sal* I digests further indicate tandem integration for all four integrants by the presence of the 6.5 kb plasmid bands (see Figure 4.11), which should only be visualised if a tandem integration event had occurred (lanes 9, 12, 15, and 18, Figure 4.14). The 1.2 kb band was also there its intensity being weak in the case of I₂, SI and SII, (lanes 9, 12 and 15, Figure 4.14), but clearly visible for SIV (lane 18, Figure 4.14). In all four integrants there was a high molecular weight band observed upon digestion with *Sal* I. This band also occurred when DBY746 was digested with *Sal* I which suggests that the uracil portion of the plasmid probe pFAMY is hybridising to uracil genomic DNA. This band may represent the junction fragment which is of indeterminate size because of its high molecular weight >23 kb (lanes 6, 9, 12 and 15, Figure 4.14).

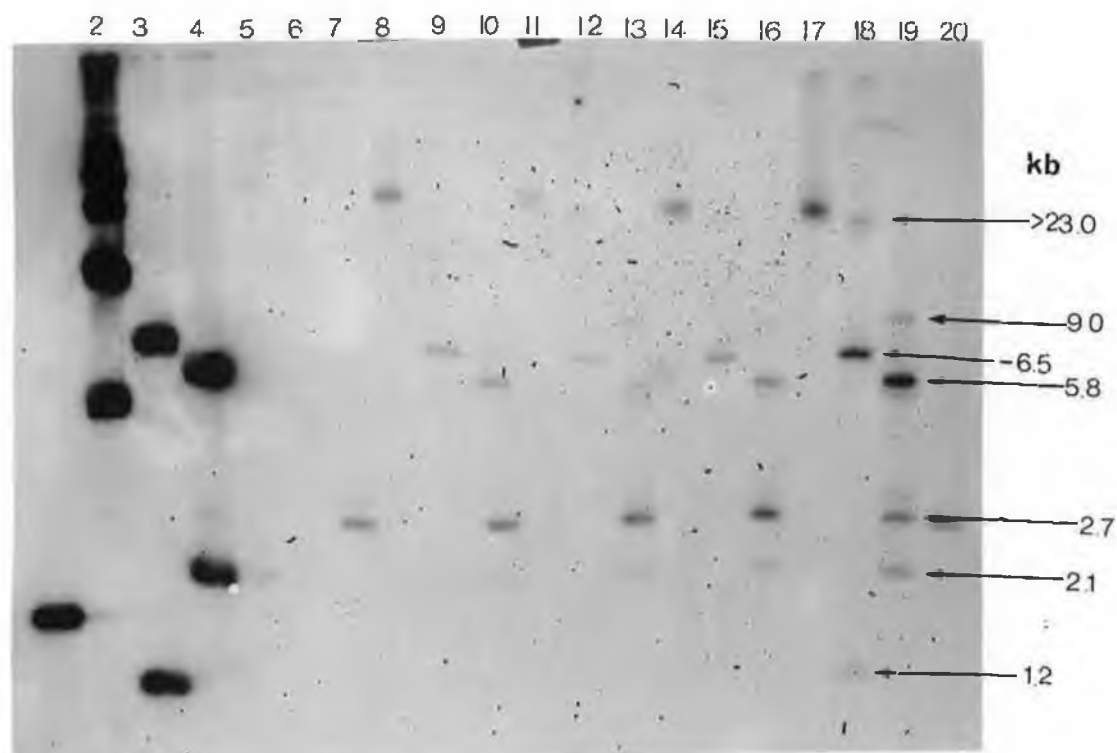


Figure 4.14 Southern blot of genomic DNA from DBY746 and the integrants I₂, SI, SII and SIV, electrophoresed unrestricted and restricted with *Sal* I and *Kpn* I, and probed with ³²P-labelled *Eco*R I linearised pFAMY.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** pFAMY digested with *Sal* I (6,480; 1,220 bp)
- Lane 4:** pFAMY digested with *Kpn* I (5,560; 2,140 bp)
- Lane 5:** DBY746 undigested
- Lane 6:** DBY746 digested with *Sal* I
- Lane 7:** DBY746 digested with *Kpn* I
- Lane 8:** I₂ undigested
- Lane 9:** I₂ digested with *Sal* I
- Lane 10:** I₂ digested with *Kpn* I
- Lane 11:** SI undigested
- Lane 12:** SI digested with *Sal* I
- Lane 13:** SI digested with *Kpn* I
- Lane 14:** SII undigested
- Lane 15:** SII digested with *Sal* I
- Lane 16:** SII digested with *Kpn* I
- Lane 17:** SIV undigested
- Lane 18:** SIV digested with *Sal* I

Lane 19: SIV digested with *Kpn* I
Lane 20: λ DNA *Hind* III/*Eco*R I digested (21,226; 5,148; 4,973; 4,277; 2,027; 1904; 1,584; 1,330; 983; 831; 564 bp)

Finally the *Kpn* I digests also indicate tandem integration, as the plasmid bands of 5.56 kb and 2.14 kb are clearly visible for all four integrants along with a very strong band at approximately 2.6 kb (which was also present in the unintegrated DBY746, lane 7 Figure 4.14) and another band at 9.0 kb once again this may possibly represent a junction fragment (lanes 10, 13, 16 and 19, Figure 4.14).

From the results of the above experiment one can conclude that in all cases a tandem integration event was the outcome of all four transformations with the integrating vector pFAMY.

4.1.1.1.5.1 Analysis of the Restriction Spectra of genomic DNA extracted from Saccharomyces cerevisiae and restricted with Hind III and EcoR I (Figure 4.12).

Strong characteristic bands can be readily identified within the genomic DNA of *S. cerevisiae* on an agarose gel after restriction digestion with certain restriction enzymes. The proper evaluation of these restriction spectra is important for many experiments, if only to see that restriction digestion went to completion. In particular, correct interpretation of the Southern blotting data for integrated genomic DNA samples is dependent on adequate restriction digestion of the original samples. However complete digestion of the sample is ideal.

In many instances the bands which stand out following restriction digestion are due to repeated sequences. In fact, up to 15% of the nuclear DNA consists of repeated sequences, some of which can be seen as weak or strong bands after cleavage of the DNA with restriction endonucleases and separation of the fragments in agarose gels. There are four classes of repeated DNA (rDNA, 2 μ m plasmid, Ty elements, telomeric Y' sequences) within the genome of *S. cerevisiae* which are the origin of most of the bands seen in the restriction spectra after electrophoresis on a 0.7% agarose gel stained with ethidium bromide. The ribosomal DNA consists of a cluster of usually 100 to 140 tandem copies of a

9.08 kb repeat unit. The 2- μ m plasmid is present in 50 to 100 copies in many but not all *S. cerevisiae* strains. The mobile elements Ty1 and Ty2 are usually present in 30 - 35 copies dispersed throughout the genome. Ty3 and Ty4 are present in only 1 - 4 copies. These Ty elements can be lost from the chromosome by homologous recombination between their 0.34 kb terminal direct repeats. Single copies of the terminal repeats remain at the previous location of the Ty element. This explains the many copies of solo δ sequences (50 - 100), solo σ sequences (20 -30), and solo τ sequences (15 - 25) found in all strains even those with low Ty copy number. The fourth class of repeated DNA visible in restriction spectra consists of the 6.7 kb telomeric Y' sequences. These are present as single copies or short clusters of 2 - 4 copies at the ends of almost all 16 chromosomes, with about 45 copies per haploid genome. Approximately 85% of the sequences in yeast chromosomes are unique. Some genes exist in pairs, for example genes for histones, α -tubulin, translation elongation factor 1 α , mating pheromones, several ribosomal proteins and some glycolytic enzymes (Philippsen et al., 1991).

The *EcoR* I restriction spectra of DBY746 (lane 7, Figure 4.12) and the four integrants (lanes 10, 13, 16 and 19, figure 4.12) are dominated by ribosomal DNA and 2- μ m bands. Six bands are clearly distinguishable, four of these bands represent the 2- μ m plasmid the other two arise from cleavage of the ribosomal DNA. The 4.2 kb, 3.9 kb, 2.5 kb and 2.1 kb fragments arise from the 2- μ m plasmid DNA. The 2.5 kb band is in fact a doublet which comigrates with a rDNA fragment. The bands at 2.9 kb and 1.9 kb represent 25s rRNA and 18s rRNA bands respectively.

The *Hind* III digests (lanes 6, 9, 12, 15 and 18, Figure 4.12) contain a strong band at 6.9 kb which originates from the rDNA, as does the band at 2.6 kb. The remaining four bands represent the 2- μ m plasmid at 4.2 kb, 2.7 kb, 2.1 kb and 1.3 kb respectively. In the undigested lanes (lanes 5, 8, 11, 14 and 17, Figure 4.12) one band stands out clearly, at approximately 3.6 kb this band most likely represents the covalently closed circular forms of the 2- μ m plasmid DNA. From viewing the banding patterns on the agarose gel after restriction digestion with both *EcoR* I and *Hind* III it is clear that the restriction digestion went to completion.

4.1.1.1.6 *Southern Blot analysis III - Further proof of integration*

Genomic DNA from the four integrated strains I₂, SI, SII and SIV was once again digested with *Sal* I but this time the probe used was the plasmid pRB149 (Appendix II) which contains the *URA3* gene and β -actin gene from *S. cerevisiae*. The probe was radiolabelled with ³²P. Analysis of Figure 4.16 shows DBY746 (lane 9) hybridising to the probe at a position of approximately 23.0 kb (the uracil specific portion of the probe) and a band at approximately 9.4 kb which according to Canavan (1994), is the β -actin band. In the integrated strains (lanes 10, 11, 12 and 13, Figure 4.16) there is a shift in size of the 23.0 kb band for all four integrants offering further proof of an integration event. This band cannot be accurately sized as it is of high molecular weight. Also the 6.5 kb plasmid band, which incidentally contains the uracil gene is present in the lanes of the integrants only, (lanes 10, 11, 12 and 13, Figure 4.16), as expected.

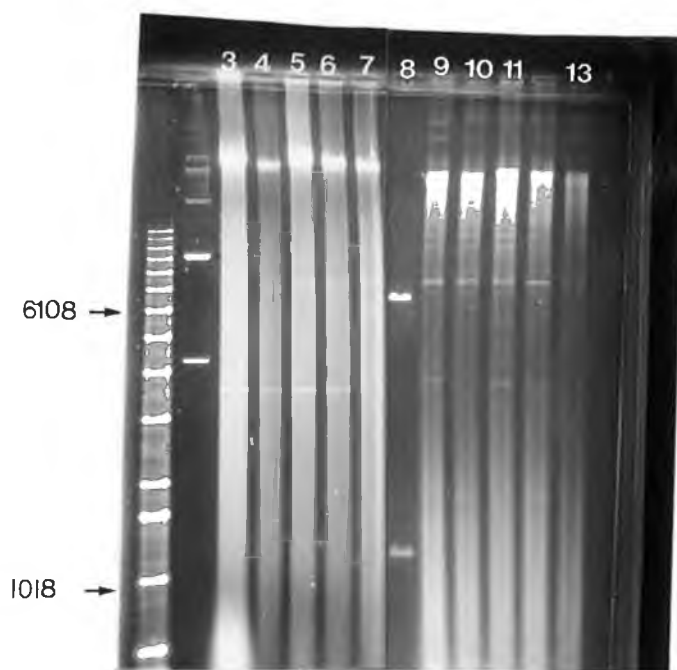


Figure 4.15 Agarose gel of genomic DNA from DBY746 and the integrants I₂, SI, SII and SIV, electrophoresed unrestricted and restricted with *Sal* I.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** DBY746 undigested
- Lane 4:** I₂ undigested
- Lane 5:** SI undigested
- Lane 6:** SII undigested
- Lane 7:** SIV undigested
- Lane 8:** pFAMY digested with *Sal* I (6,480; 1,220 bp)
- Lane 9:** DBY746 digested with *Sal* I
- Lane 10:** I₂ digested with *Sal* I
- Lane 11:** SI digested with *Sal* I
- Lane 12:** SII digested with *Sal* I
- Lane 13:** SIV digested with *Sal* I

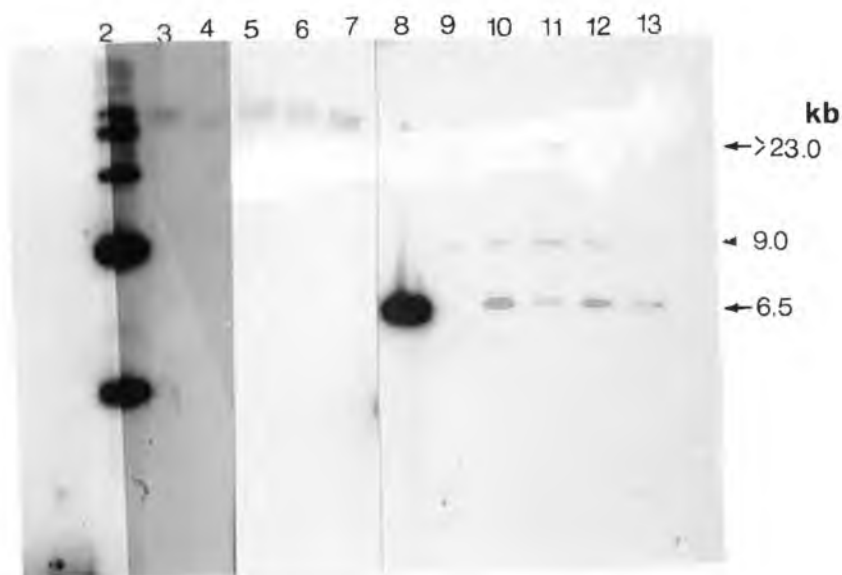


Figure 4.16 Southern Blot of genomic DNA from DBY746 and the integrants I₂, SI, SII and SIV, electrophoresed unrestricted and restricted with *Sal* I and probed with a uracil and β -actin probe.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** pFAMY undigested
- Lane 3:** DBY746 undigested
- Lane 4:** I₂ undigested
- Lane 5:** SI undigested
- Lane 6:** SII undigested
- Lane 7:** SIV undigested
- Lane 8:** pFAMY digested with *Sal* I (6,480; 1,220 bp)
- Lane 9:** DBY746 digested with *Sal* I
- Lane 10:** I₂ digested with *Sal* I
- Lane 11:** SI digested with *Sal* I
- Lane 12:** SII digested with *Sal* I
- Lane 13:** SIV digested with *Sal* I

4.1.1.1.7 Pulse-field gel electrophoresis of DBY746 and the integrated strains I₂, SI, SII and SIV.

The purpose of this experiment was to confirm that integration had occurred at a single site in the genome, this being the *ura3-52* locus on chromosome V. The chromosomal site of integration of the pFAMY plasmid was confirmed for the four transformants, I₂, SI, SII and SIV by Southern blotting of intact chromosomes separated by Pulse-field gel electrophoresis. This method is capable of separating out the 16 chromosomes of *S. cerevisiae* into clearly resolved bands on an agarose gel with band sizes ranging from 200 kb to 2,200 kb, which cannot be resolved by conventional agarose gel electrophoresis.

The gel was prepared and the running conditions were as described in section 2.10.3. The apparatus used was a BioRad CHEF Mapper. After electrophoresis the gel was stained with ethidium bromide, destained and photographed. Following this the gel was blotted and probed with a non-radioactive DIGoxigenin α -amylase labelled probe. Figure 4.17 shows *S. cerevisiae* chromosomal DNA markers, (BioRad 170-3605, lane 1), DBY746 as the negative control (lane 2) and the 4 integrated strains, I₂, SI, SII and SIV (lanes 3 to 6). Southern blot analysis (Figure 4.18) shows hybridisation to a single chromosome corresponding to the chromosome V on the gel. Interestingly, in the integrated strains I₂, SII and SIV a size increase in chromosome V was noted in comparison to the control strain DBY746, suggesting that these three integrants contain multiple copies of the plasmid. This increase in size was not so obvious for the integrant SI. This result correlates well with the data from copy number analysis (section 4.1.1.1.9) which predict that the integrants I₂, SII and SIV contain 5, 4 and 5 copies respectively of the plasmid. A tandem integration of 5 copies of this plasmid would result in an increase in chromosome size of approximately 38.5 kb, which is in keeping with the observed band shift. In contrast, SI is the result of a double integration event (corresponding to a size increase of 15.4 kb which is not very noticeable on a pulse-field gel).

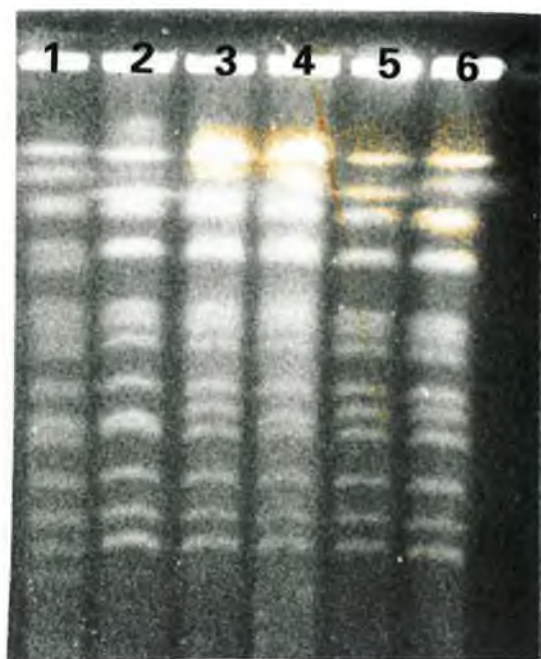


Figure 4.17 Pulse-field Gel showing the individual chromosomes of *Saccharomyces cerevisiae* strain DBY746 and the four integrated strains I₂, SI, SII and SIV.

Lane 1: CHEF *Saccharomyces cerevisiae* DNA size markers (Biorad, 170-3605)

225; 285; 365; 450; 565; 610; 680; 750; 785; 825; 945; 1,020; 1,125; 1,600; 2,200 kb

Lane 2: DBY746

Lane 3: I₂

Lane 4: SI

Lane 5: SII

Lane 6: SIV

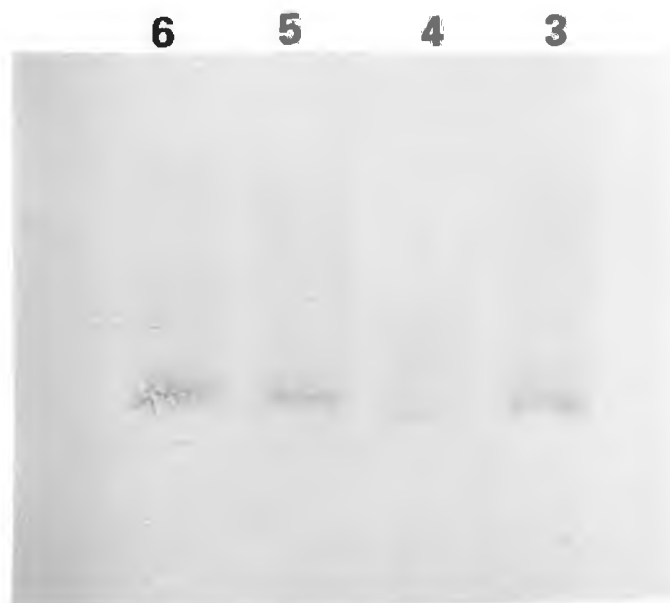


Figure 4.18 Southern blot of the Pulse-field Gel shown in figure 4.17.

Lane 1: CHEF *Saccharomyces cerevisiae* DNA size markers (Biorad, 170-3605)

225; 285; 365; 450; 565; 610; 680; 750; 785; 825; 945; 1,020; 1,125; 1,600; 2,200 kb

Lane 2: DBY746

Lane 3: I₂

Lane 4: SI

Lane 5: SII

Lane 6: SIV

4.1.1.1.8 *Activity Analysis of Integrated Strains, I₂, SI, SII and SIV.*

To accurately quantify the amount of α -amylase activity produced by the integrants the dinitrosalicylic acid (DNS) assay was employed (Miller et al., 1960). This assay measures the levels of reducing sugars produced as a result of the digestion of starch by α -amylase. In order to ensure accurate quantitation of activity in cell supernatants, glucose in the growth medium had to be removed before assaying by dialysis (2.13.2.1). Figure 4.19 shows a typical standard curve used for the determination of α -amylase activity. One unit of enzyme activity was defined as that amount of enzyme which liberates glucose or other reducing carbohydrates with a reduction power corresponding to 0.15 μ moles D-glucose in 30 minutes in the standard assay.

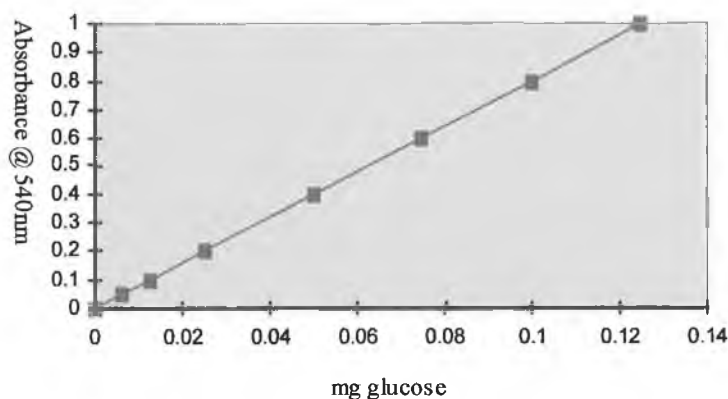


Figure 4.19 Standard Curve for DNS Assay.

The units of α -amylase activity were calculated as follows:

$$\text{Units of enzyme activity: } \frac{\text{mg glucose/ml per 30 minutes} \times D \times RV}{180.2 \times 10^{-6}}$$

where D = Dilution factor

RV = Reaction Volume

180.2 = Molecular weight of glucose

The optimum assay conditions for the *B. licheniformis* α -amylase gene are a pH of 6.9 and a temperature of 93°C (Ortlepp et al., 1983). The optimum pH and temperature conditions of the *S. cerevisiae* recombinant α -amylase strain, DBY746::pAAMY-01 were also determined and the findings were in agreement with those of Ortlepp (see figures 4.20 and 4.21 below). The α -amylase activity was once again quantified using the DNS assay. Culture supernatants of stationary phase (36 hours) DBY746::pAAMY-01 were dialysed against 1 x PBS prior to assaying. The pH profile was determined over the range pH 2 to 10 and the temperature profile over the range 30 to 100°C. All assays were carried out in duplicate and the mean values are expressed as the relative percentage activity with the optimal conditions giving 100% activity.

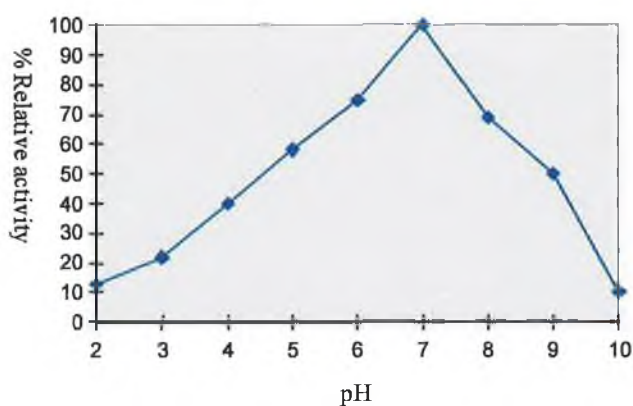


Figure 4.20 pH profile of the *B. licheniformis*' α -amylase gene activity in *S. cerevisiae* DBY746::pAAMY-01.

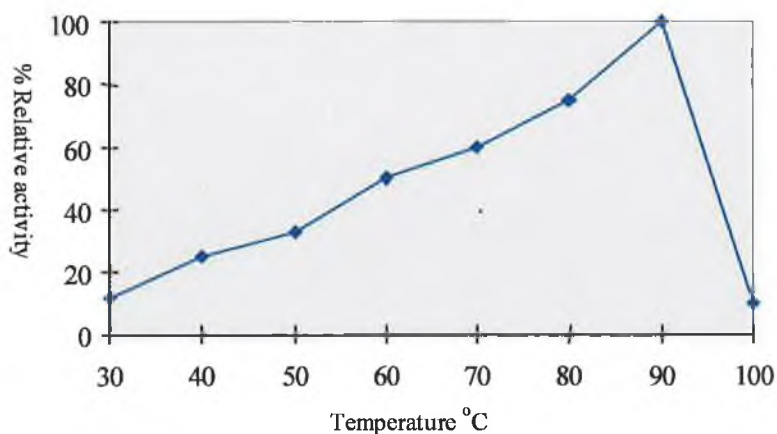


Figure 4.21 Temperature profile of the *B. licheniformis*' α -amylase gene activity in *S. cerevisiae* DBY746::pAAMY-01.

The level of extracellular α -amylase produced by the 4 integrated strains I₂, SI, SII and SIV are shown in table 4-3 below. DBY746 was used as a negative control in this experiment.

Table 4-3 Levels of α -amylase expressed by the Integrants.

Strain	Units of secreted amylase	Cell no.	Units/cell
I ₂	569.37	3.35×10^7	1.69×10^{-5}
SI	329.6	3.3×10^7	1.0×10^{-5}
SII	494.5	3.4×10^7	1.36×10^{-5}
SIV	464.48	3.4×10^7	1.45×10^{-5}

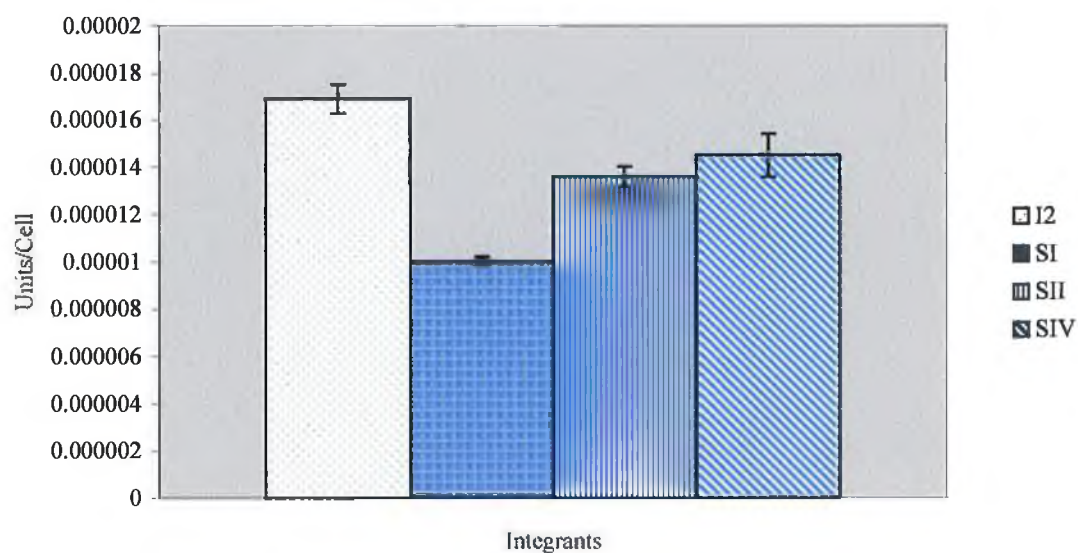


Figure 4.22 Bar Chart depicting the levels of extracellular α -amylase activity as displayed by the integrants.

4.1.1.1.9 *Determination of the copy number of integrated α -amylase genes*

The method chosen for determining plasmid copy number (PCN) was based on Southern hybridisation and it depended on an appropriate probe containing sequences which would be capable of binding to both the plasmid

DNA and at the same time to some endogenous chromosomally located sequence (reference DNA) whose copy number is known. PCN can then be calculated from the intensity of hybridisation to the plasmid specific fragment, relative to the intensity of hybridisation to the reference DNA. To estimate the copy number of an integrated plasmid it was decided to let one of the junction fragments represent the reference DNA as these sequences should only occur once. pUC-19 was chosen as the probe since both pUC-19 and the integrating plasmid pFAMY contain homologous pBR322 sequences (in the cases of these plasmids the homologous region is the ampicillin resistance gene). When the integrated strains are digested with *EcoR* I one would expect hybridisation of the ³²P-radiolabelled pUC-19 probe to hybridise to a 7.7 kb band, which represents the number of integrated copies of the plasmid (*EcoR* I cuts once within the plasmid pFAMY) and to a junction fragment 3' to the site of integration of unknown size as the region had not been sequenced prior to the design of this experiment. There should be only one junction fragment to which this probe will hybridise as the ampicillin resistance gene is after the *EcoR* I site in the plasmid (see Figure 4.23).

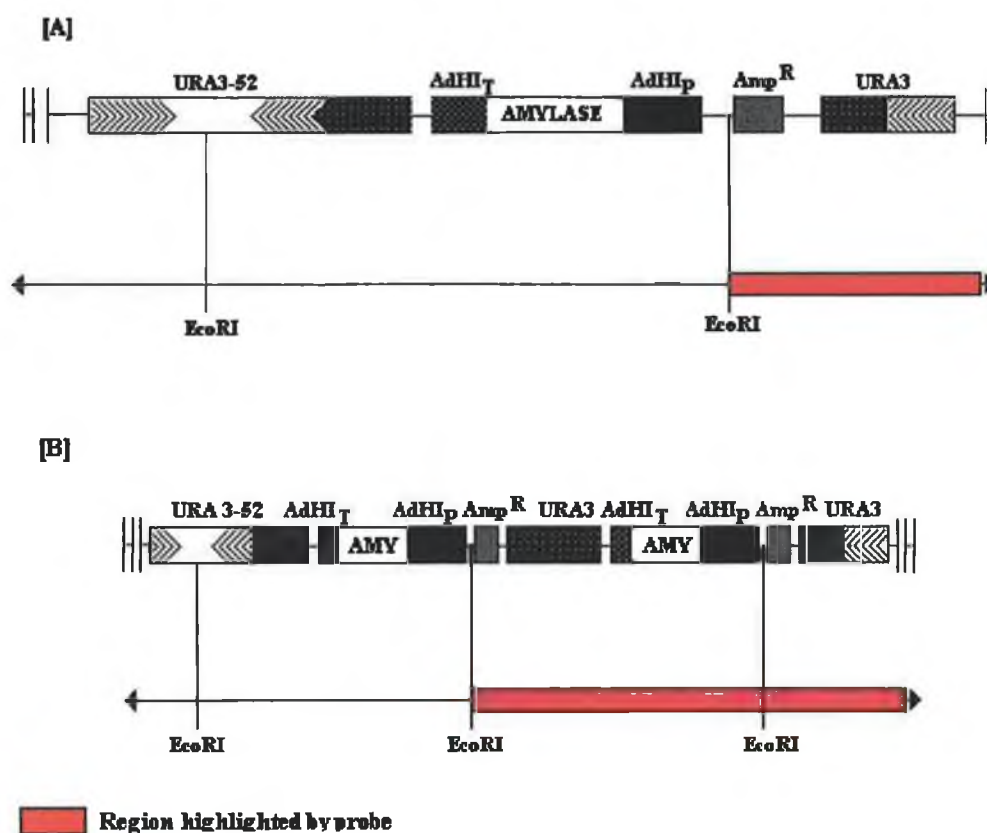


Figure 4.23 Schematic representation of where the pUC-19 probe is expected to bind if [A] a single copy or [B] if multiple copies of the plasmid pFAMY integrate into the *ura3-52* locus of DBY746.

Genomic DNA was extracted from DBY746 and the 4 integrants, I₂, SI, SII and SIV. Following extraction, the DNA was restricted (section 2.6.11.1) with *EcoR* I and the samples were electrophoresed on a 0.8% agarose gel (Figure 4.24). This gel was blotted and probed with a ³²P-labelled pUC 19 probe (Figure 4.25).

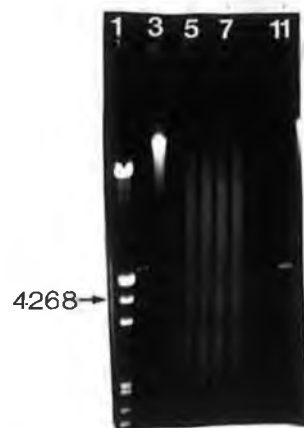


Figure 4.24 Agarose Gel of genomic DNA digested with *EcoR* I.

- Lane 1:** λ DNA *Hind* III/*EcoR* I digested (21,227; 5,148; 4,973; 4,268; 3,530; 2,027; 1,904; 1,584; 1,375; 974; 831; 564; 125 bp)
- Lane 2:** pRB149 digested with *EcoR* I (5,500; 3,800 bp)
- Lane 3:** DBY746 undigested
- Lane 4:** DBY746 digested with *EcoR* I
- Lane 5:** I₂ digested with *EcoR* I
- Lane 6:** SI digested with *EcoR* I
- Lane 7:** SII digested with *EcoR* I
- Lane 8:** SIV digested with *EcoR* I
- Lane 9:** -----
- Lane 10:** -----
- Lane 11:** pFAMY digested with *Hind* III (5,350; 1,900; 450 bp)

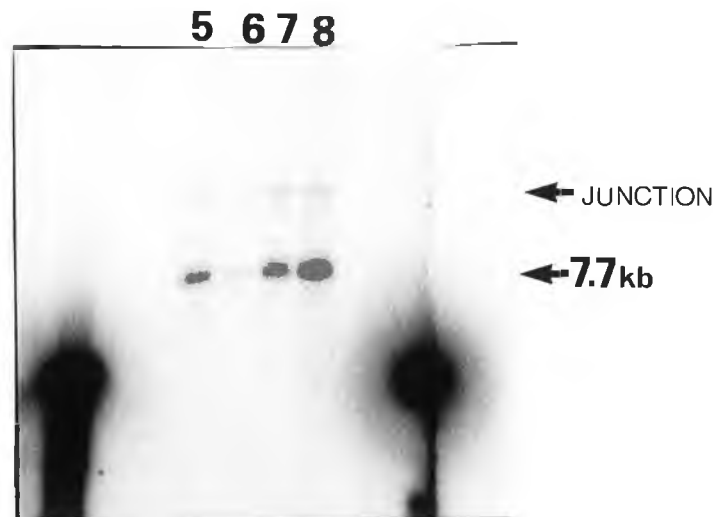


Figure 4.25 Southern blot of genomic DNA digested with *EcoR* I, electrophoresed and probed with a ^{32}P -labelled pUC 19 probe.

Lane 1:	λ DNA <i>Hind</i> III/ <i>EcoR</i> I digested (21,227; 5,148; 4,973; 4,268; 3,530; 2,027; 1,904; 1,584; 1,375; 974; 831; 564; 125 bp)
Lane 2:	pRB149 digested with <i>EcoR</i> I (5,500; 3,800 bp)
Lane 3:	DBY746 undigested
Lane 4:	DBY746 digested with <i>EcoR</i> I
Lane 5:	I ₂ digested with <i>EcoR</i> I
Lane 6:	SI digested with <i>EcoR</i> I
Lane 7:	SII digested with <i>EcoR</i> I
Lane 8:	SIV digested with <i>EcoR</i> I
Lane 9:	-----
Lane 10:	-----
Lane 11:	pFAMY digested with <i>Hind</i> III (5,350; 1,900; 450 bp)

The intensities of the bands were analysed using a Leica Q 500MC Image Processing and Analysis System (Cambridge, England). The areas under the curve were integrated using Sigma Plot (Jendal Scientific). The ratio of the intensity of the plasmid DNA to the reference was tabulated (table 4-4). The copy number of the integrants I₂, SI, SII and SIV was estimated to be 5, 2, 4 and 5 respectively according to the method employed. SI was the integrant chosen as it possessed the lowest copy number, but more importantly if there was a reduction in the activity of SI this could still be measured.

Table 4-4 Comparison of the intensity of the plasmid bands to the reference band in order to determine the copy number of integration.

Integrand	Plasmid DNA	Reference DNA	Copy Number
I ₂	1392	264	5
SI	241	148	2
SII	1487	423	4
SIV	2201	415	5

As a further step to confirm this result, the copy number of each of the integrants and an episomal plasmid, pAAMY-01 (which contains a functional α -amylase gene) were plotted against their relative α -amylase activities. As expected the graph (Figure 4.26) indicates that the integrant with the least copy number of α -amylase genes gave the lowest activity (SI). Although it might be expected that an integrant with 4 copies of the α -amylase gene should have twice the activity of an integrant with 2 copies, this was not found to be the case although the activity was higher.

The integrants SII, SIV and I₂ which possess 4, 5 and 5 copies of the α -amylase gene respectively, do not vary significantly in the levels of α -amylase produced. On the other hand the episomal plasmid, pAAMY-01, which has a copy number of 9 exhibits twice the activity of SII possessing 4 copies of the α -amylase gene.

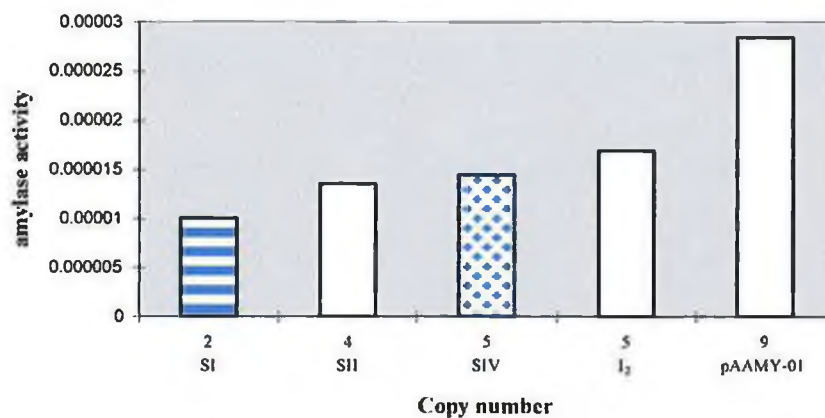


Figure 4.26 Copy number of the integrants and the episomal plasmid pAAMY-01 versus their respective amylase activities.

The α -amylase gene copy number of the integrant SI (which is the chosen strain for the introduction of the various antisense constructs in this section and in *trans* I (c)) was further proven to be present at 2 copies per cell by Northern blot analysis (Figure 4.27). When a total RNA sample of SI was probed with both an α -amylase probe and a β -actin probe (which is present in one copy per cell) the calculated ratio of α -amylase to β -actin transcripts was 2:1.

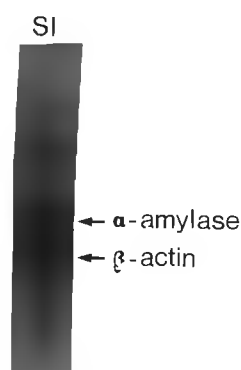


Figure 4.27 Northern blot of total RNA extracted from SI and probed with a ^{32}P -labelled α -amylase and β -actin fragment.

4.1.2 CONSTRUCTION OF A YEAST EPISOMAL VECTOR CONTAINING THE *BACILLUS LICHENIFORMIS* α -AMYLASE GENE CLONED IN ANTISENSE ORIENTATION

Plasmid pAAH5 was used to construct a YEep plasmid carrying expression cassettes capable of producing either antisense α -amylase mRNA (pSOC-01) or sense α -amylase mRNA (pAAMY-01, McMahon, 1995). The plasmid pAAH5 contains 2μ sequences, a *LEU2* selectable marker and an expression cassette composed of the *AdHI* promoter, a unique *Hind* III site for cloning and an *AdHI* terminator. The 2μ fragment includes the 2μ origin of replication which permits this plasmid and its derivatives to exist in high copy number in [*cir*⁺] yeast cells. YEep based shuttle vectors have been reported to exist at 10 - 40 copies per cell in yeast transformants (Armstrong et al., 1989).

With the expression cassette located on plasmid pAAH5, the *AdHI* promoter was used to direct transcription of antisense RNA sequences. This is a constitutive promoter. The *AdHI* transcription terminator downstream of the *AdHI* promoter provided the signals for precise termination of transcription and polyadenylation of the resulting transcripts. Any transcripts produced from this expression cassette or its derivatives were predicted to contain both a 5' cap and a 3' poly-A tail.

The construction and analysis of the effect of the antisense transcript produced from pSOC-01 on α -amylase gene expression from the yeast strain SI are discussed in this section. The plasmid pSOC-01 is represented schematically in Figure 4.28 and the details of construction are included in the legend to the figure.

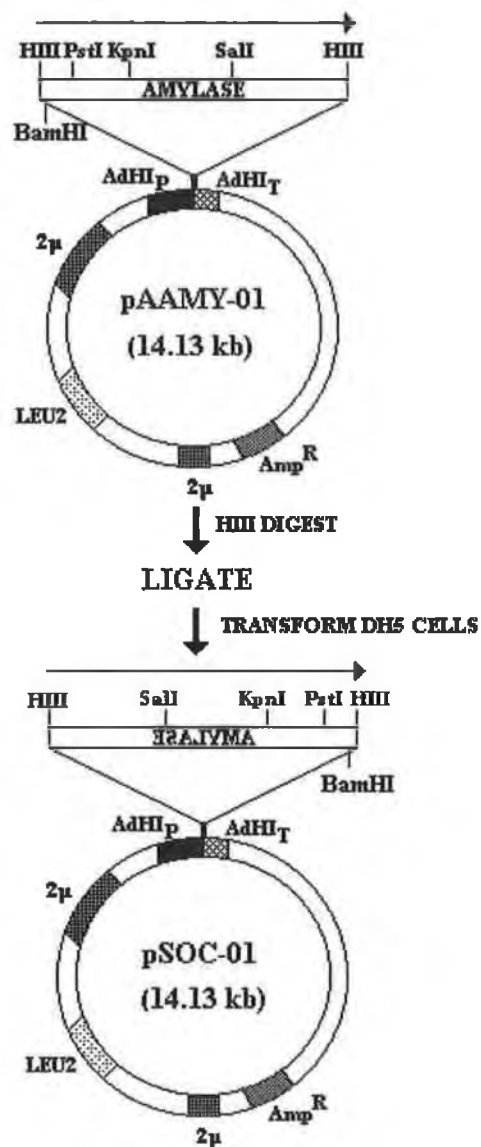


Figure 4.28 Construction of a yeast episomal vector (pSOC-01) containing the *B. licheniformis* α -amylase gene in its antisense orientation.

The plasmid pAAMY-01 was digested with *Hind* III to release a 1.9 kb fragment corresponding to the α -amylase gene. The restriction digest was then column cleaned to remove any remaining restriction enzyme and buffer. Following this the mixture was ligated at 4°C overnight, then transformed into DH5 α cells, plated on LB amp starch plates and screened.

Normally, expression of α -amylase in *E. coli* would not be expected with a yeast expression vector construct (such as pAAH5) as it lacks a prokaryotic promoter at the cloning site. However it had already been observed in our laboratory that this *Hind* III α -amylase containing fragment when introduced into *E. coli* cells, resulted in α -amylase activity irrespective of either the plasmid into which it was cloned or orientation of the fragment, suggesting the sequences on the fragment itself contained promoter activity (Figure 4.29). For this reason, one could take advantage of this finding, and screen potential pAAH5-based transformants for α -amylase activity, thereby indicating the presence/absence of the insert in the transformants under investigation.

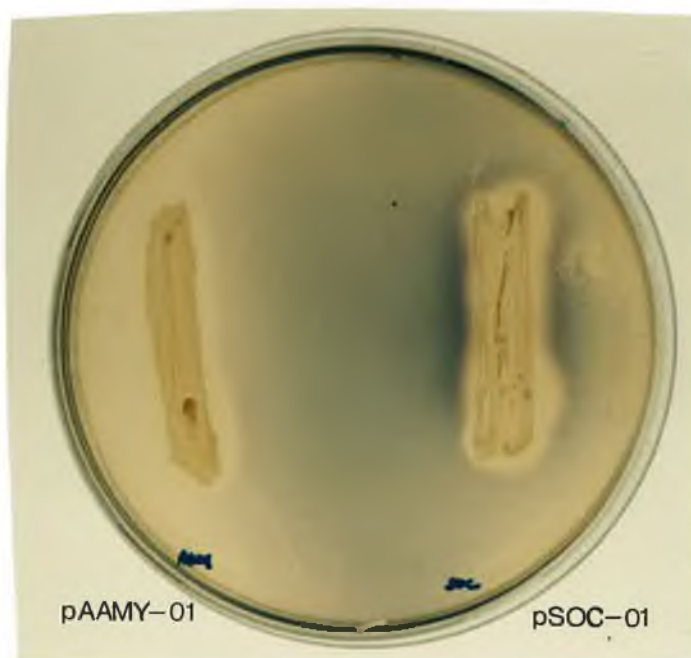


Figure 4.29 Plasmids pAAMY-01 and pSOC-01 displaying α -amylase activity after transformation into *E. coli* DH5 α cells.

Restriction analysis (Figure 4.30) of the transformants displaying α -amylase activity was carried out to check for transformants carrying single

copies of the gene. The orientation of the gene was confirmed using *Sal* I restriction analysis (Appendix III, VIII). Digestion with this enzyme gave fragment sizes of 6.75kb, 5.52kb and 1.86kb (lane 10, Figure 4.30) in the antisense orientation and fragment sizes of 6.75kb, 5.88kb and 1.5kb (lane 9, figure 4.30) in the sense orientation (plasmid pAAMY-01). The antisense construct will be referred to as pSOC-01 from now on, and it is important to note that the α -amylase gene was antisense in relation to the constitutively expressed yeast *AdHI* promoter and terminator.

The restriction enzyme *Hind* III was chosen to show that the α -amylase gene insert was the desired 1.9 kb (lane 14, Figure 4.30). *Bam*H I was chosen as a further enzyme to demonstrate the orientation of the insert. pAAH5 was also restricted with the same enzymes as pSOC-01 and pAAMY-01 in order to highlight bands corresponding to vector sequences.



Figure 4.30 Restriction analysis of plasmids pAAH5, pAAMY-01 and pSOC-01.

The approximate sizes of each of the restriction fragments are shown in kilobases in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

Lane 1:	1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)
Lane 2:	pAAH5 undigested
Lane 3:	pAAMY-01 undigested
Lane 4:	pSOC-01 undigested
Lane 5:	pAAH5 digested with <i>Bam</i> H I (10.23 kb; 2.0 kb)
Lane 6:	pAAMY-01 digested with <i>Bam</i> H I (10.23 kb; 2.35 kb; 1.55 kb)
Lane 7:	pSOC-01 digested with <i>Bam</i> H I (10.23 kb; 3.45 kb; 0.45 kb)
Lane 8:	pAAH5 digested with <i>Sal</i> I (6.75 kb; 5.48 kb; high molecular weight partial)
Lane 9:	pAAMY-01 digested with <i>Sal</i> I (6.75 kb; 5.88 kb; 1.5 kb)
Lane 10:	pSOC-01 digested with <i>Sal</i> I (6.75 kb; 5.52 kb; 1.86 kb)
Lane 11:	1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)
Lane 12:	pAAH5 digested with <i>Hind</i> III (12.23 kb)
Lane 13:	pAAMY-01 digested with <i>Hind</i> III (10.23 kb; 1.9 kb)
Lane 14:	pSOC-01 digested with <i>Hind</i> III (10.23 kb; 1.9 kb)
Lane 15:	pAAH5 digested with <i>Eco</i> R I (9.63 kb; 2.6 kb)
Lane 16:	pAAMY-01 digested with <i>Eco</i> R I (11.53 kb; 2.6 kb)
Lane 17:	pSOC-01 digested with <i>Eco</i> R I (11.53 kb; 2.6 kb)
Lane 18:	1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)

The restriction data (Figure 4.30) confirms the construction of the antisense plasmid pSOC-01 in which the α -amylase gene was cloned in antisense orientation relative to the *AdHI* promoter and terminator. The orientation was confirmed by the sizes of the *Bam*H I and *Sal* I fragments obtained (lanes 7 and 10, Figure 4.30). In the case of *Bam*H I, there are *Bam*H I sites at either end of the *AdHI* promoter, terminator cassette, in addition to an internal *Bam*H I site at the beginning of the α -amylase gene sequence. The distance from the promoter to the *Bam*H I site in the α -amylase gene in antisense orientation is 3.45 kb. The distance from the *Bam*H I site in the antisense α -amylase gene to the *Bam*H I site of the *AdHI* terminator is 0.45 kb. These bands corresponded to the band sizes observed in lane 7 (Figure 4.30). If the gene had been cloned in sense orientation (pAAMY-01) relative to the *AdHI* promoter and terminator, a *Bam*H I restriction digest should yield fragment sizes of 10.23, 2.35 and 1.55 kb respectively (lane 6, Figure 4.30).

From the restriction data it is apparent that the α -amylase fragment has been successfully cloned in antisense orientation.

4.1.2.1 Transformation of pSOC-01 into the yeast strain DBY746

The plasmid pSOC-01 was transformed into *S. cerevisiae* strain DBY746 and transformants were plated onto buffered minimal media containing 2% Lintners starch to screen for lack of α -amylase activity. The media was buffered with 0.1 M sodium phosphate buffer, pH 6.9. Previous work, carried out by McMahon (1995), showed that growth of *S. cerevisiae* for 48 hours at 30°C in unbuffered minimal media broth resulted in a drop in pH from 6.2 to 2.3. This lowering of the pH of the medium was found to result in undetectable α -amylase activity. Buffering of the media to pH 6.9 with 0.1 M sodium phosphate buffer overcame this problem and amylolytic activity was demonstrated by clear haloes around amylase positive clones. DBY746 transformed with pSOC-01 displayed no amylolytic activity on the starch supplemented minimal media plates. pAAMY-01 was also transformed into DBY746 as a positive control to demonstrate the effect of amylase activity.

4.1.2.2 Confirmation of the presence of the plasmid pSOC-01 in DBY746

4.1.2.2.1 *Growth on selective media*

The yeast strain DBY746 is auxotrophic for uracil and leucine. Transformants were plated onto minimal medium supplemented with uracil only as the plasmid pSOC-01 has a *LEU2* marker and the strain will only grow if the plasmid has been successfully transformed thus converting the strain to leucine prototrophy. Figure 4.31 shows a minimal media plate divided into 3 sections, section 1 contains uracil spread onto this region of the plate, which was then allowed to dry, similarly section 2 contains leucine and section 3 was minimal media alone unsupplemented with any amino acids. DBY746::pSOC-01 was then patched onto each of these sections of the plate. As can be seen DBY746::pSOC-01 grew only on the section of the plate supplemented with uracil. In Figure 4.32 starch was added to the media and the same application of amino acids was carried out, once again the same result was observed and this plate also demonstrated the lack of α -amylase activity when the plates were flooded with iodine vapour. DBY746::pAAMY-01 Figure 4.33 was used as a positive control for demonstrating α -amylase activity in this experiment.

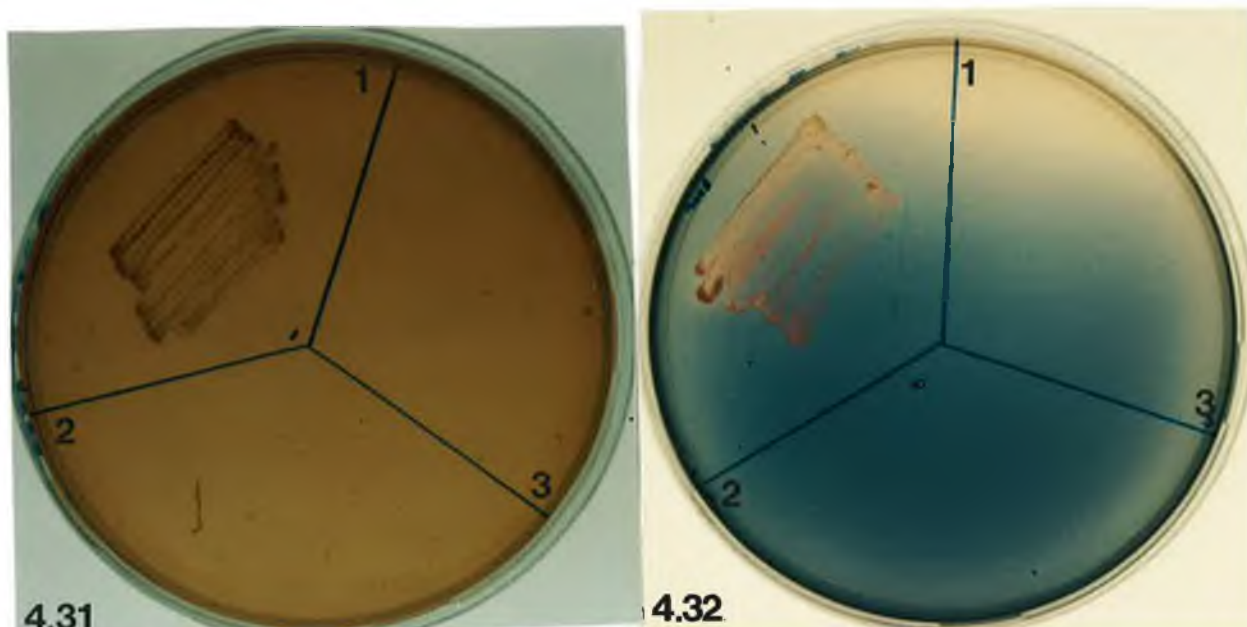


Figure 4.31 DBY746::pSOC-01 plated on each of the 3 sections of the minimal media plate containing on section 1 uracil, section 2 leucine and no amino acid supplementation on section 3.

Figure 4.32 DBY746::pSOC-01 plated on the minimal media plate with the same amino acid composition as Figure 4.31, except the media also contains 2% starch.

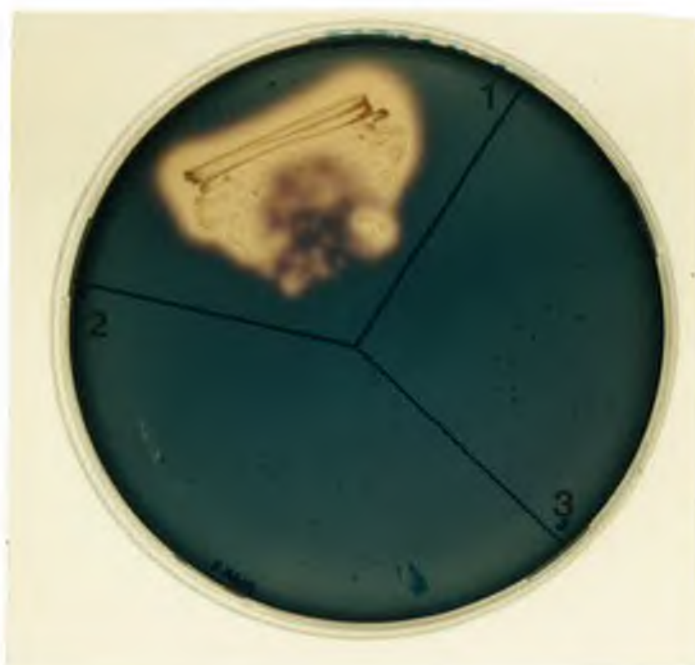


Figure 4.33 DBY746::pAAMY-01 plated on the minimal media plate with the same amino acid composition as Figure 4.31, except the media also contains 2% starch.

4.1.2.2.2 *The use of the Polymerase Chain reaction (PCR) to confirm the presence of the plasmid pSOC-01 in DBY746.*

PCR was chosen firstly to directly confirm the presence of the antisense gene in DBY746 and secondly because it is difficult to obtain good yields of plasmid DNA for restriction analysis starting from yeast cells. The experiment was designed so that one set of primers would amplify the sense α -amylase gene to give a band size of 0.98 kb and the second set of primers would give a 2 kb band if the antisense α -amylase gene was amplified (see Figure 4.34). One primer was common to both reactions and it contained sequences complementary to the pBR322 sequences present in the plasmid pAAH5. The other primers were sequences designed to hybridise to the sense or antisense α -amylase gene, shown schematically in Figure 4.34 (and Appendix III).

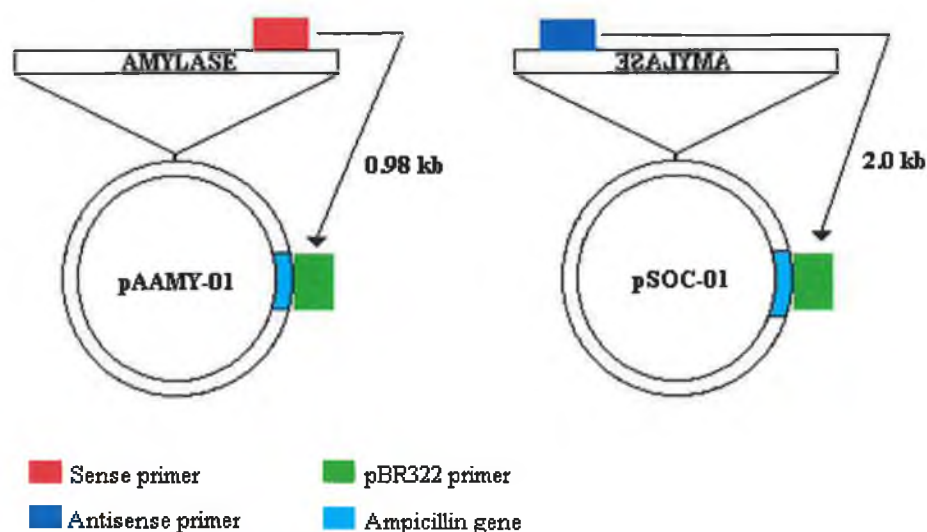


Figure 4.34 Schematic diagram to illustrate where the primers bind.

The primers selected for use in the PCR reaction are shown below:

pBR322:	5' GTG ATG TCG GCG ATA TAG 3'
Amylase sense:	5' GTG AGA CAT GGC ATG ACA 3'
Amylase antisense:	5' TGT GAT GCC ATG TCT GAC 3'

The annealing temperature was calculated to be 49°C.

The template DNA used in the PCR reactions was obtained after genomic DNA was extracted from DBY746::pAAH5 (used as a negative control), DBY746::pAAMY-01 and DBY746::pSOC-01. The annealing reactions were performed at 49°C. 5µl from the PCR reaction was run on a 0.7% agarose gel.

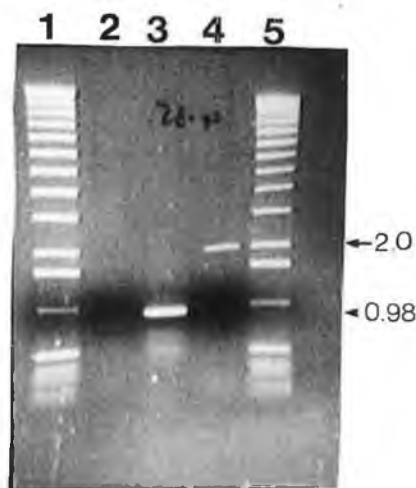


Figure 4.35 Result of the PCR reactions.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)
- Lane 2:** Negative control (pAAH5 with pBR322 primer and antisense amylase)
- Lane 3:** DBY746::pAAMY-01 (pBR322 primer and sense amylase primer) (0.98 kb)
- Lane 4:** DBY746::pSOC-01 (pBR322 primer and antisense amylase primer) (2.0 kb)
- Lane 5:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp)

As expected there was no amplification observed in the lane containing DBY746::pAAH5 (lane 2, Figure 4.35). A band of the expected size of 0.98 kb was observed in lane 3, thus indicating the presence of the α -amylase gene in sense orientation in DBY746::pAAMY-01. Finally a band of the expected size of 2 kb was observed in the lane containing DBY746::pSOC-01 (lane 4, Figure

4.35) thus indicating the successful transformation of the plasmid pSOC-01 into *S. cerevisiae*.

4.1.2.2.3 Southern blot analysis

Genomic DNA was extracted from DBY746 and DBY746::pSOC-01, both were electrophoresed undigested on a 0.7% agarose gel, then blotted and probed with a non-radioactively labelled DIGoxigenin α -amylase probe. The presence of the plasmid was confirmed by multiple bands of the various forms of undigested DNA lighting up, there was no hybridisation of the probe to the lane containing DBY746, (see Figure 4.67, section 4.3.1.5.2).

4.1.2.2.4 Northern analysis

To determine whether there was *in vivo* production of antisense α -amylase RNA from DBY746::pSOC-01, the transformant was examined using Northern hybridisation. Total RNA was isolated under the conditions described in section 2.6.10. RNA was separated on an agarose - formaldehyde gel and bidirectionally transferred to a nitrocellulose membrane. The first blot was hybridised with a ^{32}P -labelled 1.9 kb α -amylase probe. The second blot was probed with a β -actin probe in order to be able to [1] standardise loadings and [2] to show that the RNA was not degraded.

As expected, control strain DBY746 generated no signal on the autoradiograph when probed with the α -amylase probe (lane 2, Figure 4.36). The positive control, DBY746::pAAMY-01 (lane 3) expressed the full-length α -amylase transcript of the expected size of 1.9 kb and DBY746::pSOC-01 (lane 4) produced an antisense transcript which was discrete and of the same length. It should be noted that the vector contains a transcriptional stop site; the RNA transcript obtained was of a length consistent with that expected if transcription was initiated close to the *AdHI* promoter and terminated at the *AdHI* transcript terminator.

A band of 1.3 kb, which corresponds to the β -actin gene is clearly visible in the lanes containing pAAMY-01 and pSOC-01 (lanes 3 and 4, Figure 4.37). This indicates that the RNA was not degraded. A weaker β -actin band was

visualised in the lane containing DBY746 (lane 2, Figure 4.37), this was due to poor loading of this sample onto the agarose gel (data not shown).

The intensities of the bands from both blots were analysed using a Bio-Rad Imaging Densitometer (Model GS670). The areas under the curve were integrated using Sigma Plot (Jendal Scientific). The ratio of the intensity of the sense α -amylase mRNA transcripts produced by pAAMY-01 to the antisense α -amylase transcripts produced by pSOC-01 (after standardising the loadings with β -actin) was 1:1. This result demonstrates that the insertion of a full-length antisense α -amylase gene (to produce pSOC-01) into the vector pAAH5 produces a significant amount of antimessage of similar size to its' sense counterpart (pAAMY-01). The sharpness of the mRNA band on the blot also demonstrates the stability of the anti-message.

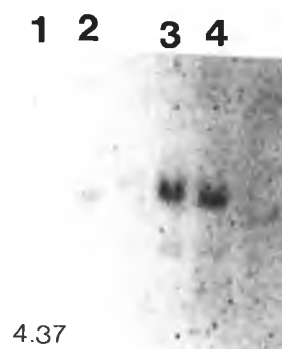


Figure 4.36 Northern blot of total RNA extracted from DBY746, DBY746::pAAMY-01 and DBY746::pSOC-01 probed with a ^{32}P -labelled α -amylase fragment.

Figure 4.37 Northern blot of total RNA extracted from DBY746, DBY746::pAAMY-01 and DBY746::pSOC-01 probed with a ^{32}P -labelled β -actin fragment.

Lane 1: RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)

Lane 2: DBY746

Lane 3: DBY746::pAAMY-01

Lane 4: DBY746::pSOC-01

4.1.2.2.5 *Growth Curves*

DBY746 and DBY746::pSOC-01 were grown in minimal media over time to examine whether or not the presence of this antisense construct had any adverse effects on growth. DBY746::pAAMY-01 was also included in this study as a further control for comparison purposes. As can be seen from the growth

profiles in Figure 4.38, there were no significant growth effects on the *S. cerevisiae* strain DBY746 transformed with the antisense plasmid pSOC-01.

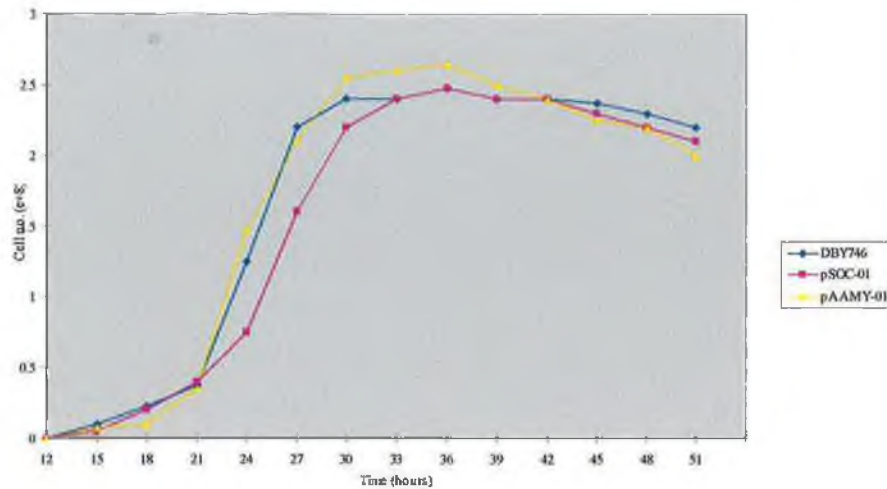


Figure 4.38 Growth Curves of yeast strains; DBY746, DBY746::pAAMY-01 and DBY746::pSOC-01.

4.1.2.3 Analysis of the effect of the YEp-antisense plasmid pSOC-01 when transformed into the integrated sense α -amylase strain SI.

To assess the effect of the antisense α -amylase RNA transcript produced from the plasmid pSOC-01 on the levels of α -amylase activity exhibited by the integrated strain SI, pSOC-01 was transformed into SI. Transformants were plated on minimal media containing 2% starch but lacking the amino acids leucine and uracil, as the integrating plasmid converts DBY746 to uracil prototrophy while the YEp-antisense plasmid pSOC-01 bears a *LEU2* marker. Hence a successful transformation event exhibited colonies growing on minimal media minus uracil and leucine.

In this study, an inhibitory effect (if any) on the expression of the target gene was assessed initially by plating of transformants (containing both the sense and antisense α -amylase gene) onto minimal media containing 2% Lintners' starch. The plates were then normally flooded with iodine vapour. The lack of a

halo surrounding a transformant would be indicative of a lack of α -amylase activity. Since all transformants displayed some level of activity, approximately 100 transformants were taken and patched onto more minimal starch containing media plates to examine more clearly the sizes of the halos produced. A selection of these transformants were then chosen for further analysis by means of DNS assay and/or Northern blot analysis.

Initial visualisation of starch containing minimal media plates after transformation of SI with pSOC-01 showed that all the transformants were capable of breaking down starch, thus indicative of α -amylase activity. Eventually, four different transformants (A, B, C and D) were chosen, because of slight variations in halo size, for analysis to determine whether or not there was any downregulation of amylase activity. The DNS assay was used to measure the amount of amylase produced in relation to the control strain SI. All samples were assayed in triplicate and the entire assay was repeated 3 times. The cumulative results of this experiment are represented by the bar chart shown below (Figure 4.39). Results are reported as the % amylase activity relative to the control strain SI (100%).

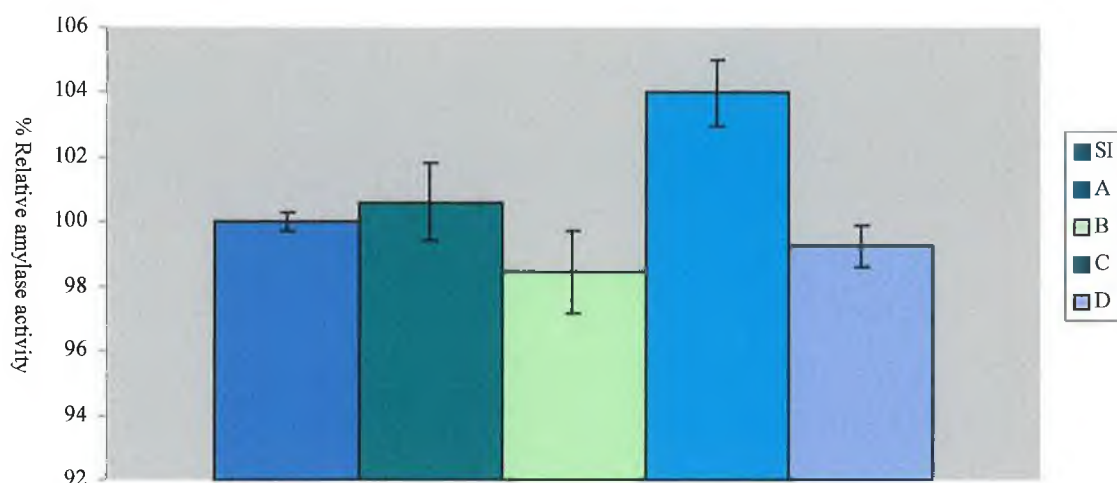


Figure 4.39 Bar chart depicting the effect of the antisense plasmid pSOC-01 on the levels of α -amylase produced by the integrated strain SI.

From the bar chart shown in Figure 4.39 one can see that the presence of the antisense RNA construct in SI did not significantly downregulate or ablate the expression of the α -amylase gene. Indeed as can be seen from transformant C there was a slight increase in the level of α -amylase activity being produced as compared to the control strain SI. These results raise the following questions assuming that both sense and antisense transcripts are being made (which shall be determined in section 4.1.2.4):

1. Is the sense mRNA accessible to the antisense RNA?
2. What is the degree of secondary folding within the α -amylase transcript?
3. Is the antisense mRNA stable?
4. Is there spatial and temporal coincidence of the two RNAs?
5. Is it possible that there is an unfavourable ratio of antisense:sense mRNA?

The Northern blot analysis performed in section 4.1.2.4 should help to address the question of antisense mRNA stability (or instability), the temporal coincidence of the two RNAs and finally the ratio of antisense:sense RNA.

4.1.2.4 Northern Analysis

In order to confirm the presence of antisense transcript in the transformants, RNA was extracted for analysis. Assuming the antisense transcript is present it should then be possible to estimate the ratio of sense:antisense mRNA. Due to the fact that both sense and antisense transcripts will be of equal size a probing system which could discriminate between a sense and antisense transcript was necessary. The system chosen was Promegas' Riboprobe. This system is designed for the *in vitro* preparation of high specific activity single-stranded RNA probes from cloned DNA inserts (Figure 4.40) in the vector pGEM7Zf. This vector has a versatile multiple cloning site (MCS) cloned between the T7 and SP6 promoters. Hence depending on the way that the DNA of interest is cloned into this MCS either a T7 or SP6 polymerase may be used to generate a probe which will bind specifically to a sense or antisense sequence on a Northern blot.

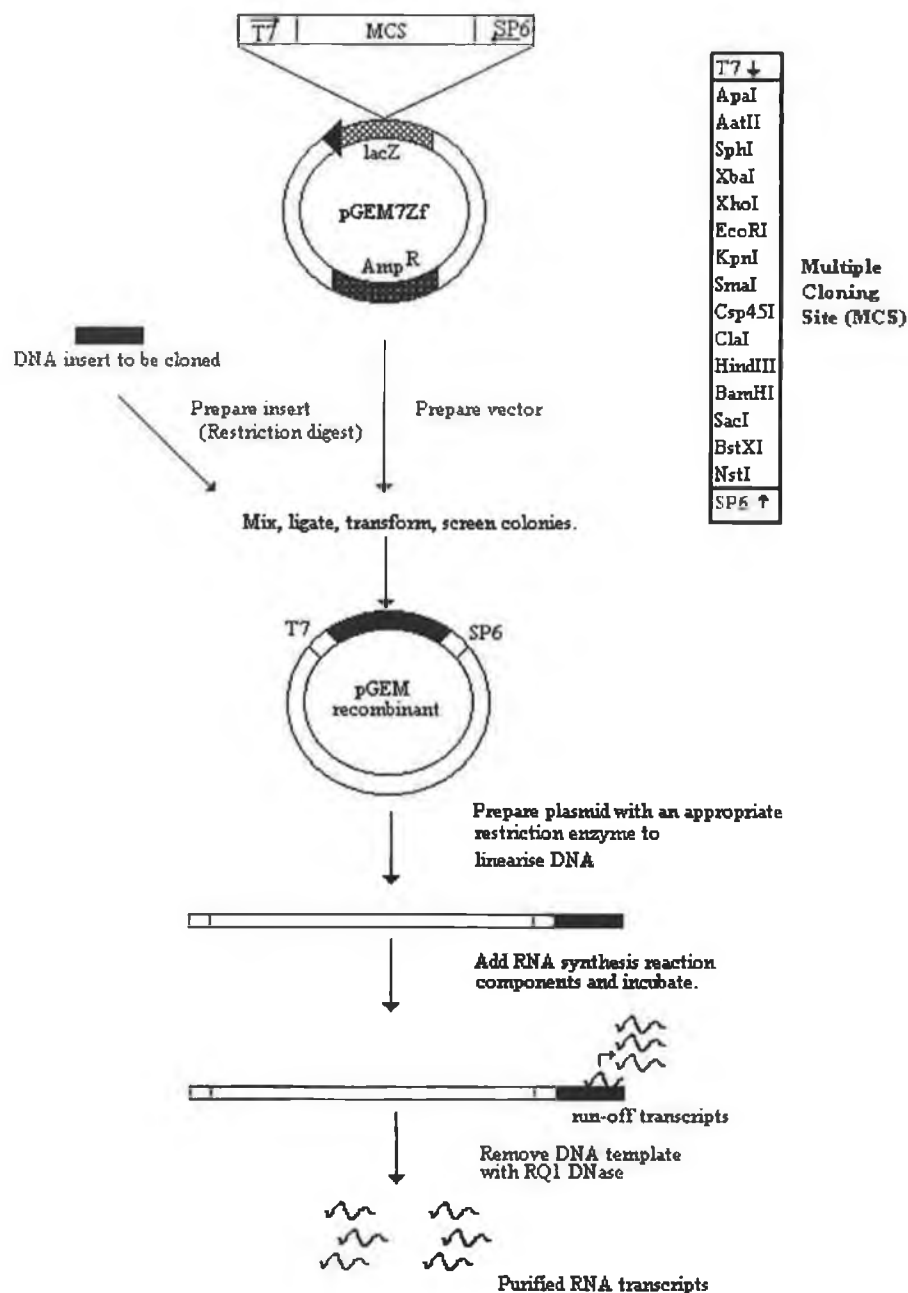


Figure 4.40 Schematic representation of how RNA transcripts are generated using Promega's Riboprobe system.

A bi-directional Northern blot was performed with one blot being probed with an antisense probe which should detect all sense α -amylase transcripts and a

β -actin probe. Conversely the second blot was probed with the sense probe to detect all the α -amylase antisense gene sequences. For preparation of the recombinant pGEM7Zf vector a 1.9 kb *Hind* III α -amylase gene fragment was gene cleaned from a 0.7% agarose gel and ligated into *Hind* III restricted pGEM7Zf. The transformants were plated on LB amp plates which also contained 2% Lintners starch and X-gal for exploitation of the *lacZ* phenotype. If the ligation and subsequent transformation resulted in the α -amylase gene sequence being cloned in one orientation only with respect to the T7 and SP6 promoters, then both polymerases would be necessary to generate the sense and antisense probes. However as the cloning resulted in the α -amylase gene being cloned in both orientations (pSP6-AMY and pT7-AMY, Figure 4.41) it was only necessary to use one of the polymerases.

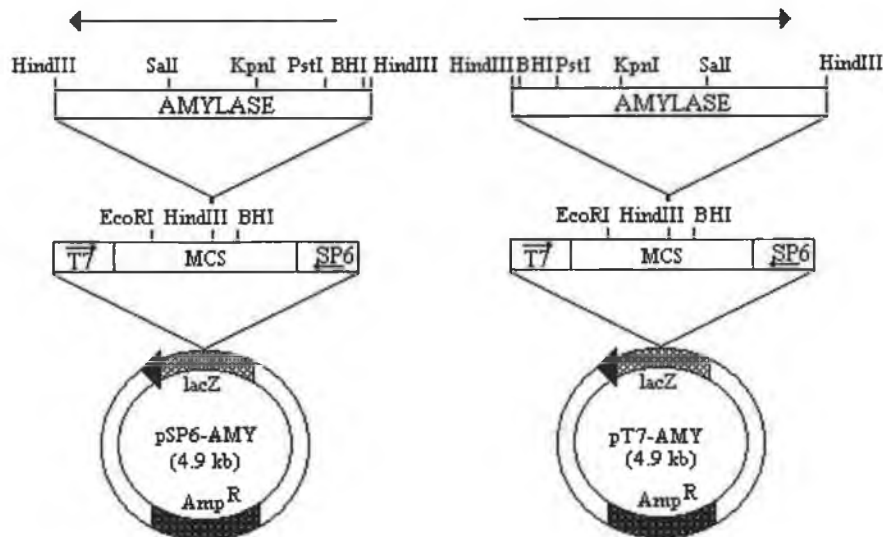


Figure 4.41 Schematic representation of the plasmids pSP6-AMY and pT7-AMY which were used for the generation of riboprobes. (BHI represents the restriction enzyme *Bam*H I).

Additionally to standardise the loadings on gels it was necessary to have a probe capable of binding to β -actin sequences. A 3.8 kb fragment containing the β -actin gene was removed from the plasmid pRB149 (Appendix II) after restriction digestion with *Eco*R I. This fragment was gene cleaned and ligated into *Eco*R I restricted pGEM7Zf. This plasmid was named p β ACTSP6 as the

insert was in sense orientation relative to the SP6 promoter (Figure 4.42). Restriction analysis of each of these 3 plasmids is shown in Figure 4.43.

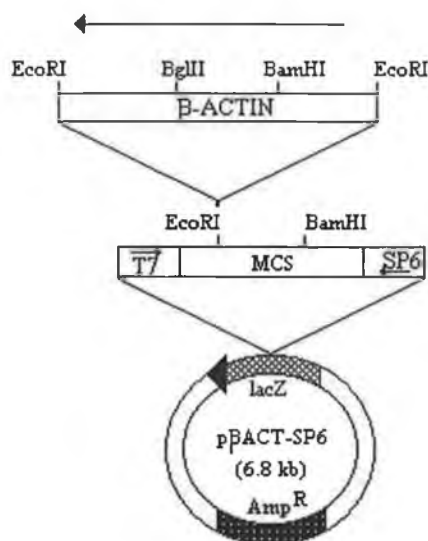


Figure 4.42 Schematic representation of the plasmid pβACTSP6 used for the generation of a riboprobe.

To confirm the construction of pSP6-AMY, pT7-AMY and pβACTSP6, plasmid DNA was extracted from DH5α cells and analysed by restriction enzyme digestion. The plasmids pSP6-AMY and pT7-AMY were restricted with *Hind* III (to prove an insert of 1.9 kb corresponding to the α-amylase gene) and *Bam*H I was chosen for the purposes of orientation, i.e. whether the α-amylase gene was cloned in the sense orientation relative to the T7 or SP6 promoter. The restriction enzyme *Eco*R I was chosen as this enzyme should linearise the plasmid. The plasmid pβACTSP6 was restricted with *Eco*R I (to confirm an insert of 3.8 kb) and *Bam*H I was chosen for purposes of orientation i.e. whether the β-actin gene sequence was cloned in sense orientation relative to the SP6 or T7 promoter. *Bgl* II was chosen as this restriction enzyme should linearise the plasmid as there is a *Bgl* II restriction site in the cloned β-actin fragment but not within the pGEM vector.



Figure 4.43 Restriction analysis of pSP6-AMY, pT7-AMY and pβACTSP6.

- Lane 1:** 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)
- Lane 2:** pSP6-AMY undigested
- Lane 3:** pSP6-AMY digested with *Hind* III (3.0; 1.9 kb)
- Lane 4:** pSP6-AMY digested with *Bam*H I (4.9 kb)
- Lane 5:** pSP6-AMY digested with *Eco*R I (4.9 kb)
- Lane 6:** pT7-AMY undigested
- Lane 7:** pT7-AMY digested with *Hind* III (3.0; 1.9 kb)
- Lane 8:** pT7-AMY digested with *Bam*H I (3.0; 1.9 kb)
- Lane 9:** pT7-AMY digested with *Eco*R I (4.9 kb)
- Lane 10:** pβACT-SP6 undigested
- Lane 11:** pβACT-SP6 digested with *Eco*R I (3.8; 3.0 kb)
- Lane 12:** pβACT-SP6 digested with *Bam*H I (6.0; 0.8 kb)
- Lane 13:** pβACT-SP6 digested with *Bgl* II (6.8 kb)
- Lane 14:** 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)

From Figure 4.43 it can be seen that the *Hind* III digests of pSP6-AMY and T7-AMY (lanes 3 and 7) confirm an insert of 1.9 kb. The *Bam*H I digest of pSP6-AMY (lane 4, Figure 4.43) yielding a fragment size of 4.9 kb indicates that

the α -amylase gene was cloned in sense orientation relative to the SP6 promoter, whereas the *Bam*H I digest of pT7-AMY (lane 8, Figure 4.43) yielded the expected 2 bands of 3.0 and 1.9 kb bands to prove that the α -amylase gene was cloned in sense orientation relative to the T7 promoter. Restriction digestion of these two plasmids with *Eco*R I (lanes 5 and 9) yielded the expected single band of 4.9 kb.

Restriction digestion of p β ACT-SP6 with *Eco*R I demonstrates an insert of the expected size of 3.8 kb (lane 11, Figure 4.43). The *Bam*H I digest yielding bands of 6.0 and 0.8 kb respectively (lane 12, Figure 4.43) indicates that the β -actin gene sequences had cloned in sense orientation relative to the SP6 promoter. If this gene had cloned in sense orientation relative to the T7 promoter band sizes of 3.8 and 3.0 kb would have been expected. The *Bgl* II restriction data (lane 13, Figure 4.43) linearised the plasmid to give a fragment size of 6.8 kb. In conclusion, the restriction data obtained (Figure 4.43) confirms the construction of the plasmids pSP6-AMY, pT7-AMY and p β ACT-SP6.

Total RNA was isolated from DBY746, DBY746::pSOC-01, SI and SI::pSOC-01 under the conditions described in section 2.6.10. The RNA was separated on an agarose - formaldehyde gel and bidirectionally transferred to a nitrocellulose membrane. The first blot (Figure 4.45) was probed with a 32 P-labelled sense α -amylase riboprobe and the second blot with both a 32 P-labelled antisense α -amylase riboprobe and a β -actin riboprobe.

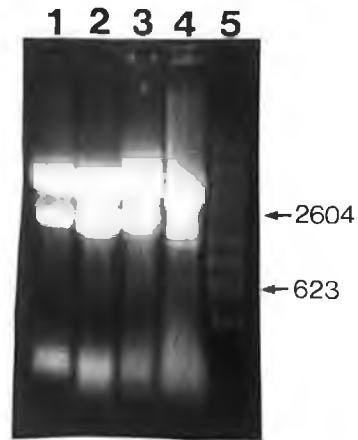


Figure 4.44 Formaldehyde agarose gel of total RNAs.

Lane 1:	DBY746
Lane 2:	SI
Lane 3:	DBY746::pSOC-01
Lane 4:	SI::pSOC-01 (B)
Lane 5:	RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)

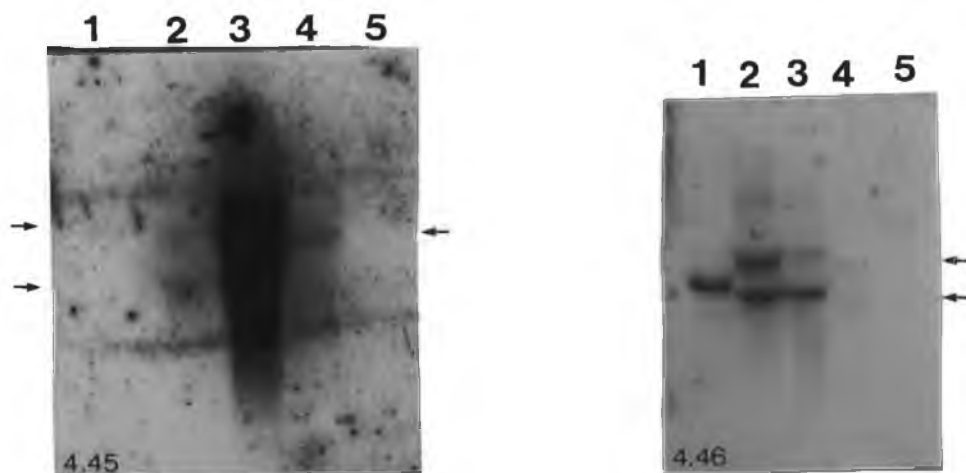


Figure 4.45 Bi-directional Northern blot of total RNAs probed with a ^{32}P -labelled sense α -amylase riboprobe.

Figure 4.46 probed with a ^{32}P -labelled antisense α -amylase and β -actin riboprobe.

Lane 1:	DBY746
Lane 2:	SI
Lane 3:	DBY746::pSOC-01
Lane 4:	SI::pSOC-01 (B)
Lane 5:	RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)

The Northern blot analysis was performed as previously mentioned to answer the following questions;

1. Is there anti-message being made?
2. Is there temporal coincidence of the two transcripts?
3. Are both RNAs stable? And finally,
4. What are the relative ratios of antisense:sense RNA?

After hybridisation with the sense probe (Figure 4.45) the following observations were made. Firstly, and most importantly a band of the expected size for the antisense α -amylase transcript (1.9 kb) can be seen in the lane containing the

integrated yeast strain SI::pSOC-01(B) (lane 4, equivalent in size to that seen in lane 3 containing DBY746::pSOC-01). However for some unexplained reason there appears to be far less antisense α -amylase transcripts in SI::pSOC-01 compared with DBY746::pSOC-01. As expected, DBY746 and SI strains alone (lanes 1 and 2, Figure 4.45) do not have the 1.9 kb antisense α -amylase transcript. It should be noted that 2 non-specific bands of sizes (2.25 and 1.15 kb) appear in lane 2 (Figure 4.45). These 2 bands have appeared on other Northern blots in samples extracted from yeast samples containing plasmid DNA.

When the blot was probed with an antisense α -amylase probe, a band of the expected size for the sense transcript (i.e. 1.9 kb) is visible in lanes 2 and 4 (SI, SI::pSOC-01, figure 4.46), but is not present in lanes 1 and 3, (Figure 4.46 DBY746 strains rather than SI), however a non-specific band of 2.1 kb does appear in lane 3. The presence of the β -actin band is visible in lanes 1 to 3 (Figure 4.46), but has not hybridised to the sample in lane 4 (Figure 4.46). It might be possible to explain the absence of this band simply in terms of poor hybridisation overall of the RNA in this lane to all probes used. In summary the following overall conclusions can be made;

1. anti-message is being made,
2. there was temporal coincidence of the two transcripts,
3. both the sense and antisense transcripts are discrete bands of the expected sizes of 1.9 kb respectively.

Finally, although the β -actin probe did not bind strongly in lane 4 (Figure 4.46), on measuring the band intensities of the sense and antisense transcripts produced from SI::pSOC-01 on a Pharmacia-Biotech (ImageMaster 1D) Imaging densitometer (taking into account the backgrounds) an estimation of the ratio of sense : antisense transcripts was obtained and calculated to be 1:4. Based on the known copy number of pSOC-01 and the integrated sense gene number in SI (10:2), this observed transcript ratio of 4:1 does reflect the copy number data.

Copy number analysis has been performed on the plasmid pSOC-01 based on Southern hybridisation (data not shown) and the result obtained showed that the plasmid pSOC-01 was present in DBY746 between 9 - 11 copies per cell. In agreement with this result was the result obtained from copy number analysis of a similar plasmid, pJG317, (which has a β -glucanase gene inserted

into the vector pAAH5) it was found to be present in DBY746 at 10 - 11 copies per cell (Canavan, 1994). Also Korpela et al., (1987) measured the plasmid copy number of pAAH5 as 10 copies per cell. Although the plasmid copy number was lower than expected there is still an excess of antimessage to message being produced. However the amount of antimessage being produced is not as high as that recommended for studies of this kind (Izant and Weintraub, 1984, Melton, 1985). In order to try and increase the levels of antisense transcript, two alternative approaches were taken. These are described in *trans*-Experiment I (b) (this chapter) and *trans*-Experiment II (chapter 5).

4.1.3 DISCUSSION

The first half of *trans*-Experiment I (a) dealt with the construction of a yeast strain containing an integrated sense α -amylase gene in as low a copy number as possible. This was achieved successfully and the integrated copy number of the α -amylase gene was 2. A full-length antisense α -amylase gene was introduced into the integrated strain on the plasmid pSOC-01, this plasmid being based on the yeast shuttle vector pAAH5. pAAH5 was chosen initially as Armstrong et al., (1989) reported that yeast-shuttle vectors which contain a 2 μ origin of replication can exist in high copy numbers (from 10 - 40 copies per cell) in [*cir*⁺] yeast cells. It was hoped therefore to that by using this system we would be able to produce antisense RNA in 10 - 20 times excess of the sense message assuming that no transcription factors are limiting. However copy number analysis of pSOC-01 in yeast strain DBY746 revealed a copy number of between 9 - 11 plasmids per cell. This number was lower than what was anticipated and even though there was an excess of antisense mRNA to mRNA (4:1) perhaps the excess was inadequate to mop up all the sense transcripts and hence this is one of the possible explanations for the lack of inhibition of α -amylase gene activity.

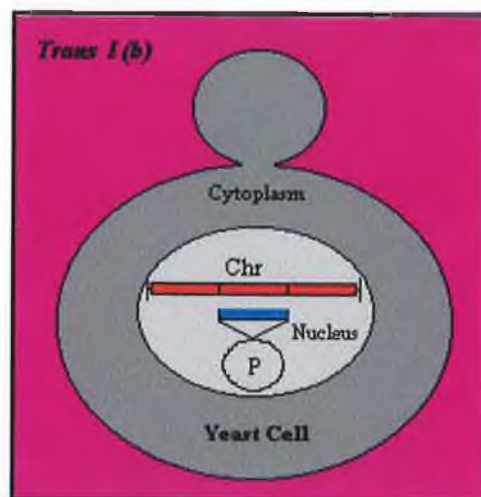
In many cases, the lack of inhibition by antisense genes has been attributed to the instability of the transcribed antisense RNA (Kim and Wold, 1985; Nishikura and Murray, 1987; Delauney et al., 1988; van der Krol et al., 1988b). In *trans*-Experiment I (a), Northern blot analysis was used to examine the steady state level of antisense α -amylase RNA and sense mRNA in the



appropriate yeast transformants. This analysis suggested that the antisense RNAs were stable but possibly the excess of antimessage to message was inadequate to produce a regulatory effect on α -amylase gene expression.

Temporal coincidence of the antisense and sense α -amylase gene transcripts was monitored directly in this experimental system. This was achieved by northern analysis as a coincident hybridisation signal for both antisense and target RNA on a northern blot was assumed to indicate temporal coincidence. Following on from this point it was essential that liquid cultures were sampled appropriately and that growth and comparison between individual samples was carefully controlled. For analysis of all transformants each sample was taken at mid-log phase (an OD 0.8 - 1.0, usually after 36 hours) to ensure that all strains were at the same level of growth before comparisons were made. Time course analysis (both DNS and Northern) was also performed (data not shown) to assess the best time point to take samples for DNS and Northern analysis.

The results obtained from *trans*-Experiment I (a) led to the design of *trans*-Experiment I (b) where the main concern was to increase the amount of antisense RNA relative to sense RNA. From the early studies of Izant and Weintraub (1984) and Melton (1985) to more recent studies based on oligonucleotides, the emphasis has been on creating a situation in the cell where there is an excess of antisense to sense RNA. Izant and Weintraub (1984) co-injected an antisense thymidine kinase (TK) gene with a wildtype TK gene-containing plasmid into mouse TK⁻ L cell nuclei at a ratio of 100 to 200:1, respectively. Under these conditions there was a 4- to 5- fold reduction in the TK⁺ cell transformants compared to controls. Melton (1985) showed that injection of *Xenopus* oocytes with a 50-fold greater amount of antisense β -globin RNA resulted in complete blockage of β -globin mRNA translation. Therefore in this study, by increasing the antisense:sense message ratio it was hoped that a critical threshold would be reached after which α -amylase activity would begin to be down-regulated.

Trans-Experiment I (b)



 Sense amylase sequences
 Antisense amylase sequences

P; Plasmid
Chr, Chromosomal location

4.2 TRANS-EXPERIMENT I (b) - INTRODUCTION

Like the *trans* I (a) experimental system, the *trans* I (b) system also sought to generate a favourable anti-mRNA:mRNA ratio, as the importance of having excess antimessage to message has been stressed in many review articles (Daugherty et al., 1989). Although an excess of anti-mRNA was being produced in *trans* Experiment I (a), this ratio of 4:1, of anti-mRNA:mRNA was not sufficient to down-regulate expression of the α -amylase gene. It was decided to increase the amount of anti-mRNA being produced by the integration of the α -amylase antisense gene into the ribosomal DNA (rDNA) cluster of the *S. cerevisiae* strain DBY746. This region of the genome was chosen as it contains 140 tandem repeats of a 9.1 kb rDNA unit on chromosome XII. According to Lopes et al., (1989) who designed a vector that can integrate into the rDNA locus of *S. cerevisiae*, it is possible to integrate up to 140 copies of the vector which are stably maintained over long periods of growth and non-selective conditions. Furthermore by introducing the sense α -amylase gene on a low-copy number plasmid bearing an ARS-CEN origin of replication (ARS-CEN vectors are present in only 1 - 2 copies per cell) it was hoped that the amount of antimessage being made would be in far greater excess than the sense mRNA being produced from this plasmid. Once again the sense and antisense α -amylase genes were under the control of the same promoter to ensure temporal coincidence of the two RNAs.

Various different approaches based upon integration into reiterated chromosomal DNA have been adopted to generate stable multi-copy integrants. These strategies include integration into TY elements, delta elements and reiterated ribosomal DNA (rDNA) sequences. Sakai et al., (1991), developed a δ -integration system which utilises the δ sequences of the TY element on yeast chromosomes as a homologous recombination site. No less than 80 copies of δ -sequences are estimated to be present on the yeast chromosomes and integration of multiple copies of a heterologous gene enabled a stable overproduction of the encoded protein in rich medium, these integrations being mitotically stable. Unlike the 2μ -based, YEp-type multi-copy plasmid used to introduce

heterologous DNA into yeast which are not mitotically stable and tend to be lost during cultivation in rich media without a selective force. Using the δ -integration system Sakai et al., (1991), succeeded in overproducing human nerve growth factor at a level 3-4 times higher than that achieved by a 2 μ -based plasmid (Mochizuki et al., 1994).

The multiple integration vector chosen was pMIRY (for Multiple Integration in the Ribosomal DNA of Yeast) developed by Lopes et al., (1989). Lopes et al., integrated genes encoding *PGK* (phosphoglycerate kinase), *SOD* (superoxide dismutase) and thaumatin into a non-transcribed spacer region of the reiterated rDNA. These integrative transformations resulted in *LEU*⁺ transformants carrying 100 - 200 copies of the aforementioned genes.

Yeast vectors suitable for high level expression of heterologous proteins should combine a high copy number with a high mitotic stability under non-selective conditions. High stability can be ensured by integration of the vector into chromosomal DNA and the high copy number is ensured by targeting the vector to the reiterated rDNA yeast sequence. The ribosomal yeast DNA sequences present in the pMIRY2 vector are composed of part of the 26S rRNA gene, NTS1 (non-transcribed spacer), 5S RNA gene and NTS2 (see Figure 4.47 [rDNA alone]). Additionally, in order for amplification to occur, a strong selection pressure is required, which was created in the pMIRY vector (Appendix VI), by using as a selection marker an expression deficient gene (*LEU2d*) involved in the production of the basic nutrient leucine. The pMIRY vector chosen for this part of the study, fulfils all of the above criteria. Additionally, this vector can also be linearised at the unique *Kpn* I site present in its leucine deficient gene, to direct integration to the *LEU2* locus present on chromosome III. This would result in a lower copy number of plasmid integrations.



Figure 4.47 Genetic organisation of an rDNA unit from *Saccharomyces cerevisiae* (Lopes et al., 1996).

The first half of *trans*-Experiment I (b) describes the cloning of the *Bacillus licheniformis* α -amylase gene into the ARS-CEN vector pFL38 with subsequent analysis of its' α -amylase activity in comparison to the sense α -amylase integrants and the 2 μ based sense α -amylase plasmid pAAMY-01 (from *trans*-Experiment I (a)). The second half of this section will deal with the construction of the multiple integrating plasmid pMISOC, which contains the entire α -amylase gene cloned in antisense orientation relative to the *AdHI* promoter and terminator. Following this, will be a description of how this plasmid was integrated into DBY746 to produce the integrated strain MI, ensued by a characterisation of this integration event. The important results as with *trans*-Experiment I (a) come in this section with the introduction of the pFL38-AMY plasmid into this integrated yeast strain, MI. *Trans*-Experiment I (b) will conclude with a brief discussion on the results obtained.

4.2.1 CLONING OF THE *BACILLUS LICHENIFORMIS* α -AMYLASE GENE INTO A LOW COPY VECTOR

Once again the objective in this experimental system as with *trans*-Experimental system I (a), was to keep the sense α -amylase gene copy number low, (low relative to the antisense genes), but not too low so as to result in an insensitive assay. This objective was achieved by cloning the sense α -amylase gene into a low-copy vector (by virtue of its' ARS-CEN origin of replication, these plasmids are present in only 1 - 2 copies per cell), the assumption here being that the level of message will be reflected in the copy number. The vector chosen was pFL38 (Bonneaud et al., 1991, Appendix VII), as in addition to the

ARS-CEN origin of replication, this vector also possesses a different selection marker to the multiple integration vector, pMISOC. *URA3* being the marker present on pFL38. The α -amylase gene was cloned into the unique *Bam*H I site of pFL38 on an *AdHI* promoter - sense α -amylase gene - *AdHI* terminator cassette. By virtue of the fact that both the sense and antisense genes were under the control of the same promoter this should ensure temporal coincidence of both RNAs. The construction of this plasmid, which will be referred to as pFL38-AMY from now on, was constructed as outlined in the legend to Figure 4.48.

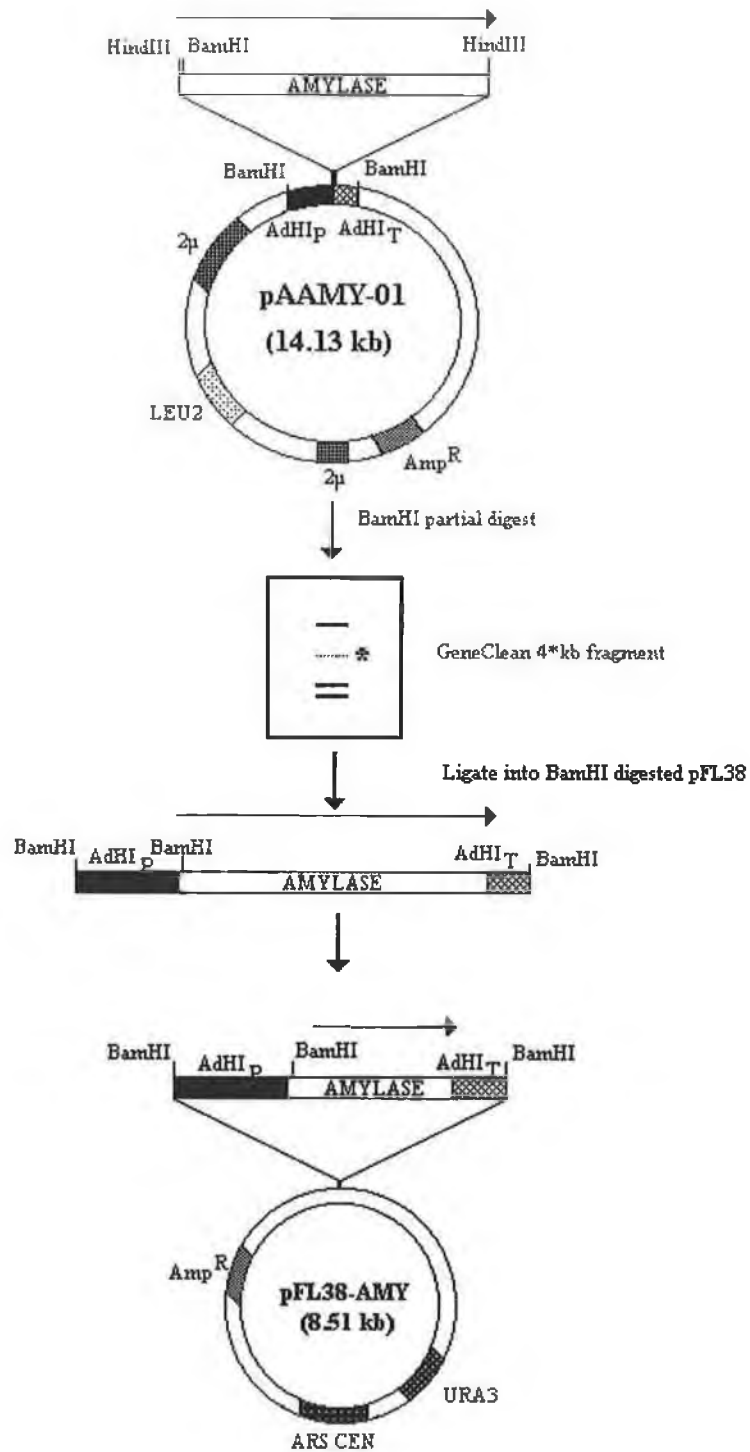


Figure 4.48 Construction of pFL38-AMY.

A 4.0 kb *Bam*H I partial containing the *AdHI* promoter - sense α -amylase gene - *AdHI* terminator from the plasmid pAAMY-01 was gene cleaned from a 0.7% agarose gel and ligated into the *Bam*H I digested pFL38 vector. The ligation mix was transformed into DH5 α *E. coli* cells and plated on LB amp plates containing 2% starch. With this clone orientation does not matter as the gene is in the correct orientation with respect to the *AdHI* promoter and terminator. This plasmid was named pFL38-AMY and is 8.51 kb in size.

Plasmid DNA was extracted from DH5 α *E. coli* transformants, which exhibited α -amylase activity. Restriction enzyme analysis was then carried out (Figure 4.49). Restriction of pFL38-AMY with the enzyme *Bam*H I should produce 3 bands, corresponding to the parental vector pFL38 (4.61 kb), the α -amylase gene and *AdHI* terminator (2.35 kb) and the *AdHI* promoter (1.55 kb). The DNA was also restricted with *Hind* III and *Sal* I to further confirm the construction of the plasmid pFL38-AMY.

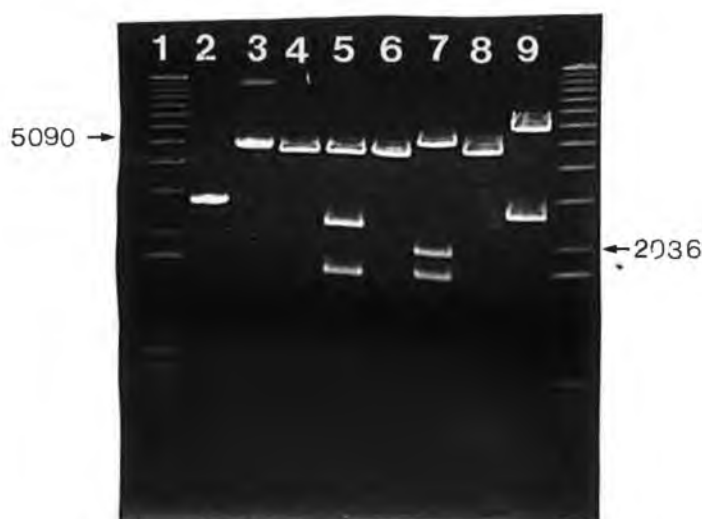


Figure 4.49 Restriction analysis of pFL38 and pFL38-AMY.

The approximate sizes of each of the restriction fragments is shown in base pairs in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 2:** pFL38 undigested
- Lane 3:** pFL38-AMY undigested
- Lane 4:** pFL38 digested with *Bam*H I (4,610 bp)
- Lane 5:** pFL38-AMY digested with *Bam*H I (4,610; 2,350; 1,550 bp)
- Lane 6:** pFL38 digested with *Hind* III (4,610 bp)
- Lane 7:** pFL38-AMY digested with *Hind* III (5,060; 1,900; 1,550 bp)
- Lane 8:** pFL38 digested with *Sal* I (4,610 bp)
- Lane 9:** pFL38-AMY digested with *Sal* I (5,830; 2,680 bp)
- Lane 10:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).

The *Bam*H I restriction digest of pFL38-AMY (lane 5, Figure 4.49) produced the expected bands of 4.61, 2.35 and 1.55 kb respectively. The *Hind* III digest (lane 7, Figure 4.49) released the expected 1.9 kb α -amylase gene fragment. The *Sal* I

restriction digest (lane 9, Figure 4.49) produced 2 bands of 5.83 and 2.68 kb consistent with the cloning of the 3.9 kb *AdHI* promoter -sense α -amylase gene - *AdHI* terminator cassette into pFL38.

4.2.1.1 Activity analysis of pFL38-AMY

This plasmid was transformed into DBY746 to estimate the level of α -amylase activity. Transformants were plated on minimal media supplemented with 2% Lintners starch. Visualisation of the plates after exposure to iodine vapour demonstrated that this plasmid does indeed express a functional product in *S. cerevisiae* strain DBY746. The actual level of amylolytic activity was assessed for 3 of these transformants by performing a DNS assay. The level expressed by these transformants, DBY746::pFL38-AMY1, 2 and 3 was compared to the levels expressed in the recombinant pAAMY-01 which carries the α -amylase gene on a higher copy number 2 μ episomally based vector. A comparison was also made with the integrated strains I₂, SI, SII and SIV (constructed in section 4.1.1) the copy numbers of which, in particular SI, should be similar to that of pFL38-AMY. DBY746 was used as a negative control. The results of this assay are tabulated in Table 4-5.

Table 4-5 Levels of α -amylase activity as produced by different plasmids and from various integrated strains.

Strain	Activity U/ml	Cell Number	Activity/Cell
DBY746	0	0	0
DBY746::pAAMY01	985.2	3.3×10^7	2.98×10^{-5}
DBY746::pFL38-AMY1	345.2	3.4×10^7	1.02×10^{-5}
DBY746::pFL38-AMY2	321.8	3.45×10^7	0.93×10^{-5}
DBY746::pFL38-AMY3	307.9	3.3×10^7	0.93×10^{-5}
I ₂	547.3	3.3×10^7	1.65×10^{-5}
SI	304.9	3.2×10^7	0.95×10^{-5}
SII	503.7	3.3×10^7	1.50×10^{-5}
SIV	578.6	3.5×10^7	1.65×10^{-5}

4.2.2 CONSTRUCTION AND ANALYSIS OF A MULTIPLE INTEGRATING PLASMID PRODUCING ANTISENSE RNA COMPLEMENTARY TO THE ENTIRE α -AMYLASE GENE

A pMIRY plasmid containing an expression cassette capable of expressing antisense α -amylase was constructed and named pMISOC. The plasmid is represented schematically in figure Figure 4.50 and the details of construction are included in the legend to the Figure 4.51. The plasmid construction was confirmed using diagnostic restriction enzyme analysis (Figure 4.53). Orientation of the 4.0 kb *Bam*H I insert into the parental vector pMIRY did not matter as the insert was a cassette consisting of the *AdHI* promoter - antisense α -amylase gene - *AdHI* terminator. The important factor being that the α -amylase gene was in antisense orientation relative to the *AdHI* promoter and terminator.

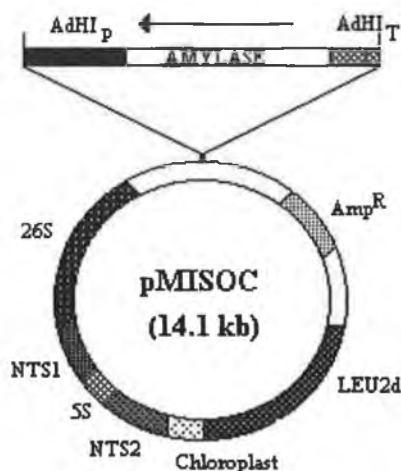
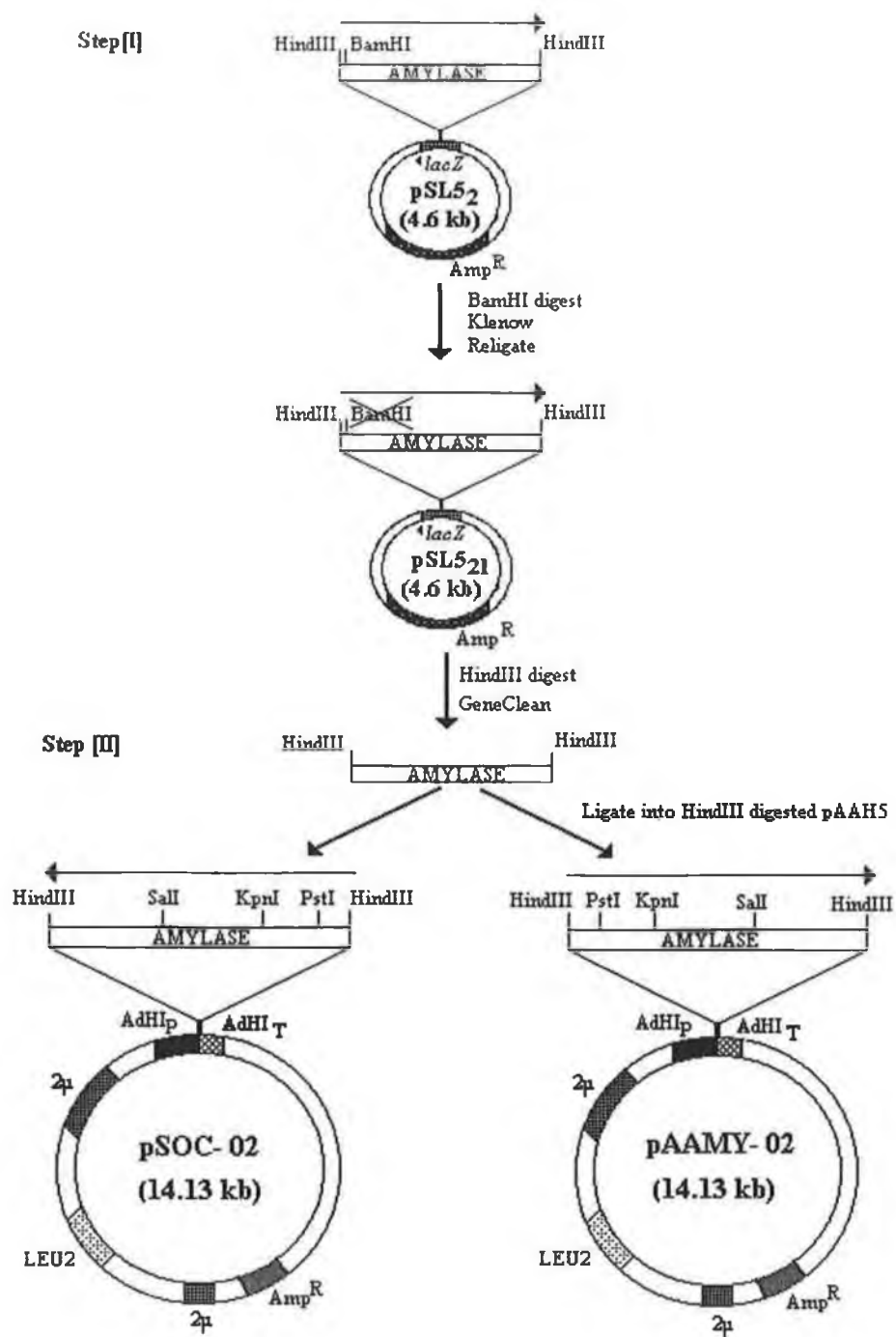


Figure 4.50 Schematic representation of the multiple integrating plasmid pMISOC which contains the α -amylase gene in antisense orientation relative to the *AdHI* promoter and terminator.



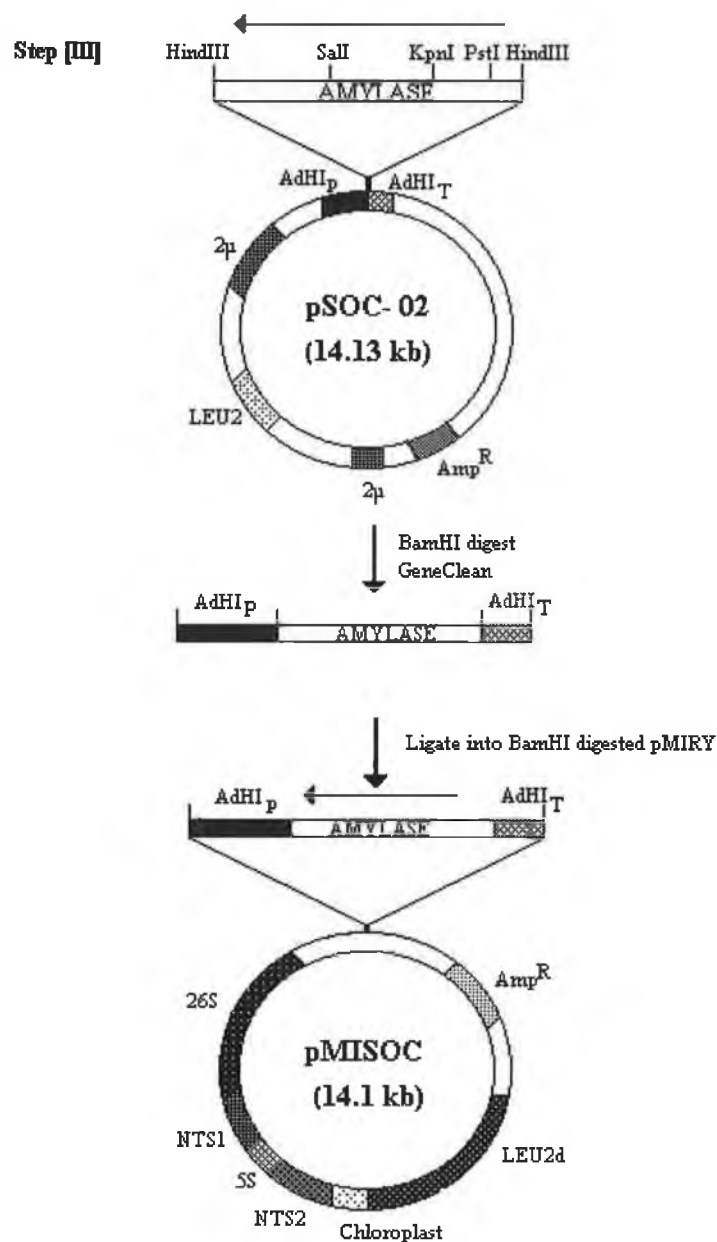


Figure 4.51 Steps involved in constructing the multiple integrating vector pMISOC.

The *AdHI* promoter - terminator cassette of pAAH5 (Appendix VIII) is on a 2.0 kb *Bam*HI I fragment, however because there is an internal *Bam*HI I site at the start of the α -amylase gene before the transcription start site, it meant partially digesting pSOC-01 to obtain a 4.0 kb *Bam*HI I fragment containing the *AdHI* promoter - antisense gene - *AdHI* terminator, which could then be cloned into the unique *Bam*HI I site of pMIRY. The partial digest proved to be somewhat refractory, therefore another approach was adopted.

Step I: Involved the removal of the internal *Bam*H I site from the α -amylase gene cloned into pSL5₂ by *in vitro* mutagenesis. This was achieved by digesting pSL5₂ with *Bam*H I, the ends were filled-in using the Klenow enzyme. After this the mixture was phenol extracted twice, chloroform washed once, ethanol precipitated and washed. Following this the contents of the microfuge tube were religated with T₄ DNA ligase. The mixture was transformed into *E. coli* DH5 α cells. Clones were screened by digesting with *Bam*H I to confirm the removal of this site, followed by digestion with *Hind* III to release the 1.9 kb α -amylase gene. This plasmid now being called pSL5₂₁. **Step II** involved subcloning the *Hind* III α -amylase gene from pSL5₂₁ into the unique *Hind* III site of pAAH5. The ligation mixture was transformed into *E. coli* DH5 α cells and resulting transformants were screened. Plasmids which contained the α -amylase gene in sense orientation were designated pAAMY-02; plasmids which contained the gene in the antisense orientation were designated pSOC-02. Orientation was confirmed using *Kpn* I as the diagnostic enzyme. pAAMY-02 giving fragment sizes of 7.49, 4.14 and 2.5 kb. pSOC-02 giving fragment sizes of 6.77, 4.86 and 2.5 kb (figure 4.52). Finally, in **Step III** the 3.9 kb *Bam*H I *AdHI* promoter - antisense α -amylase gene - *AdHI* terminator cassette was cloned after gene cleaning the fragment from a 0.7% agarose gel into the unique *Bam*H I site of the multiple integrating vector pMIRY to yield pMISOC. With this clone orientation obviously does not matter as the gene is in correct orientation with respect to the *AdHI* promoter and terminator.

The construction of pSOC-02 and pAAMY-02 (step [II], Figure 4.51) was verified by restriction enzyme analysis. The restriction enzyme *Kpn* I was chosen to demonstrate the antisense (pSOC-02) and sense (pAAMY-02) orientation of the α -amylase gene insert into pAAH5, whereas *Bam*H I was used to demonstrate the successful removal of the internal *Bam*H I site of the α -amylase gene.

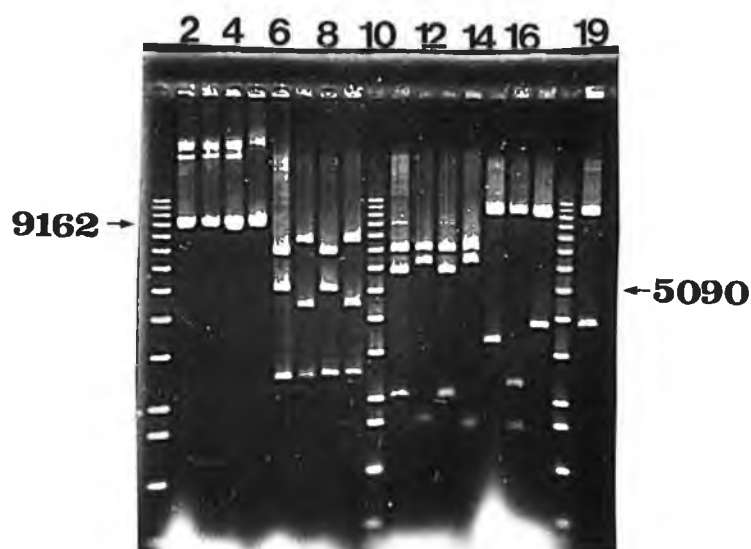


Figure 4.52 Restriction analysis of plasmids pSOC-02 and pAAMY-02.

The approximate sizes of each of the restriction fragments is shown in base pairs in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 2:** pSOC-01 undigested
- Lane 3:** pAAMY-01 undigested
- Lane 4:** pSOC-02 undigested
- Lane 5:** pAAMY-02 undigested
- Lane 6:** pSOC-01 digested with *Kpn* I (6,770; 4,860; 2,500 bp)
- Lane 7:** pAAMY-01 digested with *Kpn* I (7,490; 4,140; 2,500 bp)
- Lane 8:** pSOC-02 digested with *Kpn* I (6,770; 4,860; 2,500 bp)
- Lane 9:** pAAMY-02 digested with *Kpn* I (7,490; 4,140; 2,500 bp)
- Lane 10:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 11:** pSOC-01 digested with *Sal* I (6,750; 5,520; 1,860 bp)
- Lane 12:** pAAMY-01 digested with *Sal* I (6,750; 5,880; 1,500 bp)
- Lane 13:** pSOC-02 digested with *Sal* I (6,750; 5,520; 1,860 bp)
- Lane 14:** pAAMY-02 digested with *Sal* I (6,750; 5,880; 1,500 bp)
- Lane 15:** pSOC-01 digested with *Bam*H I (10,230; 3,450; 450 bp)
- Lane 16:** pAAMY-01 digested with *Bam*H I (10,230; 2,350; 1,550 bp)
- Lane 17:** pSOC-02 digested with *Bam*H I (10,230; 3,900 bp)

Lane 18: 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).

Lane 19: pAAMY-02 digested with *Bam*H I (10,230; 3,900 bp)

The *Kpn* I restriction data clearly demonstrates the antisense and sense orientation of the α -amylase gene, with the expected fragment sizes of 6.77, 4.86 and 2.5 kb (lane 8, Figure 4.52) and 7.49, 4.14 and 2.5 kb (lane 9, Figure 4.52) respectively. The successful removal of the internal *Bam*H I site from the α -amylase gene was demonstrated by restriction digestion of pSOC-02 and pAAMY-02 with *Bam*H I, which gave the expected band sizes of 10.23 and 3.9 kb respectively (lanes 17 and 19, Figure 4.52). In contrast, the plasmids pSOC-01 and pAAMY-01 which contain the internal *Bam*H I site gave fragment sizes of 10.23, 3.45 and 0.45 kb (lane 15, Figure 4.52) and 10.23, 2.35 and 1.55 kb (lane 16, Figure 4.52) respectively.

Finally, verification of the cloning of the 3.9 kb *AdHI* promoter - antisense α -amylase gene - *AdHI* terminator into the multiple integrating vector pMIRY was also achieved by restriction enzyme analysis (Figure 4.53).

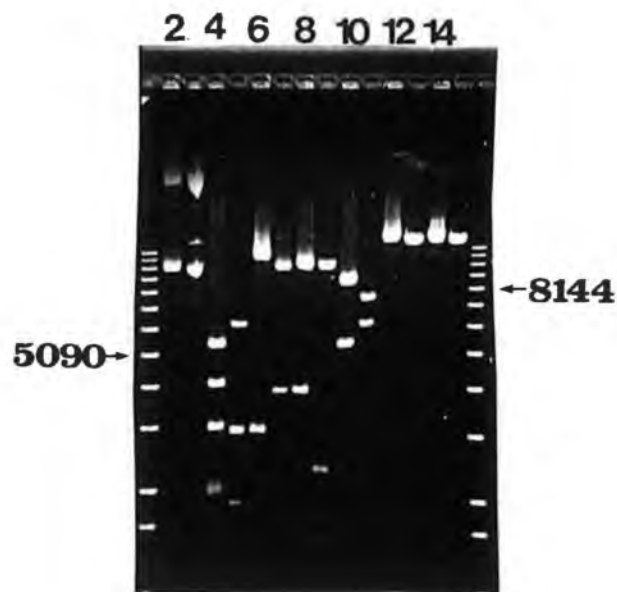


Figure 4.53 Restriction analysis of pMISOC (and pMIAMY, which does contain a *Bam*H I site in the α -amylase gene insert).

The approximate sizes of each of the restriction fragments is shown in base pairs (bp) in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- Lane 1:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 2:** pMISOC undigested
- Lane 3:** pMIAMY undigested
- Lane 4:** pMISOC digested with *Hind* III (5,350; 3,950; 2,900; 1,900 bp)
- Lane 5:** pMIAMY digested with *Hind* III (6,450; 2,900; 2,850; 1,900 bp)
- Lane 6:** pMISOC digested with *Pst* I (11,200; 2,900 bp)
- Lane 7:** pMIAMY digested with *Pst* I (10,100; 4,000 bp)
- Lane 8:** pMISOC digested with *Bam*H I (10,200; 3,900 bp)
- Lane 9:** pMIAMY digested with *Bam*H I (10,200; 2,350; 1,550 bp)
- Lane 10:** pMISOC digested with *Kpn* I (8,800; 5,300 bp)
- Lane 11:** pMIAMY digested with *Kpn* I (7,700; 6,400 bp)
- Lane 12:** pMISOC digested with *Sal* I (14,100 bp)
- Lane 13:** pMIAMY digested with *Sal* I (14,100 bp)
- Lane 14:** pMISOC digested with *Sma* I (14,100 bp)
- Lane 15:** pMIAMY digested with *Sma* I (14,100 bp)
- Lane 16:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).

The band of 3.9 kb visualised on the ethidium bromide stained agarose gel following restriction digestion of pMISOC with *Bam*H I (lane 8, Figure 4.53) constitutes proof of the cloning of the *AdHI* promoter - antisense α -amylase - *AdHI* terminator into pMIRY. Linearisation of the plasmid with *Sal* I (lane 12, Figure 4.53) or *Sma* I (lane 14, Figure 4.53) also gave the predicted band size of 14.1 kb. Orientation of the antisense α -amylase gene cloned between the *AdHI* promoter - terminator cassette in the plasmid pMISOC was demonstrated by restriction with either *Hind* III (lane 4, Figure 4.53), *Pst* I (lane 6, Figure 4.53) or *Kpn* I (lane 10, Figure 4.53). For comparison purposes the plasmid pMIAMY which contains the sense α -amylase gene cloned between the *AdHI* promoter - terminator cassette was restricted with the same enzymes as the plasmid pMISOC.

4.2.3 INTEGRATION OF THE ANTISENSE α -AMYLASE GENE INTO THE RIBOSOMAL DNA OF *SACCHAROMYCES CEREVISIAE* TO PRODUCE THE STRAIN MI.

Plasmid pMISOC was transformed into *S. cerevisiae* DBY746 cells after linearisation of this plasmid at the unique *Sma* I site (Figure 4.54) which cuts within the rDNA sequences on the vector and serves to direct integration into the rDNA loci. After integrative transformation the cells were plated on selective media minus leucine. The plates were also supplemented with 2% Lintners starch and the plates were analysed for a lack of amylolytic activity. As expected the transformants displayed no haloes when subjected to iodine vapour.

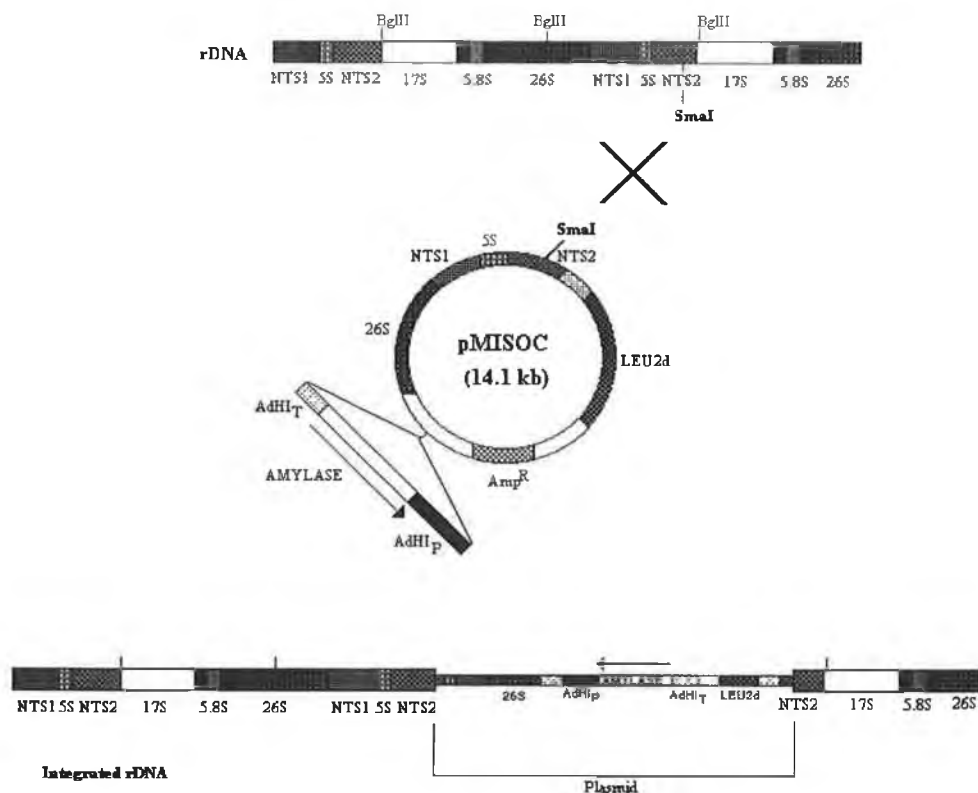


Figure 4.54 Schematic representation of most likely integration event.

4.2.3.1 Characterisation of the integration event

4.2.3.1.1 *Growth on selective plates*

Only the transformants which received the *LEU2d* genes from the plasmid pMISOC which complements the *LEU2* mutations of the host cell would be capable of growth on leucine minus minimal media. As the plasmid pMISOC lacks an origin of replication, in addition to possessing a leucine deficient marker, growth of transformants on media lacking leucine would strongly suggest the presence of the integrated plasmid. As DBY746 is auxotrophic for both uracil and leucine, growth on minimal media containing only uracil constitutes proof of the presence of the integrated plasmid.

4.2.3.1.2 Stability of the Integrated strain

Yeast transformants were grown initially in minimal medium, then they were transferred to YEPD media where they were kept growing for over 100 generations by repeatedly transferring them into fresh YEPD medium (see methods section 2.9). The percentage of cells with the *LEU*⁺ marker was then determined by plating single colonies on YEPD and checking for the loss of the plasmid associated marker by replica plating onto selective medium. The episomal plasmid pAAMY-01 was used as a control. The number of *URA*⁺ cells harbouring the YE_p-type plasmid pAAMY-01 which contains the α -amylase gene, showed a decrease over the first few generations and dropped to approximately 10.0% after 50 generations. In contrast, no decrease in the number of *LEU*⁺ cells was observed among the rDNA integrants after 100 generations. These results indicated that the rDNA integrated sequences were maintained very stably during mitotic growth in rich medium. The high stability of the *LEU*⁺ phenotype was also another indication that integration into the yeast genome had occurred.

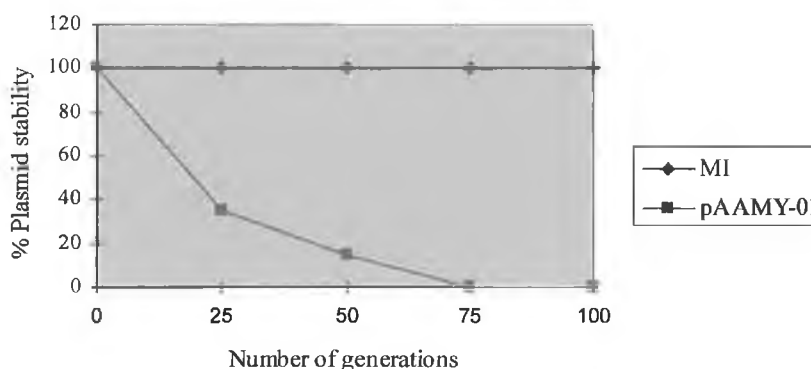


Figure 4.55 Graph depicting the mitotic stability of the integrated strain, MI.

4.2.4 ANALYSIS OF THE EFFECT OF THE ANTISENSE α -AMYLASE RNA TRANSCRIBED FROM THE INTEGRATED STRAIN, MI, ON THE SENSE α -AMYLASE RNA TRANSCRIBED FROM pFL38-AMY

After transformation of pFL38-AMY into MI, the cells were plated onto leucine and uracil minus minimal media (*leu*⁻ and *ura*⁻, leucine being provided

by the plasmid pMISOC and uracil from the pFL38-AMY plasmid) supplemented with 2% starch. From the initial visualisation of the plates it appeared that the α -amylase gene was still active as clear haloes were seen surrounding the transformants after exposure to iodine vapour (Figure 4.56). Three transformants were taken for further analysis and a DNS assay was performed on the supernatants after 36 hours growth in minimal media at 30°C, to assess if any down regulation of the enzymes' activity had occurred. The positive control used was DBY746 transformed with pFL38-AMY (DBY746::pFL38-AMY) alone and the negative control was DBY746.



Figure 4.56 α -amylase plate activity assays for several DBY746 transformants.

The three transformants were assayed in triplicate and the entire assay was repeated 3 times. The cumulative results of this experiment are represented by the bar chart shown below (Figure 4.57). Results are reported as the % relative amylase activity in comparison to the control strain DBY746::pFL38-AMY (100% activity).

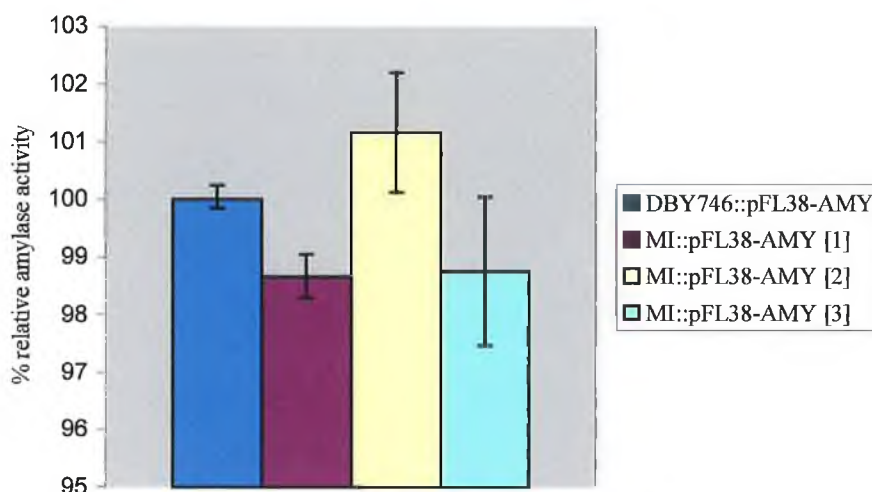


Figure 4.57 Bar Chart depicting the effect of the antisense plasmid pMISOC on the levels of α -amylase produced by the plasmid pFL38-AMY.

From the bar chart (Figure 4.57) one can see that the presence of the integrated antisense RNA construct, pMISOC, did not downregulate to any significant extent, or ablate the expression of the α -amylase gene, produced from pFL38-AMY.

4.2.5 RNA ANALYSIS

One of the transformants was analysed using Northern hybridization, to determine the level of antisense transcripts in this transformant. Total RNA was isolated under the conditions described in section 2.6.10. RNA was separated on an agarose - formaldehyde gel and bidirectionally transferred to a nitrocellulose membrane. The first blot was probed with a sense α -amylase riboprobe (section 4.1.2.4) to detect all antisense transcripts (Figure 4.58) and the second blot was probed with an antisense α -amylase riboprobe (section 4.1.2.4) to detect all the sense transcripts (Figure 4.59). This second blot was also probed with a β -actin riboprobe (section 4.1.2.4) to enable a comparison of the intensities of loadings and secondly to allow a comparison between the sense transcripts produced by SI and DBY746::pFL38-AMY (lane 2 and 7, Figure 4.59). Northern analysis revealed that no antisense RNA was being produced from the integrated strain MI (lane 6 and 8, Figure 4.58). However as was expected due to the visualisation

of starch breakdown on agar plates, a 1.9 kb sense mRNA was being produced by the transformants DBY746::pFL38-AMY and MI:: pFL38-AMY (lane 7 and 8, Figure 4.59). This Northern was repeated 3 times and on each occasion the result was the same.

DBY746 (lane 1, Figure 4.58 and Figure 4.59) was loaded as a negative control in this experiment, as expected no bands were visualised in this lane when probed with both the sense and antisense riboprobes. SI (lane 2, Figure 4.59) was run as a positive control for the antisense riboprobe and also so that a comparison of its' mRNA levels to the mRNA levels produced from the low copy number plasmid pFL38-AMY could be made. DBY746::pSOC-01 (lane 3, Figure 4.58) was run as a positive control to confirm that the sense riboprobe was capable of binding to antisense transcripts.

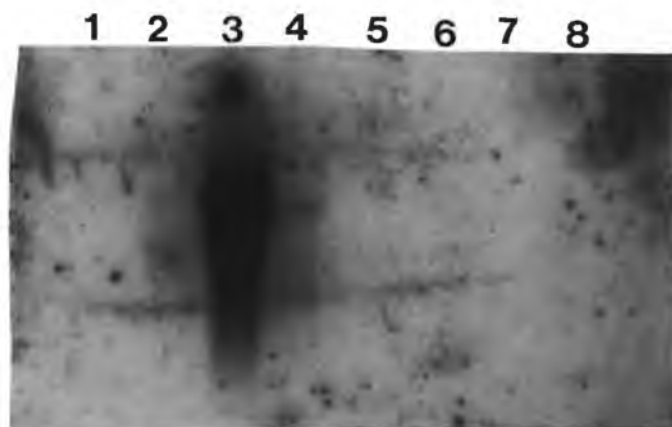


Figure 4.58 Northern blot of total RNAs probed with a ^{32}P -labelled sense α -amylase riboprobe.

- Lane 1:** DBY746
- Lane 2:** SI
- Lane 3:** DBY746::pSOC-01
- Lane 4:** SI::pSOC-01
- Lane 5:** RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)
- Lane 6:** MI
- Lane 7:** DBY746::pFL38-AMY
- Lane 8:** MI:: pFL38-AMY

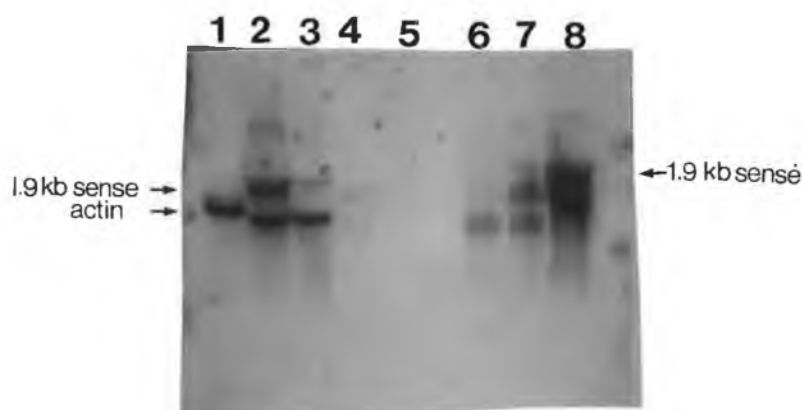


Figure 4.59 Northern blot of total RNAs probed with a ^{32}P -labelled antisense α -amylase and β -actin riboprobe.

Lane 1:	DBY746
Lane 2:	SI
Lane 3:	DBY746::pSOC-01
Lane 4:	SI::pSOC-01
Lane 5:	RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)
Lane 6:	MI
Lane 7:	DBY746::pFL38-AMY
Lane 8:	MI::pFL38-AMY

The fact that a 1.3 kb discrete β -actin band was present in MI and MI::pFL38-AMY (lanes 6 and 8, Figure 4.59) indicates that the RNA samples were undegraded. Thus if antisense RNA was produced it must be selectively degraded. Temporal coincidence as with *trans*-Experiment I (a) should have been ensured by the presence of the *AdHI* promoter on both the integrating and episomal plasmids.

Hybridisation of the antisense riboprobe to the sense transcript in the lanes containing DBY746::pFL38-AMY and MI::pFL38-AMY (lane 7 and 8, Figure 4.59) whose transcripts should have been present in lower copy numbers

than the antisense transcripts may indicate that the antisense plasmid did not integrate in the high copy numbers expected. Although integration did appear to take place as ascertained by growth of the strain on selective minimal media plates and also by the mitotic stability of the strain.

A further point to note here was that the copy number of the episomal plasmid pFL38-AMY which should be similar to the copy number of the integrant SI (from *trans*-Experiment I (a)) was indeed reflected in the mRNA levels. The ratio of sense transcript produced from SI in comparison to pFL38-AMY was 1.17:1. The fact that copy number of the sense integrated α -amylase gene in SI was 2 indicates that the copy number of pFL38-AMY was similar as reflected by its' RNA levels and also by its activity (section 4.2.1.1).

4.2.6 COPY NUMBER ANALYSIS OF THE INTEGRANTS

Due to the fact that no antisense RNA band was visualized on the Northern blots, it was decided to carry out further characterisation of the integrant, MI. Plasmid copy number was determined using the approach of Lopes et al., (1996). To perform this, total DNA was isolated from the integrant and digested with *Bgl* II. *Bgl* II digestion of the genomic ribosomal DNA produces two 4.5kb fragments, while this enzyme linearises the plasmid. The copy number of the plasmid according to Lopes et al., (1996) should have been able to be determined by electrophoresis of the digest on an 0.8% agarose gel and visual comparison of the intensities of the plasmid and rDNA bands after staining with ethidium bromide taking into account the relative lengths of the various fragments. If the plasmid had integrated at the rDNA loci one would expect to see the 14.1 kb band clearly on the agarose gel and then a comparison of intensities could have been made with the 4.5 kb rDNA band, however no strong band equivalent to the plasmid was visualised after ethidium bromide staining of the gel. This result may suggest once again the low copy number of the integrated antisense gene sequences.

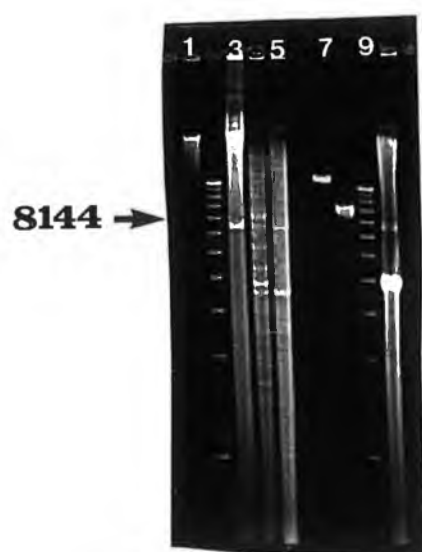


Figure 4.60 Agarose gel to allow determination of the plasmid copy number of integrations of the plasmid pMISOC.

- Lane 1:** DBY746 undigested
- Lane 2:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 3:** MI undigested
- Lane 4:** DBY746 digested with *Bgl* II
- Lane 5:** MI digested with *Bgl* II
- Lane 6:** -----
- Lane 7:** pMISOC digested with *Bgl* II
- Lane 8:** pMISOC undigested
- Lane 9:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 10:** MI::pFL38-AMY undigested

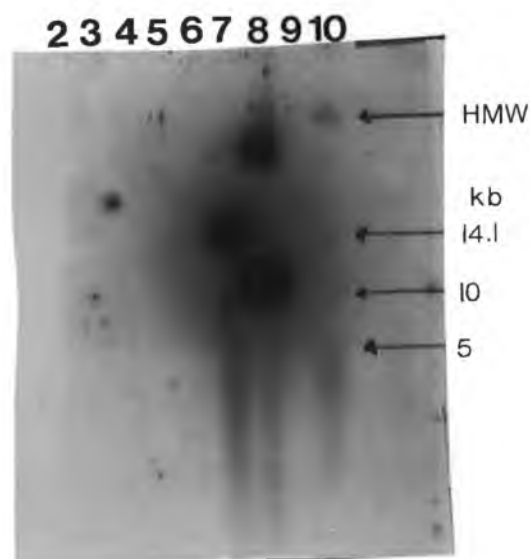


Figure 4.61 Southern blot to determine plasmid copy number of integrations of the plasmid pMISOC.

- Lane 1:** DBY746 undigested
- Lane 2:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 3:** MI undigested
- Lane 4:** DBY746 digested with *Bgl* II
- Lane 5:** MI digested with *Bgl* II
- Lane 6:** -----
- Lane 7:** pMISOC digested with *Bgl* II
- Lane 8:** pMISOC undigested
- Lane 9:** 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).
- Lane 10:** MI::pFL38-AMY undigested

The above gel was also blotted and probed with a ^{32}P -labelled α -amylase probe, unfortunately there was no hybridisation to the lanes containing MI (lanes 3 and 5, Figure 4.61). This indicates either that integration of the α -amylase gene was very low or that it did not occur at all. The fact that the integrant was able to grow in the absence of leucine however suggests that possibly that there may

have been integration of the *leu2-d* gene but loss of the remaining vector sequences.

The lane containing MI::pFL38-AMY undigested (lane 10, Figure 4.61) produced three weak bands and possibly a fourth “fuzzy” band marked by an asterisk. Two of these bands were of high molecular weight. One of these high molecular weight bands may represent the integrated plasmid pMISOC, while the other band may represent the linear form of the undigested plasmid pFL38-AMY. The remaining 2 bands may correspond to nicked and covalently closed circular forms of the undigested episomal plasmid pFL38-AMY. However because of the omission of running a sample containing undigested pFL38-AMY plasmid DNA this cannot be said for certainty. To overcome this shortcoming, if one sizes the linear, nicked and covalently closed circular forms of the plasmid pFL38-AMY from lane 3 (Figure 4.49), then make a comparison with the band sizes obtained in the lane containing MI::pFL38-AMY (lane 10, Figure 4.61). The band sizes of the linear, nicked and covalently closed circular forms of pFL38-AMY, from lane 3 (Figure 4.49) are of high molecular weight (> 12.2 kb), 11.0 and 5.0 kb respectively. If this data is applied to the lower 3 bands in lane 10 (Figure 4.61) containing MI::pFL38-AMY which are of sizes 14.1 kb (this band is the same size as that of the band visualized in lane 7, Figure 4.61), 10.0 and 5.0 kb respectively, on inference one could conclude that these 3 bands in lane 10 (Figure 4.61) do indeed represent the undigested pFL38-AMY bands. If the highest molecular weight band represents the integrated pMISOC plasmid and the remaining 3 bands the episomal pFL38-AMY, the fact that the intensities of the 3 bands are similar may suggest the low copy number of integration for pMISOC.

Finally, from Figure 4.60, there is an intense band of approximately 5 kb in lane 10 containing MI::pFL38-AMY, which was not highlighted by the probe, which indicates that this band does not contain any α -amylase gene sequences. It appears to be an anomalous band not related to either the integrating plasmid pMISOC (as it was not visualized in lane 3 containing MI), or the episomal plasmid pFL38-AMY (lane 10, MI::pFL38-AMY).

4.2.7 DISCUSSION

The failure to observe any inhibition of α -amylase activity in this system may have simply been due to the absence of the antisense construct. However, a successful integration event was implied by the growth of transformants on minimal media plates lacking leucine as the integrating vector possesses a *leu2-d* marker. In order for amplification to occur a strong selection pressure was required which it was hoped was created by using this selection marker. This marker expresses a deficient gene involved in the production of the nutrient leucine. In some systems where antisense RNA is successful in down-regulating or ablating the expression of a particular gene it has sometimes been the case that no anti-mRNA could be detected in the cell. In these cases this could be due to the fact that the anti-mRNA:mRNA hybrid has been recognised by nucleases specific for double-stranded RNAs (such as RNase H) and been degraded. This was not however, the situation in this experimental system as activity assays showed no reduction in α -amylase activity.

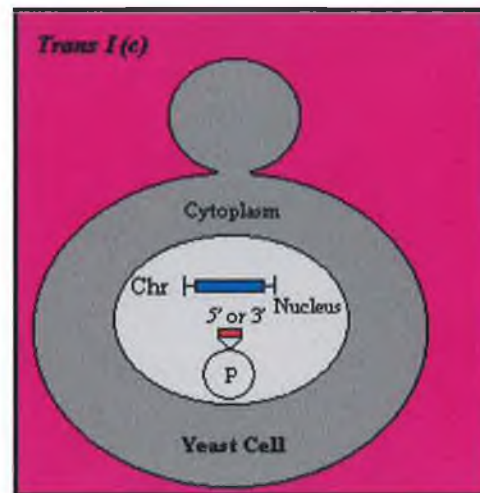
Although integration was targeted to the rDNA locus by linearising the plasmid within its' rDNA sequence it is possible that due to the large size of the plasmid (14.1 kb) that this may have led to instability and hence no amplification of the integrated sequences to the high copy numbers that have been reported by Lopes et al., (1996). Lopes et al., (1996) also reported that targeting of pMIRY to the *LEU2* locus by linearising the plasmid within the *leu2-d* gene never resulted in HCN transformants. Instead as for the more common type of YIP vectors only cells carrying a small number of plasmid copies were recovered from such transformants. Since these cells grew normally on medium lacking leucine, the result also demonstrates that 2 - 5 copies of the *leu2-d* gene are sufficient to provide the cells with an adequate supply of this amino acid, (Lopes et al., 1991). Hence a copy number of 2 for the integrating plasmid pMISOC would provide a sufficient amount of leucine for the cells to survive.

Analysis of the integrated plasmid copy number by a method described by Lopes et al., (1991 and 1996) failed to indicate a high copy number of integrations for the plasmid pMISOC. One theory as to why high copy number integration was unsuccessful may be the large size of the plasmid pMISOC. According to Lopes et al., (1996) plasmid length seems to influence the initial

copy number of pMIRY-type vectors. The copy number of pMIRY2-D (6.7 kb) was about 180, some 50% higher than the copy number typically observed for pMIRY2 (10.2 kb) transformants. Therefore a smaller size of integrated plasmid may also increase PCN, (Lopes, 1991). In addition stable maintenance is only observed when the complete plasmid has a size no larger than that of the rDNA unit (Lopes et al., 1996). It seems reasonable to assume therefore that this inverse correlation between plasmid length and copy number reflects a limitation in the amount of extra DNA that can be accommodated by chromosome XII.

Lopes et al., (1991) hypothesised that the utility of this vector may be reduced by its' location in the nucleolus where transcription is mainly dedicated to the rRNA (by RNA polymerase I) and not to protein genes. However it has been demonstrated that protein genes can be transcribed in the nucleolus (Lopes et al., 1991 got good expression of superoxide dismutase, *SOD* and thaumatin and McMahon, 1995 demonstrated the expression of the α -amylase gene cloned into this pMIRY vector) although the efficiency of such transcription might be limited to a greater or lesser extent by sub-optimal concentrations of RNA polymerase II and/or transcription factors (Lopes et al., 1991).

***Trans*–Experiment I (c)**



Sense amylose sequences
 Antisense amylose sequences

P; Plasmid
Chr, Chromosomal location

4.3 TRANS-EXPERIMENT I (c) – INTRODUCTION

An important consideration in the design of an antisense expression system, is the choice of the gene fragment which will provide the optimal effects for gene silencing. In such studies, antisense genes have been directed towards all regions of target genes, such as the 5' untranslated region (5' UTR), the AUG start codon, the coding sequence and the 3' UTR. Furthermore, various studies demonstrate that different fragments of the same gene may vary significantly in their ability to bring about an antisense effect. Phenotypic variation ranging from wild-type to a complete block of development have been seen using different fragments of the *MIHC* gene for antisense expression (Scherczinger and Knecht, 1993). A fragment overlapping the 5' end seems to be essential for the inactivation of the calmodulin gene (Liu et al., 1992), whereas for the *MIHC* gene a 3' fragment was found to be most effective (Scherczinger and Knecht, 1993). The influence of construct composition on the effectiveness of antisense inhibition has been studied for the suppression of the chalcone synthase (CHS) gene in *Petunia* (van der Krol et al., 1990), the granule bound starch synthase gene (GBSS) in potato (Kuipers et al., 1995) and the CAT enzyme activity in carrot protoplasts that transiently expressed both sense and antisense CAT genes introduced simultaneously by co-electroporation (Bourque and Folk, 1992). For *petunia*, inhibition of CHS gene expression, resulting in inhibited flower pigmentation, has been achieved with antisense genes based on either the full-length CHS cDNA or 3' cDNA. Antisense genes based on 5' fragments of the CHS cDNA were shown not to affect flower pigmentation (van der Krol et al., 1990).

Antisense inhibition of granule bound starch synthase (GBSS), in potato, was achieved using full-length cDNA constructs. Complete inhibition of GBSS gene expression could also be achieved with a construct based on a 0.7 kb internal fragment of the GBSS cDNA, and with a construct based on the 0.6 kb 3' end of the GBSS genomic DNA (gDNA). The latter fragment comprises transcribed GBSS sequences and contains the 3' end of the GBSS coding region (0.3 kb) including one intron. In carrot protoplasts, reduced CAT activity has been observed after co-electroporation of a sense CAT gene and an antisense gene based on either a 5' fragment, a 3' fragment, or the complete CAT gene

(Bourque and Folk, 1992). The inhibition was found to be the greatest after introduction of an antisense gene based on the 3' CAT gene fragment.

Arndt et al., (1995, section 1.5.3.6) constructed antisense genes to target the entire exogenous *lacZ* gene its' 5' and 3' regions, of the fission yeast *Schizosaccharomyces pombe*, to examine which, (if any), of these constructs was effective in reducing β -galactosidase activity. Olsson et al., (1997) chose to target three regions of the *S. cerevisiae MIG1* gene, comprising of the coding region alone or in combination with parts of the 5' untranslated region. Coleman et al., (1984, section 1.3.1.1), constructed several antisense genes from the *ompA* gene of *E. coli*, in order to find the most effective antisense RNA. Each of the antisense genes exerted an effect, however some antisense gene fragments were more successful than others. Daugherty et al., (1989, section 1.3.1.2), also performed many detailed experiments which examined the effects of different anti-*lacZ* RNAs of various lengths and representing different parts of the *lacZ* coding region. Once again their findings demonstrated that targeting certain regions of the gene was more successful than others.

The preceding observations demonstrate that although there have been numerous successful (and unsuccessful, Olsson et al., 1997) examples, in a diverse range of eukaryotic organisms, of where antisense genes have been used to down-regulate gene expression, the susceptible subregion of a specific target RNA for antisense RNA hybridisation, varies considerably between studies. This is due to the fact that each RNA molecule which is targeted has its' own characteristic secondary structure, location and may also be bound by ribonucleoproteins, therefore the accessibility of an RNA will vary from gene to gene, cell type to cell type and organism to organism. Due to these variables each antisense gene that is designed is designed on an empirical basis. In *trans*-Experiments I (a) it may have been that the intramolecular secondary structures of the two interacting RNAs, hindered an efficient interaction between the RNAs and it is possible that this problem may be overcome by using a different and/or shorter part of the target gene for the antisense construct. In the context of these findings, *trans*-Experiment I (c) was undertaken.

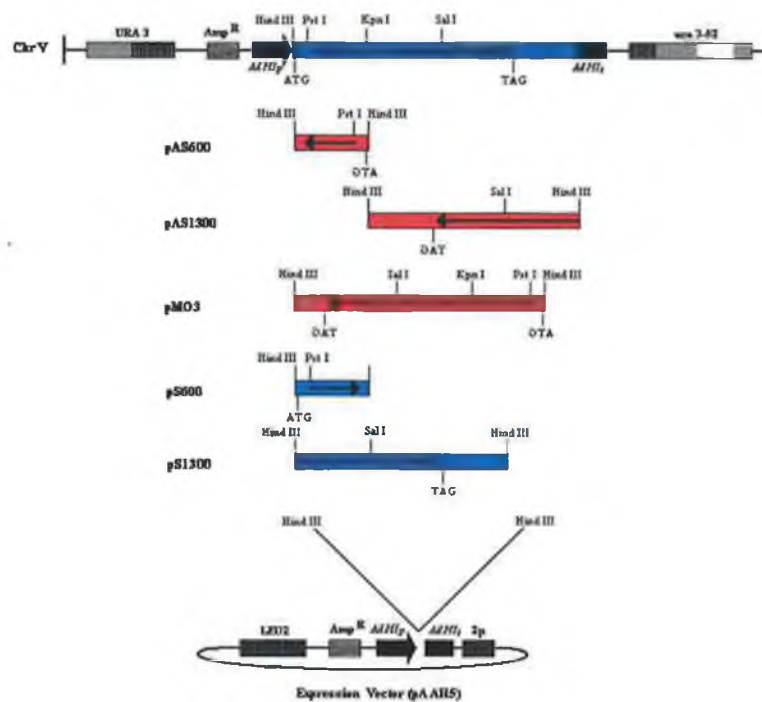
This experimental system focuses on the construction (and subsequent analysis) of truncated α -amylase antisense genes designed to target different regions of the gene. These regions included the following:

1. Antisense genes directed towards the 5' UTR for example, are expected to change the secondary or tertiary structure of an mRNA in order to inhibit the initiation of translation. The most common approach to inhibition of protein translation is to direct the antisense gene towards the AUG start codon because theoretically once antisense compounds are successfully hybridised in this region the ribosome will no longer be able to recognise the translation initiation site. Besides this region is also usually free of secondary structure presumably to allow the ribosome to bind.
2. If the coding region is targeted, it is possible that the antisense compounds may block the movement of the ribosome along the mRNA molecule or this double-stranded structure, formed between the sense and antisense genes, may provide a substrate for RNaseH cleavage.
3. Finally by targeting the 3' UTR this may increase the instability of the mRNA by removing the poly(A) tail (Mitsubishi, 1997).

The *trans* configuration in this experimental system is more or less identical to *trans* I (a), in that the sense α -amylase RNA was transcribed from the chromosomally located α -amylase gene in SI (where the gene copy number of the sense α -amylase gene was 2) and the newly constructed antisense genes were introduced on the higher copy number yeast episomal vector, pAAH5.

4.3.1 CONSTRUCTION OF YE_p PLASMIDS PRODUCING ANTISENSE RNA COMPLEMENTARY TO VARIOUS REGIONS OF THE *BACILLUS LICHENIFORMIS* α -AMYLASE GENE.

Since it is difficult to predict the most susceptible region of target mRNA for antisense RNA hybridisation, different regions of the α -amylase gene were inserted between the *AdHI* promoter and terminator (in the vector pAAH5) to produce a series of truncated constructs. For each antisense plasmid a corresponding control plasmid was constructed (see Figure 4.62). The antisense genes were constructed to produce antisense RNAs of varying sizes, complementary to different regions of the target message.



- Sense amylase sequences
- Antisense amylase sequences

Figure 4.62 Schematic representation of the α -amylase target gene and antisense and control plasmids.

The *Bacillus licheniformis* α -amylase gene, under the control of the *AdHI* promoter and terminator is shown (top) integrated at the *ura3-52* locus in *S. cerevisiae* chromosome V. DNA fragments of the α -amylase gene cloned into the *Hind* III site on the expression vector pAAH5 are shown below the α -amylase target gene. Arrows indicate the normal direction of transcription for a given segment of DNA. The plasmid designations for each insert are given beside each specific DNA fragment.

4.3.1.1 Construction of pAS600 and pS600.

A YE_p plasmid containing an expression cassette capable of expressing antisense α -amylase mRNA complementary to the 5' end of the sense amylase mRNA was constructed (see Appendix III for the complete sequence of the α -amylase gene, the exact positions of the truncated inserts and relevant restriction sites are also shown). This plasmid, pAS600 is represented schematically in Figure 4.63 and the details of construction are included in the legend to this figure. Plasmids pAS600 and pS600 differed only in the orientation of the 0.6 kb *Hind* III - α -amylase DNA insert relative to the *AdHI* promoter and *AdHI* terminator sequences. Transcription from an *AdHI* start site within the expression cassette located on plasmid pAS600 was expected to produce an antisense α -amylase-*AdHI* fusion transcript composed of antisense α -amylase RNA and a portion of the *AdHI* terminator mRNA. The antisense α -amylase RNA was complementary to 31 nucleotides of the 5' upstream region, including the ribosome binding site and 557 nucleotides of the coding part of the α -amylase RNA. Plasmid pS600 was constructed as a control plasmid in order to examine non-specific effects of high-level *AdHI* promoter driven transcription on the α -amylase levels. These plasmid constructions were confirmed using diagnostic restriction enzyme analysis. *Bam*H I digestion of pAS600 resulted in the production of three bands of sizes, 10.2 kb, 2.15 kb and 0.45 kb, while the same analysis of pS600 yielded 10.2 kb, 1.55 kb and 1 kb fragment sizes (Figure 4.64).

4.3.1.2 Construction of pAS1300 and pS1300.

In an attempt to determine whether the 3' end of the sense mRNA would be an effective template for antisense control, YE_p plasmids were constructed in which the middle to 3' end of the α -amylase gene and the 3' UTR region (600 - 1847 base pairs, Appendix III) were cloned into the unique *Hind* III site of the vector pAAH5 (see legend to Figure 4.63 for details of cloning). Plasmid pAS1300 (antisense) contained the insert in the antisense orientation relative to

the *AdHI* promoter and terminator, while plasmid pS1300 (sense) contained the insert in the sense direction. Transcription from the *AdHI* promoter on pAS1300 was expected to produce an antisense α -amylase-*AdHI* fusion transcript in which the antisense RNA sequences were complementary to the 3' coding and non-coding region of the *B. licheniformis* α -amylase mRNA.

A number of restriction digests were performed to confirm the plasmid constructions for pAS1300 and pS1300. Firstly a *Hind* III digest was carried out to confirm the presence of the insert which should release a 1.3 kb fragment from both plasmids and the vector band of 12.2 kb. Secondly, the orientation of the inserts was determined using *Sal* I as the diagnostic enzyme (Figure 4.64). *Sal* I digestion of pAS1300 resulted in three bands with molecular sizes of 6.75 kb, 5.45 kb and 1.27 kb. The same digest of pS1300 also produced three restriction fragments of the following sizes: 6.75, 5.30 and 1.4 kb.

STEP I

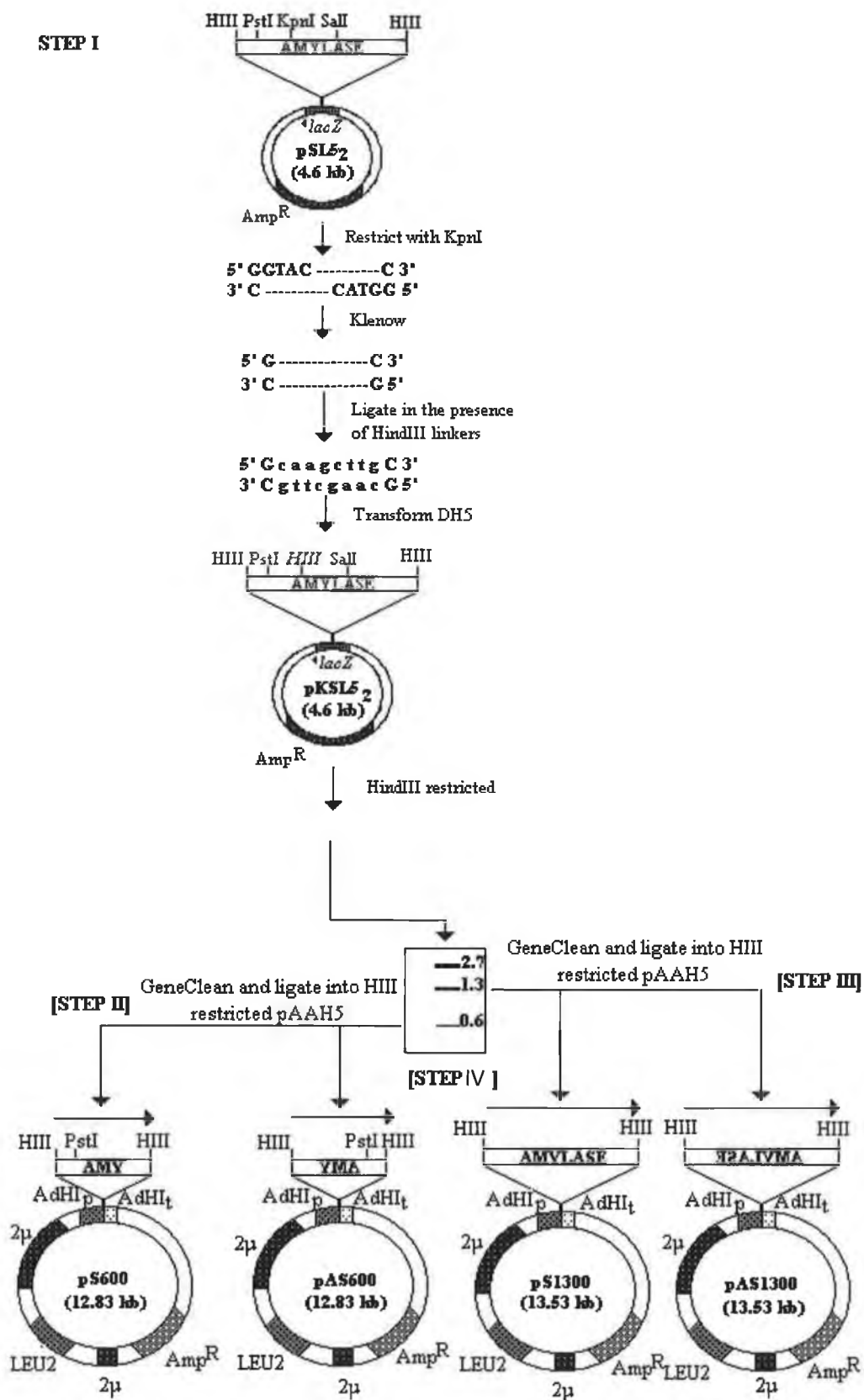


Figure 4.63 Construction of the YEp-antisense plasmids, pAS600 and pAS1300 harbouring the 5' end and the mid - 3' end respectively of the *B. licheniformis* α -amylase gene.

Step I: The plasmid pSL5₂ (4.6 kb, McMahon, 1995) which contains the sense α -amylase gene cloned in as a 1.9 kb *Hind* III fragment into the vector pUC8 (2.7 kb, Appendix V) was digested with the restriction enzyme *Kpn* I. This site was present approximately 557 base pairs into the 5' coding region of the α -amylase gene, (the pUC8 vector contains no *Kpn* I site). After digestion with *Kpn* I the 5' overhang was blunt-ended using the Klenow enzymes 3' - 5' exonuclease activity and an 8 mer (5'CAAGCTTG 3') *Hind* III linker was ligated onto the molecule, the plasmid was recircularised using T₄ DNA ligase and the ligation mix was transformed into DH5 α *E. coli* cells. After the Klenow reaction and *Hind* III linker addition the *Kpn* I site was destroyed, and this also abolished α -amylase activity. The transformants were screened for the presence of an additional *Hind* III site, the plasmid now possessing *Hind* III fragments of 2.7 kb, 1.3 kb and 0.6 kb respectively. This plasmid was named pKSL5₂ and the 0.6 kb [step II] and 1.3 kb [step III] *Hind* III fragments were then gene cleaned from a 0.7% TAE agarose gel.

Step IV: The yeast shuttle vector pAAH5 which possesses a unique *Hind* III site for cloning situated between the *AdHI* promoter and terminator was linearised with this restriction enzyme to produce a 12.2 kb band which was column cleaned (section 2.6.11.8) to remove any restriction enzyme buffer. It was then dephosphorylated with CIP (section 2.6.11.3) and this linearised vector fragment was ligated in the presence of the gene-cleaned 0.6 kb and 1.3 kb *Hind* III fragment of pKSL5₂ to produce the following plasmids pAS600, which has the 5' end of the α -amylase gene cloned in reverse orientation relative to the *AdHI* promoter and terminator; pS600, the sense version of this plasmid, contains the 5' end of the α -amylase gene cloned in sense orientation relative to the *AdHI* promoter and terminator; pAS1300, spanning the mid - 3' region of the α -amylase gene and pS1300, differing only in the orientation of the α -amylase DNA insert relative to the *AdHI* promoter and terminator.

4.3.1.3 Construction of pMO3.

The plasmid pMO3 was constructed by Olsen, (1995, Appendix IV, for reasons which will become clear in chapter 6). This plasmid contains a truncation of the α -amylase gene from *Bacillus licheniformis* inserted in antisense orientation relative to the *AdHI* promoter and terminator. The truncation involved the removal of a number of base pairs from the 3' flanking region of the α -amylase gene including the transcription termination signal. This truncated version of the α -amylase gene was 1.6 kb and its' transcripts would target the 5'UTR and 5' to 3' coding region of the sense α -amylase gene.

4.3.1.4 Confirmation of the constructions of pAS600, pS600, pAS1300, pS1300 and pMO3.

Plasmid DNA was extracted from *E. coli* DH5 α cells to confirm the construction of each of the above plasmids. The diagnostic restriction enzymes chosen were *Hind* III (to confirm the correct size of the α -amylase gene inserts), *Bam*H I was used to confirm the orientation of the inserts in pS600 and pAS600, finally *Sal* I was used to orientate the inserts in the plasmids pS1300, pAS1300 and pMO3 respectively.



Figure 4.64 Restriction Analysis of pAS600, pS600, pAS1300, pS1300 and pMO3.

The approximate sizes of each of the restriction fragments are shown in kilobases in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- Lane 1:** 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)
- Lane 2:** pS600 undigested
- Lane 3:** pAS600 undigested
- Lane 4:** pS1300 undigested
- Lane 5:** pAS1300 undigested
- Lane 6:** pMO3 undigested
- Lane 7:** pS600 digested with *Hind* III (12.23 kb; 0.6 kb)
- Lane 8:** pAS600 digested with *Hind* III (12.23 kb; 0.6 kb)
- Lane 9:** pS1300 digested with *Hind* III (12.23 kb; 1.3 kb)
- Lane 10:** pAS1300 digested with *Hind* III (12.23 kb; 1.3 kb)
- Lane 11:** pMO3 digested with *Hind* III (12.23 kb; 1.6 kb)
- Lane 12:** pS600 digested with *Bam*H I (10.23 kb; 1.55 kb; 1.05 kb)
- Lane 13:** pAS600 digested with *Bam*H I (10.23 kb; 2.15 kb; 0.45 kb)
- Lane 14:** pS1300 digested with *Sal* I (6.75 kb; 5.29 kb; 1.45 kb)
- Lane 15:** pAS1300 digested with *Sal* I (6.75 kb; 5.45 kb; 1.27 kb)
- Lane 16:** pMO3 digested with *Sal* I (6.75 kb; 5.22 kb; 1.86 kb)
- Lane 17:** 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506kb)

The restriction data obtained confirms the construction of each of the above mentioned plasmids. The *Hind* III digests confirmed a 0.6 kb insert for the plasmids pS600 and pAS600 (lanes 7 and 8, Figure 4.64), an insert of 1.3 kb for each of the plasmids pS1300 and pAS1300 (lanes 9 and 10, Figure 4.64) and an insert of 1.6 kb for the plasmid pMO3. Finally, the enzymes chosen to prove orientation gave the expected band sizes for each plasmid. Each of the plasmids constructed were transformed into DBY746 and plated on minimal media supplemented with 0.25 % uracil and 2% Lintners starch to demonstrate that none of these constructs expressed an active α -amylase gene (Figure 4.65).

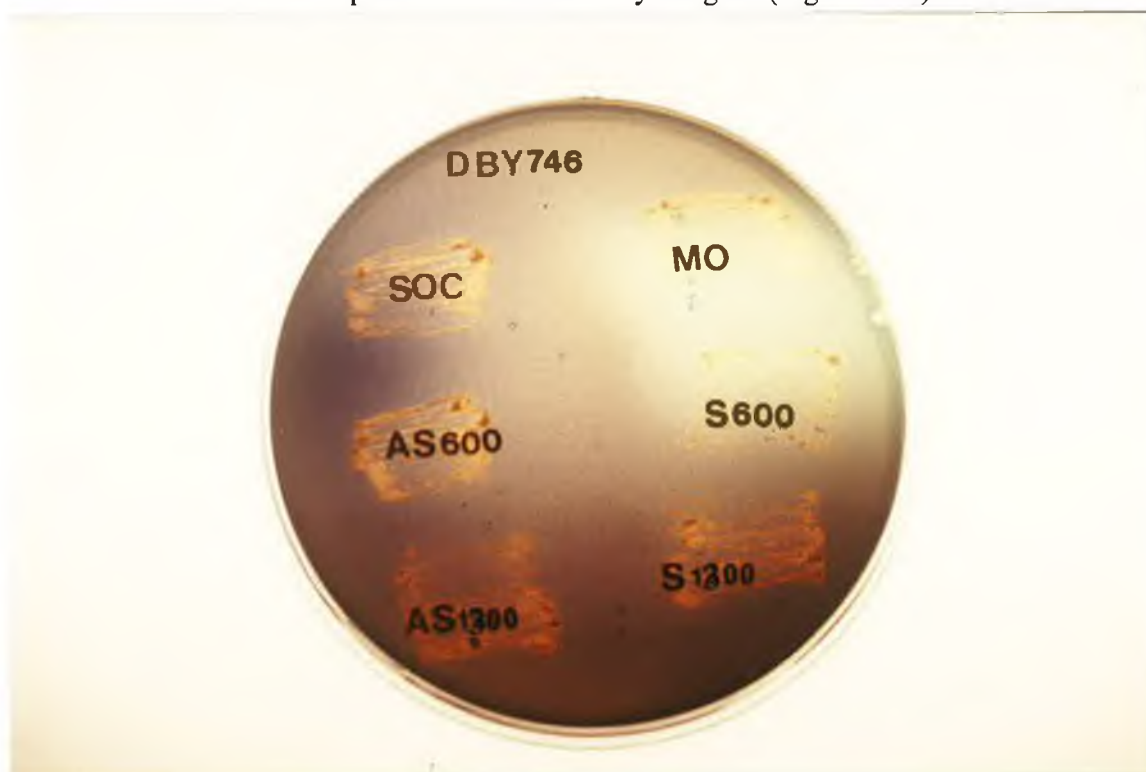


Figure 4.65 Strains DBY746::pSOC-01, DBY746::pAS600, DBY746::pS600, DBY746::pAS1300, DBY746::pS1300 and DBY746::pMO3 all patched on to minimal media supplemented with 0.25% uracil and 2% starch.

4.3.1.5 Confirmation of the presence of the various antisense and sense plasmids in SI.

4.3.1.5.1 *Growth on selective media*

After transformation of SI with the antisense and sense plasmids confirmation of their presence in the integrated strain SI was established by their ability to grow on minimal media plates lacking uracil and leucine. This is because the integrating plasmid (pFAMY) has converted DBY746 to uracil prototrophy while the YEp-antisense and sense plasmids bear a *LEU2* marker. Hence a successful transformation event into the yeast strain SI exhibited colonies growing on minimal media minus uracil and leucine (data not shown).

4.3.1.5.2 *Southern Blot Analysis.*

Genomic DNA was extracted from DBY746, SI, SI::pAS600, pS600, pAS1300, pS1300 and pMO3. All were electrophoresed undigested on a 0.7% agarose gel (Figure 4.66), then blotted and probed with a non-radioactively labelled DIGoxigenin α -amylase probe (Figure 4.67). The presence of the plasmid was confirmed by the appearance of multiple bands of the various forms of undigested DNA on the blot. It was also evident from the blot, as expected, that the plasmids transformed into this yeast strain were of different sizes as demonstrated by the covalently closed circular bands (Figure 4.67).

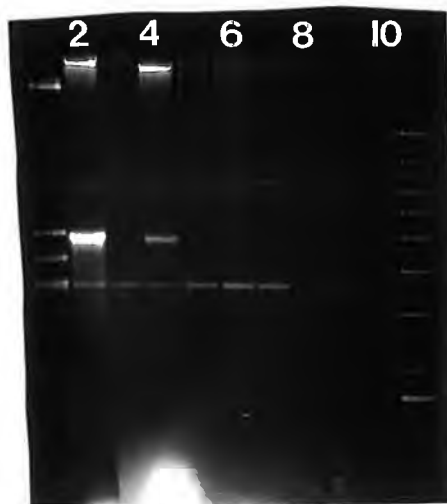


Figure 4.66 Agarose gel of undigested genomic DNA extracted from various yeast strains.

Lane 1:	λ DNA <i>Hind</i> III/ <i>Eco</i> R I digested (21,226; 5,148; 4,973; 4,277; 2,027; 1904; 1,584; 1,330; 983; 831; 564 bp)
Lane 2:	DBY746
Lane 3:	DBY746::pSOC-01
Lane 4:	SI
Lane 5:	SI::pAS600
Lane 6:	SI::pS600
Lane 7:	SI::pAS1300
Lane 8:	SI::pS1300
Lane 9:	SI::pMO3
Lane10:	SI::pSOC-01
Lane 11:	1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)

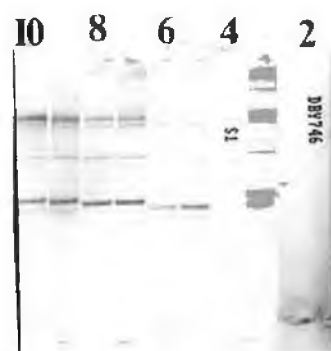


Figure 4.67 Southern blot to confirm the presence of the various sense and antisense plasmids in the integrated strain SI, probed with a non-radioactively labelled DIGoxigenin α -amylase probe.

Lane 1:	λ DNA <i>Hind</i> III/ <i>Eco</i> R I digested (21,226; 5,148; 4,973; 4,277; 2,027; 1904; 1,584; 1,330; 983; 831; 564 bp)
Lane 2:	DBY746
Lane 3:	DBY746::pSOC-01
Lane 4:	SI
Lane 5:	SI::pAS600
Lane 6:	SI::pS600
Lane 7:	SI::pAS1300
Lane 8:	SI::pS1300
Lane 9:	SI::pMO3
Lane10:	SI::pSOC-01
Lane 11:	1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb)

A single high molecular weight band was clearly visible in lane containing SI (lane 4, Figure 4.67). This band should also have been visible in each of the lanes 5 to 10, Figure 4.67. The reason for the lack of visualisation of

this band possibly was because the genomic DNA for SI was extracted from a culture which was grown up in rich media (YEED) hence a higher OD of growth than the other strains, SI::pAS600, pS600, pAS1300, pS1300 and pMO3, which were all grown in minimal media. The OD of growth was lower hence not as high a concentration of genomic DNA was extracted from these strains. Also from plasmid copy number analysis of the strain SI there are only 2 copies of the amylase gene present per cell whereas the episomal plasmids are in higher copy numbers hence visualisation of these bands but not of the SI α -amylase.

4.3.1.6 Analysis of the *in vivo* activity of the *trans*-acting antisense transcripts from the plasmids pAS600, pAS1300 and pMO3 on the expression of α -amylase from SI.

The inhibitory effect of antisense RNA on the target gene was assessed initially by plating transformants on media containing 2% starch and searching for colonies with either no haloes or reduced halo size. These colonies were then chosen for further analysis by the DNS assay. This approach could be used to identify those transformants in which antisense RNA was functional in downregulating the level of target enzyme. The integrated strain SI transformed with the antisense plasmids pSOC-01, pAS600, pAS1300 and pMO3 were grown up in minimal media until cell growth had reached mid-log phase. The cultures were then spun down at 5,000 rpm for 5 minutes at 4°C and the supernatants were analysed for a change in α -amylase activity in comparison to the control strain SI. All samples were assayed in triplicate and the entire assay was repeated 3 times. The cumulative results of this experiment are represented by the bar chart shown below (Figure 4.68). Results are reported as the % relative amylase activity in comparison to the control strain SI (100% activity). The corresponding sense plasmids of pS600 and pS1300 were also transformed into the integrated strain SI as controls, and were demonstrated to have no significant effect on the levels of α -amylase produced.

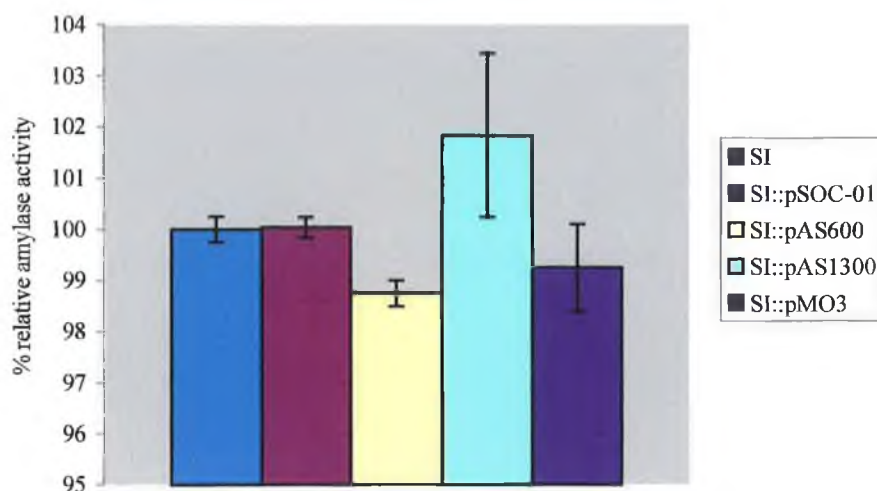


Figure 4.68 Effect of the presence of the various antisense plasmids on α -amylase activity.

From the bar chart shown in Figure 4.68 one can see that the presence of the antisense RNA constructs (pAS600, pAS1300 and pMO3) did not significantly downregulate or ablate the level of α -amylase activity being produced as compared the control strain SI. These findings, taken together with that observed for SI::pSOC-01 (*trans*-Experiment I (a)) clearly show that regardless of which part of the gene that is used, either whole or truncated, no antisense effect is observed. In order to confirm that antisense was being produced in these strains, Northern analysis was undertaken.

4.3.1.7 Northern analysis

Northern blot analysis was undertaken to show firstly, that antisense RNA was being made by the cell, secondly, that the antisense transcripts produced from the constructs SI::pAS600, SI::pAS1300 and SI::pMO3, were of different sizes. Thirdly, to obtain a ratio for the level of antisense to sense transcripts and finally fourthly, to prove the existence of temporal coincidence of both transcripts, in each of these strains. Total RNA was isolated from SI::pAS600, SI::pAS1300 and SI::pMO3 (in addition to various controls) under the conditions described in section 2.6.10, from cells grown to mid-log phase (OD 0.8 - 1.0).

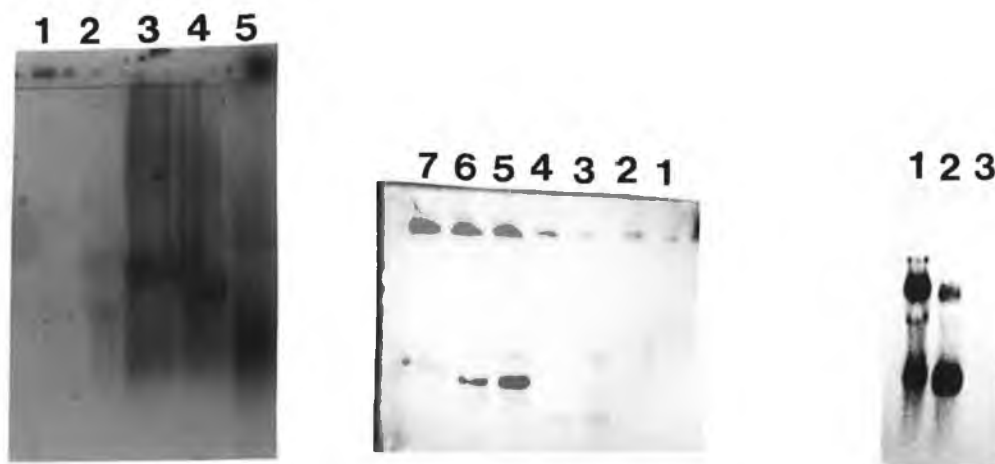


Figure 4.69 Northern blot hybridized with [a] a ^{32}P -labelled sense α -amylase riboprobe, [b] and [c] with a digoxigenin labelled α -amylase probe.

[a]	[b]	[c]
Lane 1: RNA Markers*	Lane 1: RNA Markers	Lane 1: SI::pS600
Lane 2: SI	Lane 2: DBY746	Lane 2: SI::pAS600
Lane 3: SI::pMO3	Lane 3: SI::pS600	Lane 3: DBY746
Lane 4: SI::pAS1300	Lane 4: SI::pAS600	
Lane 5: SI::pAS600	Lane 5: SI::pAS1300	
	Lane 6: SI::pS1300	
	Lane 7: SI::pMO3	

*RNA Markers: (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)

For the blot shown in figure 4.69 [a], the RNA was separated on an agarose - formaldehyde gel and bidirectionally transferred to a nitrocellulose membrane. The blot shown here was hybridised with a ^{32}P -labelled sense α -amylase riboprobe (for the detection of all antisense transcripts). Analysis of this blot, demonstrates the presence of antisense RNA in SI::pMO3, SI::pAS1300 and SI::pAS600 (lanes 3, 4 and 5, Figure 4.69 [a]), with transcript sizes of 1.7, 1.3 and 0.65 kb respectively. These were approximately the expected sizes considering how the antisense expression cassettes were constructed.

Confirmation of the temporal coincidence of both antisense and sense transcripts in the strains SI::pAS600 and SI::pAS1300 is demonstrated in the blots shown in Figure 4.69 [b] and [c]. These blots were probed with a non-radioactive digoxigenin labeled α -amylase probe, which is capable of hybridizing to both sense and antisense transcripts. The presence of both transcripts is clearly visible for SI::pAS600 (lane 4, Figure 4.69 [b] and lane 2, Figure 4.69 [c]) and SI::pAS1300 (lanes 5, Figure 4.69 [b]). Although, only the antisense transcript was visible in the lane containing SI::pMO3 (lane 7, Figure 4.69 [b], possibly due to poor loading of the sample, and the fact that the antisense transcript is expected to be present in a higher copy number than the sense transcript), temporal coincidence has been confirmed in another Northern blot analysis. In fact, the blot shown in Figure 4.69 [a] was taken from a gel that was bi-directionally blotted, the second blot (data not shown) was hybridized with an antisense α -amylase riboprobe, together with a β -actin riboprobe. This blot demonstrated the presence of the sense α -amylase transcript and also allowed for calculation of the antisense:sense transcript ratio. All ratios were obtained by measuring the band intensities of the sense and antisense transcripts on a Pharmacia-Biotech (ImageMaster 1D) Imaging densitometer (taking into account the backgrounds). The ratio of sense to antisense RNA being produced from the above transformants is tabulated in Table 4-6.

Table 4-6 Ratio of antisense to sense RNA transcripts.

	ANTISENSE mRNA	SENSE mRNA
SI::pMO3	3.5	1
SI::pAS1300	3.5	1
SI::pAS600	4.0	1

As a negative control for blots [a], [b] and [c] (data not shown for [a]) total RNA was extracted from DBY746 (lane 2, Figure 4.69 [b] and lane 3, Figure 4.69 [c]). As expected, this strain generated no signal on the autoradiograph when probed with either the sense α -amylase riboprobe or α -amylase probe.

Finally, these blots demonstrate the presence and stability of antisense transcripts of the expected sizes, the existence of temporal coincidence and also that there was an excess of antisense to sense RNA being produced.

4.3.2 DISCUSSION

When the full-length antisense α -amylase transcript from pSOC-01 in *trans*-Experiment I (a) failed to produce downregulation of α -amylase gene expression (even though there was temporal coincidence and stability of the two RNAs, and an excess of antisense to sense mRNA) it was decided then to target specific regions within the α -amylase gene, as many papers have cited the importance of targeting the antisense sequences to particular areas within the gene (Coleman et al., 1984; Daugherty et al., 1989; Liu et al., 1992; Scherzinger and Knecht, 1993; Kuipers et al., 1995). In addition to the effectiveness of an antisense RNA being dependent on the region of the gene to which it is targeted, the length of an antisense gene may also be contributing factor to either the success or failure of an antisense experiment. However, there is conflicting evidence, regarding the optimum length, as there is with the region of the gene targeted. For example Knecht, (1989) reasons that the larger the antisense molecule, the faster and more stable the duplex formed. However this author also states that, results regarding the length of an antisense molecule have been so variable that no absolute size limit can be stated. Olsson et al., (1997) reasoned that the failure of their antisense experiments was possibly due to the fact that the antisense constructs were designed to produce large RNAs which may have in turn lead to the opportunity for secondary structure formation. Therefore, these antisense RNAs may not bind to the sense mRNA with a high enough efficiency to prevent translation. Furthermore, Rittner et al., (1993) hypothesises that small changes in the length of an antisense RNA may significantly alter secondary structure and thus lead to dramatic changes in the gene silencing capacity.

In this study, antisense α -amylase RNA was expressed against the 5' upstream, RBS and 5' coding sequences of the α -amylase gene (pAS600); in addition to the mid - 3' coding and untranslated regions of the α -amylase gene (pAS1300) and to the entire gene minus its 3' non-coding regions (pMO3). As well as targeting different subregions of the target mRNA, various lengths of

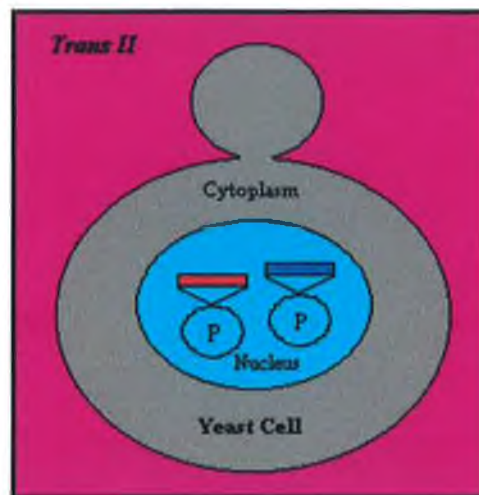
complementarity between the antisense α -amylase transcripts and the target mRNA were also used in the present study. The antisense α -amylase RNA expressed from the plasmid pAS600 complementary to only the 5' upstream region, RBS and base pairs of the coding region was predicted to cover only 585 nucleotides of the target mRNA. In the case of the antisense RNA complementary to the mid to 3' and terminal coding sequences of the target mRNA, the length of complementarity was 1,247 base pairs (pAS1300) and 1,613 base pairs in the case of pMO3 complementary to the entire coding sequence. There was however no significant reduction in the level of α -amylase produced from transformants expressing any of the truncated antisense transcripts, regardless of size or region targeted.

Once again, as was the case with pSOC-01, the antisense transcripts derived from pAS600, pAS1300 and pMO3 all exhibited stability and temporal coincidence with respect to both sense and antisense RNAs, in addition to an excess of antisense to sense mRNA (4.0:1, 3.5:1 and 3.5:1 respectively). However, all of these constructs failed to reduce or abolish α -amylase gene activity. It may be important to note here that, each of the antisense α -amylase RNAs was expressed as a chimeric transcript, which included non-complementary *AdHI* sequences. It is always possible that the *AdHI* portion of these antisense RNAs interfered with successful hybridisation between the antisense α -amylase sequences of the chimeric transcript and the target mRNA.

Furthermore, although temporal coincidence was ensured in *trans*-Experiment I (c), it is possible that failure to achieve antisense inhibition in this experimental system could be due to the fact that the two RNAs are not present in the same physical space in the cell. If this is so, they would not be able to interact with one another. In order to try and address this potential problem *trans*-Experiment II (described in chapter 5) was undertaken. In summary, the main focus here was to improve the spatial coincidence of the two RNAs and, if possible, increase the amount of antisense relative to sense RNA.

CHAPTER 5

Trans-Experiment II



■ Sense amylase sequences
■ Antisense amylase sequences

P; Plasmid
Chr; Chromosomal location

5. TRANS-EXPERIMENT II - INTRODUCTION

If one focuses on the potential interactions between two complementary RNA molecules *in vivo*, many factors must be considered, such as the relative stabilities of the two RNA molecules. The stability of RNA molecules is in part dependent on their individual secondary structures. But two aspects that are of critical importance are the spatial and temporal coincidence of the two interacting RNA molecules. This means that the two potential interacting molecules must be present within the same part or organelle of the cell, at the same time. From Northern analysis temporal coincidence is assumed by the coincident hybridisation signal for both RNAs. Spatial coincidence, however, is more difficult to predetermine or prove.

In a publication by Atkins et al., (1994), it appears that investigators have found a higher degree of subcellular localisation exists in addition to the simple nuclear/cytoplasmic division. The nucleus itself possesses a defined architecture and studies have revealed that mRNA molecules within the nuclear structure are assigned to discrete tracks. These tracks are formed following transcription and they extend from the gene toward the nuclear membrane. In the model system *trans*-Experiment I the antisense and sense RNAs were transcribed from different locations within the nucleus, i.e. from either an episomal or chromosomal location. An interesting question to ask is, does the transcription of genes encoded on episomes result in RNA transcripts that occupy tracks distinct from those occupied by chromosomally-encoded gene mRNA? If this were the case, there would be no contact between the two RNAs.

To investigate this hypothesis further it was decided to introduce both the sense and antisense α -amylase sequences into the yeast cell, episomally. The sense α -amylase gene was introduced into the cell on the plasmid pAAMY-01, which places the α -amylase gene under the control of the constitutively expressed *AdHI* promoter. The antisense α -amylase gene was introduced on another plasmid, pYES₂-SOC, which placed the gene under the control of the powerful *GAL1* promoter. By using this promoter it was also hoped to increase the amount of antimessage being produced. Both of these plasmids carry different selection markers, *LEU2* and *URA3*, on the plasmids pAAMY-01 and pYES₂-SOC respectively.

For proper induction of the inducible *GAL1* promoter the host cell must be genotypically *GAL*⁺. In *trans*-Experiment I (a), (b) and (c) the *S. cerevisiae* strain DBY746 was used as the host strain for integrative transformation and for the introduction of various sense and antisense plasmids. However for *trans*-Experiment II a different strain of yeast had to be used as DBY746 does not possess the necessary genes required to metabolise galactose. The *S. cerevisiae* strain MT235 was chosen for *trans*-Experiment II as it does possess the required genes to metabolise galactose.

In yeast, the regulation of the galactose genes has been extensively studied. Briefly, there are two regulatory proteins *GAL4* and *GAL80*, which calibrate transcription of several structural genes; *GAL2*, a permease; *GAL1* a kinase; *GAL7*, a transferase; *GAL10*, an epimerase and *MEL1* a galactosidase. When there is an inducer present in the media, the *GAL4* protein binds to sites within the upstream activating sequence, UAS (UAS is a generic term for sequences located upstream of yeast genes that provide for activation of transcription, presumably by binding transcriptional regulatory proteins) and activates transcription. When no inducer (galactose) is present or in the presence of glucose, *GAL80* binds to the carboxyl terminus of the *GAL4* protein, thus masking the activation domain. The *GAL80* protein therefore acts as a negative regulator, *GAL4* being a positive regulator of gene transcription (Martin and Scheinbach, 1989, Figure 5.1).

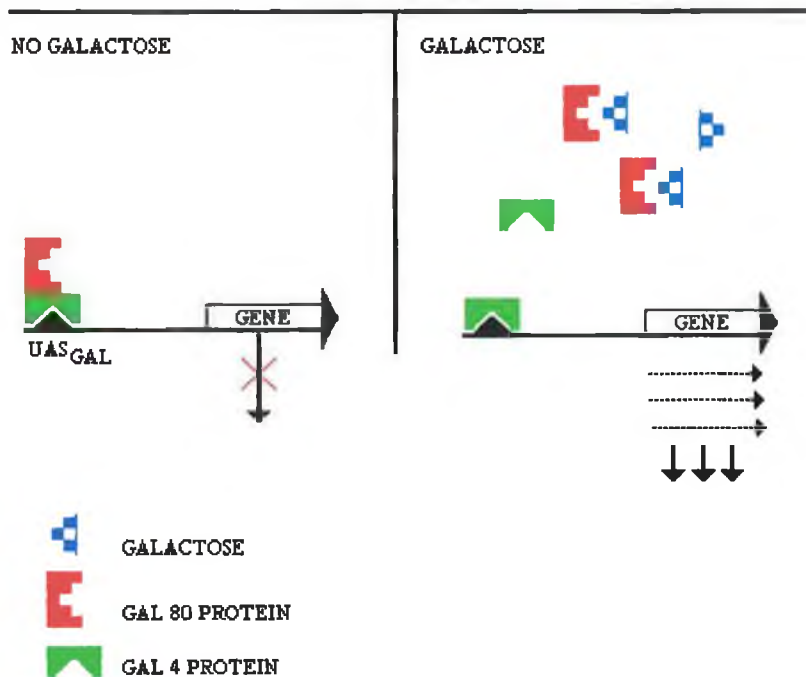


Figure 5.1 Galactose regulation in yeast.

The tight regulation of expression by carbon source makes the UAS of the *GAL1* and *GAL10* genes highly suitable for manipulating the expression of cloned genes. In addition, the extremely strong induction of transcription in the presence of galactose is useful for expressing high levels of a protein. For example expression of galactokinase (*GAL1*) is induced 1,000-fold in galactose from barely detectable levels in glucose (Martin and Scheinbach, 1989).

The first third of this chapter describes the cloning of the *B. licheniformis* α -amylase gene in both the sense and antisense orientations under the control of the *GAL1* promoter into the high-copy episomal yeast expression vector pYES₂. This is followed by a characterisation of the growth of the yeast strain MT235 on different carbon sources. The next section goes on to deal with the investigation of the optimal induction conditions for the *GAL1* promoter. Following this MT235::pAAMY-01s' α -amylase expression levels are investigated when grown on raffinose and galactose to ensure that this plasmid produces a level of α -amylase activity that can be easily monitored. Finally as with *trans*-Experiment I the important results come in the latter part of this chapter with the analysis of the effect of the antisense α -amylase transcripts from pYES₂-SOC on the levels

of α -amylase activity produced by the plasmid pAAMY-01. This chapter will conclude with a discussion on the results obtained.

5.1 PLASMIDS CONTAINING THE α -AMYLASE SENSE AND ANTISENSE GENES UNDER THE CONTROL OF THE INDUCIBLE GAL1 PROMOTER

Because of the high levels of gene expression that the *GAL* gene promoters can direct, they are attractive for use in vectors designed for high level regulated gene expression in *S. cerevisiae*. Two distinct advantages associated with using the *GAL* promoters to direct gene expression are firstly, the ability to grow the cells under conditions that almost completely repress these promoters which would allow one to express genes whose product(s) may be toxic to yeast cells. Secondly, these promoters are among the strongest in *S. cerevisiae* and even higher levels of gene expression can be obtained if these promoters are present on high copy number yeast vectors. In this study the yeast shuttle vector pYES₂ (Appendix IX) was used to construct YEp plasmids carrying expression cassettes capable of producing either antisense or sense α -amylase RNA upon galactose induction in yeast cells. The pYES₂ plasmid contains a 2 μ origin of replication (which permits this plasmid to exist in high copy number in [cir⁺] yeast cells), a *URA3* selectable marker and an expression cassette composed of the *GAL1* promoter and *CYC1* transcription terminator. Any transcripts produced from this expression cassette would be expected to contain both a 5' cap and a 3' poly(A) tail.

The sense α -amylase gene was cloned under the control of the *GAL1* promoter and *CYC1* terminator, in order to assess the optimal induction conditions for this promoter. These conditions were then implemented for the induction of the antisense α -amylase gene.

5.1.1 CONSTRUCTION OF PLASMIDS WHICH CONTAIN THE α -AMYLASE ANTISENSE (pYES₂-SOC) AND SENSE (pYES₂-AMY) GENES UNDER THE CONTROL OF THE INDUCIBLE *GAL1* PROMOTER

The plasmid constructions pYES₂-AMY and pYES₂-SOC are represented schematically in Figure 5.2. These constructs were confirmed using diagnostic restriction enzyme analysis. The 1.9 kb *Hind* III α -amylase gene contains an asymmetric *Kpn* I site located 0.6 kb from its 5' end. *Kpn* I digestion of pYES₂-SOC resulted in the production of two bands of sizes 7.2 and 0.6 kb, while the same analysis of pYES₂-AMY yielded 6.5 and 1.3 kb fragments. (Data shown for pYES₂-SOC only). Plasmids pYES₂-AMY (sense orientation) and pYES₂-SOC (antisense orientation) differ only in the orientation of the 1.9 kb *Hind* III α -amylase DNA insert relative to the *GAL1* and *CYC1* terminator sequences. Transcription from the *GAL1* start site within the expression cassette located on plasmid pYES₂-SOC was expected to produce an antisense α -amylase - *CYC1* fusion transcript composed of the antisense α -amylase RNA and a portion of the *CYC1* mRNA.

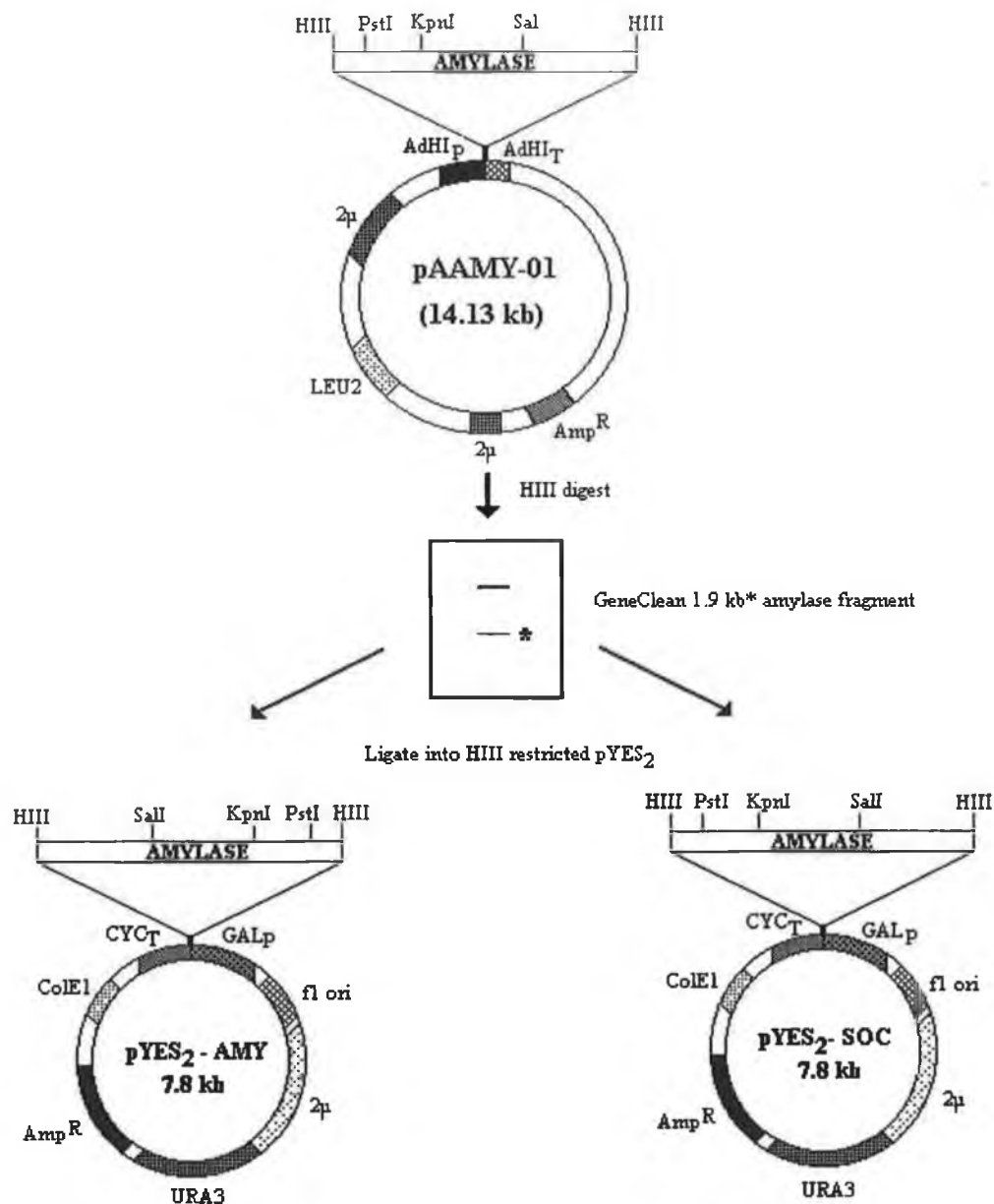


Figure 5.2 Schematic illustrating the cloning of a sense and antisense α -amylase gene into pYES₂.

The 1.9 kb *Hind* III α -amylase gene was cut from the plasmid pAAMY-01, this fragment was then gene cleaned from a 0.7% agarose gel and cloned into the unique *Hind* III site of a CIP (section 2.6.11.3) treated pYES₂ vector. The ligation mix was transformed into DH5 α and the colonies were screened on LB amp plates containing 2% Lintners starch. Once again, as in the case of pSOC-01 the amylase gene was expressed in both orientations in *E. coli*.

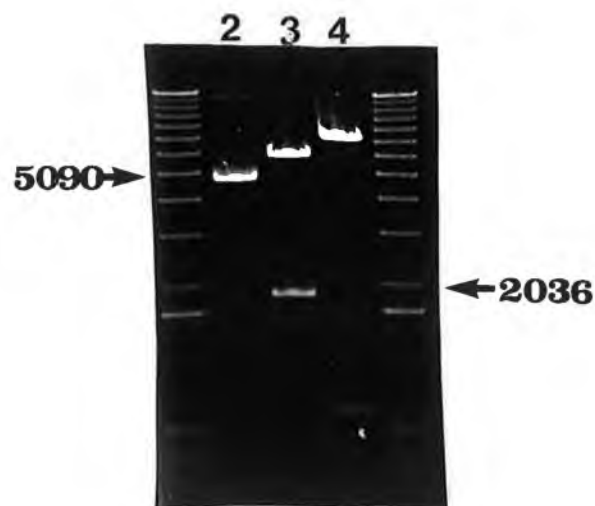


Figure 5.3 Restriction Analysis of pYES₂-SOC.

The appropriate sizes of each of the restriction fragments is shown in base pairs in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

Lane 1: 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).

Lane 2: pYES₂-SOC undigested

Lane 3: pYES₂-SOC digested with *Hind* III (5,900; 1,900)

Lane 4: pYES₂-SOC digested with *Kpn* I (7,210; 600)

Lane 5: 1 kb ladder (12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 506 bp).

**5.2 OPTIMISATION OF THE GROWTH, INDUCTION AND
EXPRESSION CONDITIONS BEFORE CO-TRANSFORMATION OF THE
ANTISENSE (pYES₂-SOC) AND SENSE (pAAMY-01) PLASMIDS INTO
MT235**

**5.2.1 ANALYSIS OF GROWTH OF THE MT235 YEAST STRAIN
WHEN GROWN IN THE PRESENCE OF DIFFERENT CARBON
SOURCES**

MT235 was grown on minimal media supplemented with adenine, leucine and uracil and either in the presence of 2% raffinose, 2% galactose or 2% glucose to examine the effect of these sugars on the growth of MT235 over a time period of 72 hours at 30°C. The reasons for choosing raffinose as a carbon source was because this is a neutral, non-inducing sugar and it is also said to have a more rapid induction of the *GALI* promoter on addition of galactose in comparison to glucose. This is because raffinose, unlike glucose, has no repression effects.

On glucose medium there is repression in addition to the absence of inducer. These repressors act between the UAS and TATA box. Galactose uptake and inducer synthesis are also inhibited. For this reason, if glucose is used as a non-inducible carbon source, induction of expression will take longer than with other carbon sources. However growth of this strain was also tested on 2% glucose.

The results indicated that MT235 growth was slower on the plates containing 2% glucose in comparison to growth of this strain on 2% raffinose or 2% galactose (Table 5-1). Also this yeast strain contains a mutation of the *ADE1* gene (*ade2-1*). The *ADE1* gene encodes an enzyme involved in *de novo* purine biosynthesis. A mutation in this gene results in the production of a red colour phenotype. Hence when MT235 was grown in either liquid or on solid media this characteristic red/pink colour was observed.

Table 5-1 Analysis of the growth of the yeast strain MT235 on different carbon sources.

CARBON SOURCES			
STRAIN	2% RAFFINOSE	2% GLUCOSE	2% GAL
MT235	###	##	###

good growth

slow growth

5.2.2 DETERMINATION OF THE OPTIMAL CONDITIONS FOR THE INDUCTION OF THE *GAL1* PROMOTER IN MT235

As the antisense transcripts from pYES₂-SOC do not express a functional product in *S. cerevisiae* this makes analysis of the optimal induction conditions of the *GAL1* promoter from this plasmid difficult to assess. However the plasmid pYES₂-AMY, which does express the functional α -amylase gene and whose expression can be easily monitored by the growth of the strain on starch containing media plates or by DNS assay was therefore used for the determination of the optimal induction conditions of the *GAL1* promoter. The optimal induction conditions could then be applied to the induction of the *GAL1* promoter on the plasmid pYES₂-SOC.

Preliminary analysis of the effect of different carbon sources on this promoter were analysed by plating MT235::pYES₂-AMY and pYES₂-SOC on minimal media containing 2% starch supplemented with different carbon sources (section 5.2.2.1). These studies were followed by growth of this strain in liquid minimal media containing a non-inducing carbon source with subsequent analysis of the levels of α -amylase activity being produced by DNS assay after the *GAL1* promoter has been induced by the addition of galactose to the growth media (section 5.2.2.2).

5.2.2.1 Analysis of the effect of different carbon sources on the *GALI* promoter monitored by either the expression or non-expression of the sense α -amylase gene from pYES₂-AMY

The yeast strains MT235:: pYES₂-AMY and MT235:: pYES₂-SOC were grown on minimal media plates supplemented with the necessary amino acids, 2% Lintners starch (BDH #30260) and either 2% raffinose, 2% glucose, 2% galactose or a combination of 0.5% raffinose/2% galactose for a period of 72 hours at 30°C. The plates were then flooded with iodine vapour. Analysis of the plates in Figure 5.4 showing MT235:: pYES₂-SOC and MT235:: pYES₂-AMY, demonstrates that as expected there was no induction of the *GALI* promoter when the strain MT235:: pYES₂-AMY was grown on media containing 2% raffinose or 2% glucose. These results were confirmed using the DNS assay. This is because raffinose is a neutral non-inducing sugar whereas in the presence of glucose the *GALI* promoter was repressed.

When performing experiments with the *GALI* promoter it is usual to initially grow the strain in a non-inducing or repressing carbon source. The reason for this is because the *GALI* promoter is so powerful if it is being continually induced by the presence of galactose in the media its' products may affect the viability of the cells. To examine this hypothesis the strain MT235::pYES₂-AMY was grown on galactose alone and on a combination of raffinose and galactose.

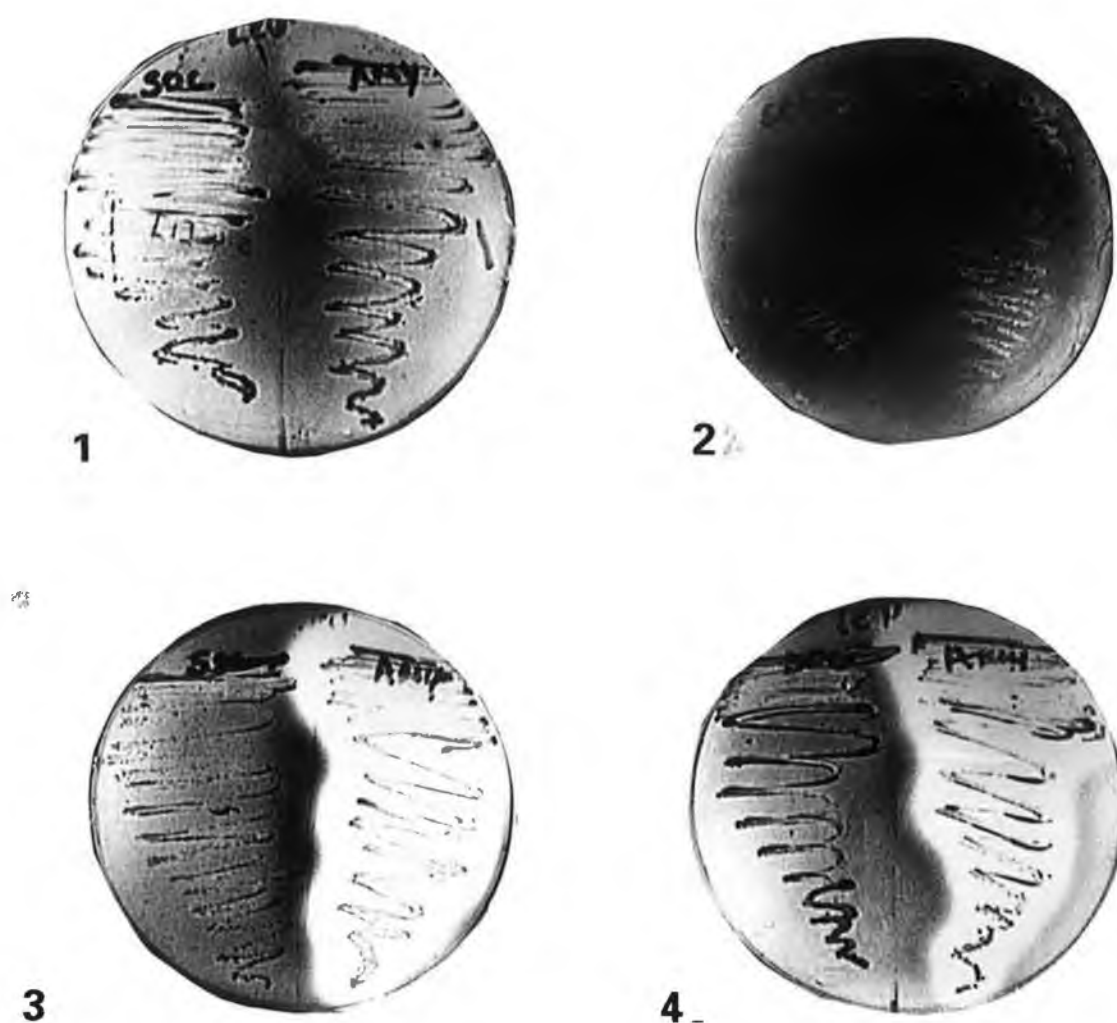


Figure 5.4 Starch containing minimal media plates inoculated with MT235::pYES₂-SOC and MT235::pYES₂-SOC. [1] Strains grown on 2% raffinose, [2] 2% glucose, [3] 2% galactose and [4] 0.5% raffinose/2 % galactose.

From analysis of the plates (Figure 5.4) it appears that growing the MT235::pYES₂-AMY and MT235::pYES₂-SOC on 2% galactose alone had no adverse effect on the viability of the cells. Expression of the α -amylase gene from MT235:: pYES₂-AMY on 2% galactose appears to be the greatest, followed by growth on 0.5% raffinose/2% galactose (Table 5-2). A further point to note from this experiment was that as expected MT235::pYES₂-SOC showed no α -amylase activity (i.e. no haloes around the patches of growth) and secondly these

strains grew least well on 2% glucose (these plates were left at 30°C for 24 hours longer). The latter finding is unimportant for the purposes of the experiments to be carried out as raffinose instead of glucose can be used as the non-inducing carbon source. The only concern is that the sense α -amylase gene (pAAMY-01) under the control of the *AdHI* promoter will be capable of expression when transformed into MT235 and grown on raffinose and/or galactose (section 5.2.3).

Table 5-2 Analysis of the expression of the α -amylase gene under the control of the *GALI* promoter when grown on different carbon sources.

CARBON SOURCES				
STRAIN	2% Raffinose	2% Glucose	2% Galactose	0.5% Raffinose/2% Galactose
MT235	### -	## -	### -	### -
MT235::pYES ₂ -AMY	### -	## -	### +	### +
MT235::pYES ₂ -SOC	### -	## -	### -	### -

good growth
 ## slow growth
 + α -amylase expression
 - no α -amylase expression

5.2.2.2 Determination of the optimal induction conditions by DNS assay

MT235::pYES₂-AMY was grown up in liquid minimal media supplemented with 2% raffinose at 30°C and grown until the culture reached an OD₆₀₀ of 0.7 - 1.0. Following this the cells were pelleted by centrifugation at 5,000 rpm for 1 minute and the supernatant was discarded. The cells were then resuspended in minimal media containing 2% galactose, pelleted again and the supernatant discarded. The cells were then resuspended in minimal media containing 2% galactose and grown for a further 18 hours. The following source was used: Sigma G-0750, as recommended.

Samples were taken at 0, 2, 4, 6 and 8 hours after the addition of galactose to the minimal media and analysed by DNS assay to find a time point at which there is good expression (induction) of the α -amylase gene. The assumption being made here is that the levels of α -amylase activity should reflect the RNA levels. All samples were assayed in triplicate and the entire assay was repeated 3 times. The cumulative results of this experiment are documented in Figure 5.5. The time at which the level of α -amylase gene expression was the greatest (4 hours after induction) was then taken as the time point for the analysis of the effect of the antisense α -amylase transcripts from pYES₂-SOC on the sense transcripts from pAAMY-01 (section 5.3.1).

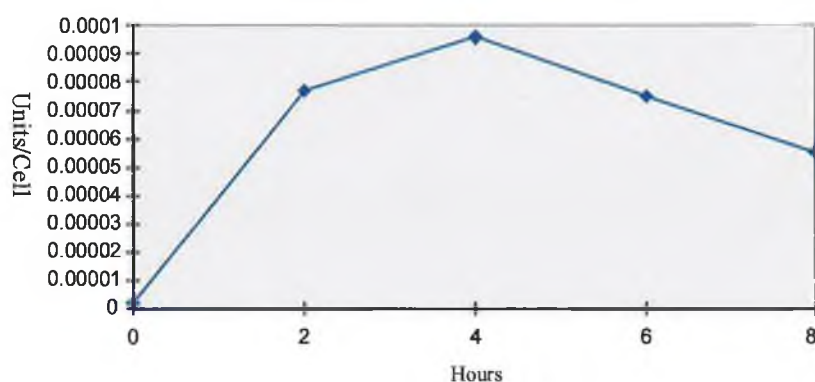


Figure 5.5 Measurement of α -amylase activity from the plasmid pYES₂-AMY at different time points after induction of the *GAL1* promoter with galactose.

5.2.3 EFFECT OF (i) HOST STRAIN, (ii) CARBON SOURCE, AND (iii) PROMOTER CHOICE ON THE LEVELS OF α -AMYLASE GENE EXPRESSION

In *trans*-Experiment I (a), the plasmid pAAMY-01 was transformed into the yeast strain DBY746 and grown on 2% glucose where there was effective expression of the α -amylase gene under the control of the constitutively expressed *AdHI* promoter. For *trans*-Experiment II pAAMY-01 was the plasmid of choice for the introduction of the sense α -amylase gene into MT235. However

it has been documented (Ammerer, 1983; Cantwell et al., 1986) that identical plasmids give variable expression of a foreign gene in different yeast strains. Hence it was essential to investigate the effect, if any, on expression of this gene under the control of the *AdHI* promoter when grown [1] in MT235 and [2] on the sugars, raffinose and galactose.

Furthermore a comparison was made between the levels of α -amylase activity produced when this gene was under the control of the *AdHI* promoter (DBY746::pAAMY-01 and MT235::pAAMY-01) and the inducible *GAL1* promoter (MT235::pYES₂-AMY). The reason for carrying out this analysis is that one of the aims of this particular experimental system was to produce a favourable antisense:sense transcript ratio. In the earlier experimental systems, *trans*-Experiment I (a) and (b), the approaches adopted to obtain a favourable transcript ratio included the choice of plasmid or chromosomal integration. In this experimental system, *trans* II, different promoters were chosen to achieve a favourable antisense:sense transcript ratio. In choosing the *GAL1* promoter, after reading the literature, it is expected that this promoter should result in a significantly higher number of transcripts than the constitutively expressed *AdHI* promoter. By analysing the levels of α -amylase activity produced by the plasmid pYES₂-AMY (where the gene is under the control of the *GAL1* promoter), it is hoped that the activity levels will reflect the RNA levels, and consequently that this will reflect the amount of RNA transcripts produced from the plasmid pYES₂-SOC, where the antisense α -amylase gene is under the control of the *GAL1* promoter. Additionally this analysis should also provide a comparison of the relative amounts of α -amylase activity produced, when the gene is under the control of the 2 different promoters. Once again it is hoped that the levels of activity will be reflected in the amount of RNA transcripts produced and this should provide an estimation of the antisense:sense transcript ratios.

All samples were grown up in minimal media supplemented with either 2% raffinose, glucose or galactose to an OD₆₀₀ of 0.7 - 1.0. The cultures were then spun down at 5,000 rpm for 5 minutes at 4°C, dialysed and the supernatants were analysed by DNS assay. All samples were assayed in triplicate and the entire assay was repeated 3 times. The cumulative results of this experiment are documented in Table 5-3.

Table 5-3 Effect of [1] host strain, [2] carbon source and [3] promoter choice on the level of α -amylase gene expression.

Strain	Carbon Source	2% Raffinose (Units/cell)	2% Glucose (Units/cell)	2% Galactose (Units/cell)
MT235::pAAMY-01		2.25×10^{-5}	2.06×10^{-5}	2.25×10^{-5}
DBY746::pAAMY-01		3.04×10^{-5}	2.82×10^{-5}	----
MT235::pYES ₂ -AMY		XXX	XXX	9.26×10^{-5}

XXX no expression of the α -amylase gene

---- no growth of strain

The important points to be noted from the results shown in Table 5-3 are as follows;

1. The *trans* II experiments were designed to use raffinose as the preferred non-inducing carbon source. A comparison of the strains MT235::pAAMY-01 and DBY746::pAAMY-01 when grown on 2% raffinose demonstrates that there is a significant reduction (approximately 26%) in the levels of α -amylase produced, in moving from the DBY746 strain to MT235. However the important point to note is that the levels of α -amylase produced in MT235 when grown on 2% raffinose are still present at a level easy for measuring increases or reductions upon the introduction of an antisense gene sequence.
2. Very importantly, upon changing the carbon source from 2% raffinose to 2% galactose there was no change in the constitutive expression of the *AdHI* promoter as demonstrated by the levels of α -amylase produced from MT235::pAAMY-01.
3. As expected expression of the α -amylase gene under the control of the *GALI* promoter (pYES₂-AMY) is far greater than the expression of this gene under the control of the *AdHI* promoter (pAAMY-01). MT235::pYES₂-AMY produces 3 times as much α -amylase activity as DBY746::pAAMY-01 and 4 times as much as MT235::pAAMY-01. It is

hoped from this finding that when the induction levels are high, that this will be reflected in the number of antisense RNA transcripts produced.

4. Finally, similar to point [1] above, when the strains MT235::pAAMY-01 and DBY746::pAAMY-01 α -amylase activities' were compared, when grown on 2% glucose, there was a significant decrease (approximately 27%) in the levels of α -amylase activity in moving from DBY746 to MT235.

5.3 CO-TRANSFORMATION OF *pYES₂-SOC* AND *pAAMY-01* INTO MT235

Initially when designing this experimental system a point to consider was the fact that the two plasmids chosen to express the sense (pAAMY-01) and antisense (pYES₂-SOC) α -amylase gene may be incompatible. Compatibility studies by Warren (1983) who examined the mitotic segregation of three 2 μ - pBR322 chimaeric plasmids in *S. cerevisiae* found that each plasmid individually displayed a characteristic rate of loss. However these loss rates were not significantly increased when two chimaeric plasmids were coresident, nor was the endogenous 2 μ plasmid itself displaced. Co-transformation of MT235 with the sense α -amylase gene (pAAMY-01) and the antisense α -amylase gene (pYES₂-SOC) bearing a *LEU2* and *URA3* selection marker respectively demonstrated the ability of the strain to grow on minimal media lacking each of the amino acids provided by the two plasmids thus indicating a successful co-transformation event.

Transformants resulting from the co-transformation were plated on minimal media supplemented with 0.25% adenine, 2% Lintners starch and 2% galactose. Initial visualisation of the plates after incubation at 30°C for 72 hours revealed individual haloes surrounding each colony indicating α -amylase activity. Ideally one might have hoped for colonies with little or no α -amylase activity. However it appeared that the haloes were of varying sizes. Approximately 100 transformants were purified and then patched out on minimal media starch plates containing 2% galactose. A certain amount of variation in α -

amylase activity was noted for these transformants. Two transformants were then chosen for further analysis, MT235::pYES₂-SOC + pAAMY-01 [1] and [2].

5.3.1 ANALYSIS OF THE EFFECT OF THE ANTISENSE α -AMYLASE TRANSCRIPTS PRODUCED FROM THE PLASMID pYES₂-SOC UNDER THE CONTROL OF THE INDUCIBLE *GALI* PROMOTER ON THE LEVELS OF α -AMYLASE ACTIVITY PRODUCED FROM THE PLASMID pAAMY-01.

Samples for determining α -amylase levels were prepared in the following way. MT235::pYES₂-SOC + pAAMY-01 [1] and [2] were grown in minimal medium containing 2% raffinose to mid-log phase of growth, the cells were washed, and then placed in 2% galactose and incubated for a further 4 hours. Samples were taken before the addition of the inducing sugar in order to quantify the levels of α -amylase enzyme activity before induction of the *GALI* promoter. Triplicate cell samples were harvested and DNS assays were completed. The complete assay procedure was carried out 3 times. Table 5-4 shows the cumulative results for the *in vivo* analysis of the efficacy of the antisense RNA transcripts produced from pYES₂-SOC in reducing α -amylase gene expression.

Table 5-4 The cumulative results (including controls) of the *in vivo* assay of the effects of the *trans*-acting antisense transcripts from pYES₂-SOC on α -amylase gene expression from pAAMY-01.

	MT235::pYES ₂ -SOC + pAAMY-01 [1]	MT235::pYES ₂ -SOC + pAAMY-01 [2]	MT235::pAAMY-01 (Control #1)	MT235::pYES ₂ -AMY (Control #2)
Levels of α -amylase activity before induction	2.26×10^{-5}	2.05×10^{-5}	2.14×10^{-5}	XXX
Levels of α -amylase activity after induction	1.96×10^{-5}	1.84×10^{-5}	2.2×10^{-5}	8.76×10^{-5}
% reduction in α -amylase activity	13	10	No reduction	Not applicable

XXX no expression of the α -amylase gene

The antisense RNA plasmid pYES₂-SOC reduced α -amylase activity by 13% in the transformant MT235::pYES₂-SOC + pAAMY-01 [1] and by 10% in MT235::pYES₂-SOC + pAAMY-01 [2] only following galactose induction of the *GAL1* promoter. This suggests that the decrease in α -amylase activity observed was due to the presence of the α -amylase antisense RNA and was not due to non-specific interference with gene expression. Two controls were carried out in conjunction with the analysis of the two co-transformants to reinforce the results obtained.

The first control involved the growth of MT235::pAAMY-01 under the same conditions as the two co-transformants examined. The result obtained for this control demonstrated that there was no reduction (in fact there was a slight increase, 0.06×10^{-5} units/cell) in α -amylase activity, on shifting the cells from growth on 2% raffinose to 2% galactose. This implies that there was little or no effect on the constitutively expressed *AdHI* promoter upon changing carbon source. This further substantiates the finding that the decrease in α -amylase

activity is due to an antisense effect and not to a reduction caused by less efficient transcription of the *AdHI* promoter upon changing the carbon source.

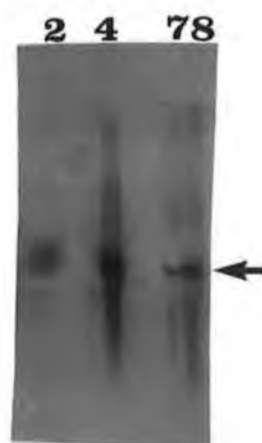
For the second control, MT235::pYES₂-AMY was also grown under the same conditions as the co-transformants. This was a control to monitor induction. The result indicates that there was a dramatic increase in the level of α -amylase gene expression, upon induction of the *GALI* promoter, achieved by changing the carbon source from the non-inducing sugar, raffinose to the inducing sugar, galactose. Hopefully, one can infer from this that the antisense plasmid pYES₂-SOC would behave in the same way with respect to the amount of transcript produced.

Because there was a reduction in α -amylase gene expression noted for both co-transformants, one would expect that there was [1] induction of the *GALI* promoter leading to high expression of the antisense α -amylase gene and [2] that there was excess antisense:sense RNA being produced. These issues will be addressed in the following section where RNA analysis was carried out. Another important point to note here, is that the observed reduction in α -amylase activity may not be because there is an excess of antisense:sense RNA, but due to the fact that both transcripts are more spatially accessible, due to transcription of the sense and antisense α -amylase genes from episomes.

5.4 RNA ANALYSIS

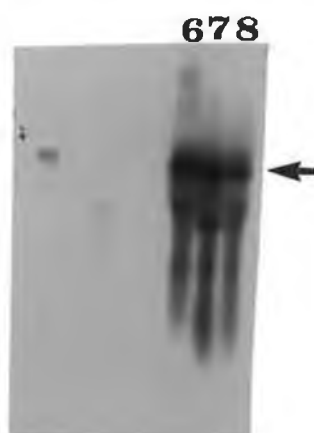
The reduction in α -amylase activity levels upon induction of the *GALI* promoter observed in the previous section (5.3.1), would suggest that antisense transcripts were being made. By carrying out Northern blot analysis, it should be possible to be able to quantify the transcripts directly and from there to calculate the relative abundancies of the messages.

The yeast strains MT235::pYES₂-AMY and MT235::pYES₂-SOC were cultured first in 2% raffinose containing media, to mid-log phase. A sample was taken and total RNA was extracted. This yielded the samples MT235::pYES₂-AMY (uninduced) and MT235::pYES₂-SOC (uninduced). The remainder of the cells were harvested by centrifugation, washed and recultured in 2% galactose containing media, (for induction) for a further 4 hours, at which time point total RNA was extracted to provide the samples MT235::pYES₂-AMY (induced) and MT235::pYES₂-SOC (induced). Total RNA was extracted from the cotransformants MT235::pYES₂-SOC + pAAMY-01 [1] and [2] after induction of the *GAL1* promoter, as described in section 2.6.10. The RNA was separated on an agarose-formaldehyde gel and bi-directionally transferred to a nitrocellulose membrane. As with *trans*-Experiment I (a) and (b), the sense and antisense α -amylase transcripts are expected to be of the same size, therefore the, Promega Riboprobe system was used (see section 4.1.2.4). The first blot (Figure 5.6) was probed with an antisense probe which should detect all sense α -amylase transcripts and conversely the second blot (Figure 5.7) was probed with a sense probe to detect all the α -amylase antisense gene sequences. One of the blots was then stripped of it's probe and reprobed with a β -actin probe (Figure 5.8) in order to standardise the loadings and to allow a comparison between the two blots.



- Lane 1:** RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)
- Lane 2:** MT235
- Lane 3:** MT235::pYES₂-AMY uninduced
- Lane 4:** MT235::pYES₂-AMY induced
- Lane 5:** MT235::pYES₂-SOC uninduced
- Lane 6:** MT235::pYES₂-SOC induced
- Lane 7:** MT235::pYES₂-SOC + pAAMY-01 [1] induced
- Lane 8:** MT235::pYES₂-SOC + pAAMY-01 [2] induced

Figure 5.6 Northern blot of total RNAs probed with a ³²P-labelled antisense α -amylase riboprobe.



- Lane 1:** RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)
- Lane 2:** MT235
- Lane 3:** MT235::pYES₂-AMY uninduced
- Lane 4:** MT235::pYES₂-AMY induced
- Lane 5:** MT235::pYES₂-SOC uninduced
- Lane 6:** MT235::pYES₂-SOC induced
- Lane 7:** MT235::pYES₂-SOC + pAAMY-01 [1] induced
- Lane 8:** MT235::pYES₂-SOC + pAAMY-01 [2] induced

Figure 5.7 Northern blot of total RNAs probed with a ³²P-labelled sense α -amylase riboprobe.

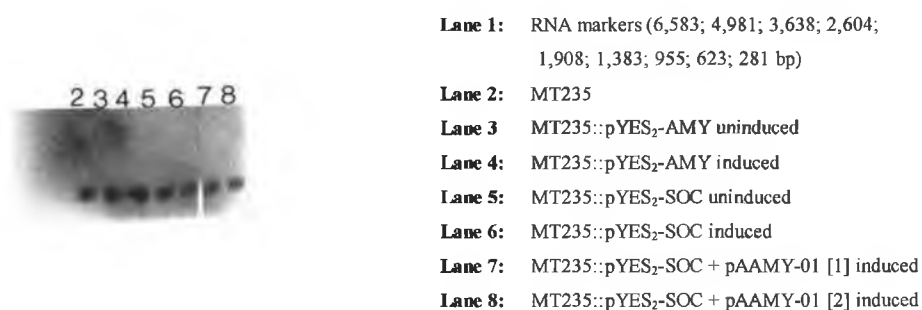


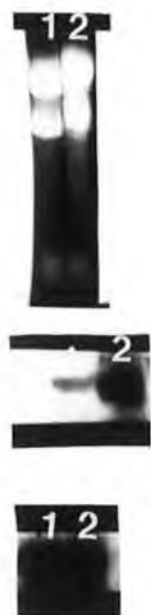
Figure 5.8 Northern blot of total RNAs, which was initially probed with the sense α -amylase probe, stripped and reprobed with a ^{32}P -labelled β -actin riboprobe.

The first point to mention from analysis of the blots are the specificity of the riboprobes. The blot shown in Figure 5.6, very specifically picks up all the sense α -amylase transcripts using the antisense riboprobe, conversely, Figure 5.7 probed with the sense riboprobe specifically picks up all of the antisense α -amylase gene transcripts. Secondly from both blots, it can be clearly seen, that the transcripts under the control the *GAL1* promoter are present only in induced cultures (compare MT235::pYES₂-AMY uninduced and induced, lanes 3 and 4, Figure 5.6 and MT235::pYES₂-SOC uninduced and induced, lanes 5 and 6, Figure 5.7). The 1.9 kb transcripts produced from both MT235::pYES₂-AMY and MT235::pYES₂-SOC are of a length consistent with that expected if transcription was initiated close to the *GAL1* promoter and terminated at the *CYC1* transcript terminator. Also the presence of these bands indicates little or no degradation of the RNA, which would imply stability of the RNAs.

On analysis of the co-transformant MT235::pYES₂-SOC + pAAMY-01 [1] (co-transformant [1], lane 7, Figure 5.6 and Figure 5.7) it appears to possess a greater abundance of antisense transcripts (Figure 5.7) relative to sense. To ascertain the relative ratios of antisense to sense RNA both blots were analysed by measuring the band intensities of the sense, antisense and

β -actin transcripts produced from MT235::pYES₂-SOC + pAAMY-01 [1] and [2]. The intensities were measured on a Pharmacia-Biotech (ImageMaster 1D) Imaging Densitometer. An antisense:sense transcript ratio of 7.5:1 was recorded for co-transformant [1]. Obviously temporal coincidence was ensured in this experimental system, as both sense and antisense transcripts of 1.9 kb were visualised, (lanes 7 and 8, Figure 5.6 and Figure 5.7). However, MT235::pYES₂-SOC + pAAMY-01 [2] (co-transformant [2]) produced a much lower antisense:sense transcript ratio of 2:1. This latter result is interesting in light of the fact that in earlier experiments (*trans* I (a) and (c)) where there was a slightly higher ratio of antisense:sense transcript recorded and yet no downregulation of the α -amylase activity levels were recorded. The difference in this particular experimental system, however, is that both sense and antisense transcripts are being transcribed from episomes in the nucleus and possibly therefore both transcripts are more spatially coincident, than in the previous experimental systems mentioned, hence the 10% decrease in α -amylase activity levels noted.

To confirm the latter results, these two co-transformants were re-analysed by the same method detailed above and the ratios produced reflected those observed above (see Figure 5.9).



Lane 1: MT235::pYES₂-SOC + pAAMY-01 [1];

Lane 2: MT235::pYES₂-SOC + pAAMY-01 [2]

Upper: Agarose-formaldehyde gel.

Middle: Hybridisation with the antisense α -amylase riboprobe.

Lower: Hybridisation with the sense α -amylase riboprobe.

Figure 5.9 Northern analysis of the sense and antisense α -amylase transcripts produced from the co-transformants, MT235::pYES₂-SOC + pAAMY-01 [1] and [2].

Another issue raised when analysing these blots, was that there was more antisense transcripts present in MT235::pYES₂-SOC (induced, lane 6, Figure 5.7) than in lane 8 containing MT235::pYES₂-SOC + pAAMY-01 [2]. Possibly, one explanation for this is that there was competition between the 2 plasmids present in the cell, which lead to a decrease in the copy number of pYES₂-SOC, hence there would be less antisense transcripts being produced.

Finally, MT235 (lane 2, Figure 5.6 and Figure 5.7) was loaded as a negative control in this experiment, as expected no bands were visualised when this sample was probed with both the sense and antisense riboprobes.

5.5 DISCUSSION

Studies involving the use of cloned antisense RNA to regulate specific gene expression usually result in partial down-regulation (Erickson and Izant, 1992). The experiments described here in *trans*-Experiment II agree with this

finding with the demonstration of the ability of an artificial antisense RNA to only partially downregulate the expression of the heterologous *B. licheniformis* α -amylase gene, by 13% and 10% in MT235::pYES₂-SOC + pAAMY-01 [1] and [2] respectively. An attractive hypothesis to account for the inability of antisense RNA to inhibit target gene expression completely is compartmentalisation of the two RNAs, with a fraction of the target mRNA unavailable for hybridisation with excess antisense RNA. In the present Experimental system care was taken to ensure temporal coincidence of target and inactivating genes. In addition, the strain was constructed to achieve an excess of antisense to sense RNA. Although both RNAs were transcribed from episomal locations in an attempt to ensure spatial coincidence the fact that the two RNAs were under the control of two different promoters may have resulted in the discrete localisation of the RNAs in separate "tracks" of the nucleus. This would rule out any antisense downregulation of gene expression in the nucleus thus restricting the usefulness of an antisense molecule to the cytoplasm where it is more likely to be degraded.

Alternatively, partial inhibition may result if a target gene is downregulated in a subset of cells rather than in the entire cell population. In some cases transgenic animals and plants containing antisense gene constructs have shown a mosaic phenotype in response to antisense RNA expression (Katsuki et al., 1988; van der Krol et al., 1988b). Within the yeast cell population it is not known whether 100% of the antisense RNA-expressing cells from MT235::pYES₂-SOC + pAAMY-01 [1] and [2] are inhibiting target gene expression by 13% and 10% respectively, or whether subsets of the cell population show differing levels of antisense RNA-mediated inhibition.

In this Experimental system similar to many other systems the antisense gene was constructed in such a way that the antisense RNA was as long as the sense mRNA molecule. The formation of intramolecular secondary structures may have possibly prevented the formation of an antisense RNA/sense mRNA hybrid. On the other hand studies of the kinetics of RNA-RNA interactions among the naturally occurring antisense RNAs found in bacteria have demonstrated that RNA hybrid formation involves an initial contact between short regions of complementarity, or nucleation sites, located in the loop regions of stem-loop structures followed by full-length hybridisation. One might predict

therefore that longer antisense RNAs, such as the antisense RNA used in this system, may have a larger number of nucleation sites and thus greater potential for RNA-RNA annealing.

Although a more favourable antisense:sense RNA ratio was achieved in this system, when compared to the *trans* I systems, one might have expected an even more favourable ratio by choosing the GAL system. The antisense transcript level obtained (7.5:1) may have been due to certain limiting factors such as the GAL4 protein. This yeast protein is a transcription factor that is required for the induction of the *GALI* promoter. The GAL4 protein normally is present at such low levels that it is rate limiting for the maximal induction of the *GALI* promoter. According to Schultz et al., (1994) the limitation is especially apparent in cells containing multicopy expression vectors with *GAL* promoters and pYES₂-SOC had a 2 μ origin of replication to allow this plasmid to exist in high copy number in yeast cells.

In this Experimental system, the physical location (or spatial coincidence) of the sense and antisense transcripts in the cell was considered. This parameter was the reason for introducing both the sense and antisense α -amylase genes into the cell on episomes. Experiments carried out by Sullenger and Cech (1993), where they utilised murine retroviral vectors to deliver both target and ribozyme RNAs have indicated that co-localising RNAs in murine cells may increase ribozyme mediated suppression of the target RNA. Also the finding that the co-transformant MT235::pYES₂-SOC + pAAMY-01 [2] produced an antisense:sense transcript ratio of 2:1, with a concomitant 10% reduction in α -amylase activity levels, possibly suggests that there was co-localisation of the transcripts. Furthermore, not all experimental systems, in which antisense transcripts have been examined require an excess of antisense:sense RNA. The occurrence of antisense inhibition with comparable amounts of antisense RNA and target mRNA has been demonstrated in several studies (van der Krol et al., 1990). For naturally occurring antisense RNAs in plants, which are thought to be involved in gene regulation, the steady-state levels of antisense RNA and mRNA do not differ greatly (Rogers, 1988). This might indicate that, in contrast to what has often been hypothesised, the formation of an excess of antisense RNA

relative to sense mRNA may not be a prerequisite for successful inhibition (Kuipers et al., 1995).

Although both the sense and antisense α -amylase genes were expressed from episomes, the fact that both genes had different promoter and terminator (or 5' and 3') sequences this may have conferred a particular nuclear export and cytoplasmic distribution, for the different terminal sequences (Gottlieb, 1992). If this situation holds true for this experimental system, then that implies that spatial coincidence has not been ensured. This thought led to the design of the final Experimental system, the *cis*-Experimental system. Spatial coincidence will be ensured in this system by the insertion of *cis*-acting antisense sequences within the 3' untranslated region of the α -amylase gene. Such an arrangement should provide an optimal situation for RNA-RNA interaction by linking both temporal and spatial distribution of the antisense and target RNA transcripts.

5.6 FUTURE WORK

After analysing the data and results from *trans*-Experiment II, there are a number of further issues which could be addressed. Firstly, due to the fact that there was such a variation between the two co-transformants examined, more co-transformants should have been taken for analysis. Also, for co-transformants [1] and [2], the plasmid copy numbers of both pAAMY-01 and pYES₂-SOC should be determined and also their relative stabilities over time should be examined.

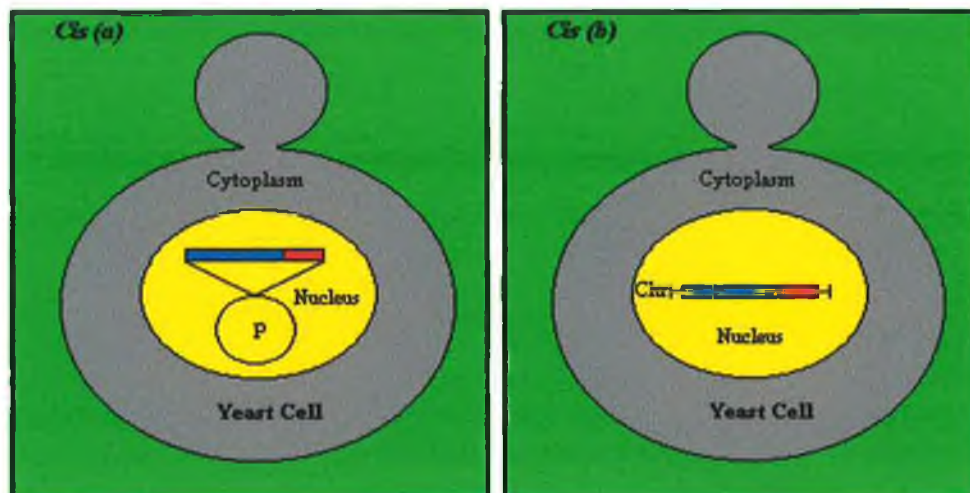
Also it was assumed, that the activity levels which produced the greatest amount of α -amylase activity, after induction of the *GALI* promoter (which was determined to be 4 hours after galactose addition to the media) would reflect the time at which there would be the highest number of transcripts being produced. However, although 4 hours was optimal for protein production, this may not be the optimal time point for transcript production. To address this, total RNA should be extracted at different time points and the transcript levels assessed.

It would also be interesting to investigate, if an arrangement where the antisense α -amylase gene was introduced into the cell under the control of a constitutively expressed promoter, to allow the establishment of the antisense transcripts in the cell, before induction of the sense α -amylase gene, (under the

control of an inducible promoter), would be more effective at reducing α -amylase activity.

CHAPTER 6

Cis-Experiment (a) and (b)



■ Sense amylase sequences
■ Antisense amylase sequences

P; Plasmid
Chr; Chromosomal location

6. CIS - EXPERIMENT (a) AND (b)- INTRODUCTION

Within the yeast *S. cerevisiae*, it appears that the failure of antisense sequences tested in a *trans* configuration, is not an uncommon situation, (Arndt, 1993; Arndt et al., 1994; Atkins and Gerlach, 1994; Olsson et al., 1997). The first report of a successful antisense mediated inhibition of gene expression in a *trans* configuration, in this organism, (Xiao and Rank, 1988) was actually retracted (Arndt et al., 1994). In fact, the earliest demonstration of a successful downregulation of an RNA sequence in *S. cerevisiae* was not from a *trans*-acting antisense RNA, but from a *cis*-acting antisense RNA (Atkins and Gerlach, 1994). In a *cis* configuration, the transcription of the sense and antisense genes are linked, thus guaranteeing spatial and indeed, temporal coincidence.

In *trans*-Experiment II, the concept of spatial coincidence was introduced, in conjunction with attempting to increase the copy number of antisense RNA transcripts in the cell, relative to the sense messenger RNA. The latter objective was achieved (7.5:1) and this resulted in a small, but significant downregulation of α -amylase gene expression. But whether this downregulation was as a result of a more favourable ratio of antisense:sense RNA, than what was achieved in *trans*-Experimental systems I (a) (4:1) and I (c) (3.5:1; 3.5:1; 4:1), or due to a more favourable spatial coincidence remains to be evaluated. From a spatial aspect, in *trans* II both interacting RNAs were transcribed from episomes, in contrast to *trans* I, where the sense and antisense transcripts were transcribed from different locations within the nucleus, i.e. either episomally or chromosomally. In the context of the above findings, it was decided to test the effect of an antisense sequence, designed to work in *cis*, on α -amylase gene expression.

The *cis* experiments involved linking the transcription of the sense and antisense genes, (both genes being transcribed by the same promoter as part of the same transcriptional unit) thus ensuring temporal and spatial coincidence. It was hoped that this configuration of sense and antisense genes should provide an optimal situation for RNA-RNA interaction. The anticipated outcome of such an arrangement would be that the antisense RNA would fold-back and bind to its' complementary 5' sense RNA and thus, either down-regulate or totally inhibit translation of the gene, due to either sequestration of the ribosome binding

site, by cleavage of the double stranded structure, or by destabilisation of the message.

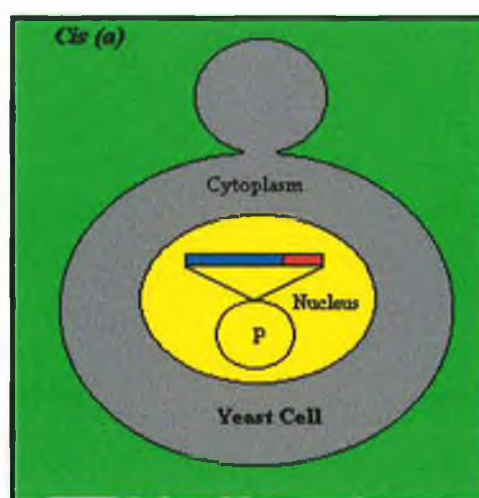
Another reason for undertaking the investigation of *cis*-acting antisense sequences comes from the analysis of the distribution of natural RNA transcripts within eukaryotic cells. Experimental work carried out by Lawrence and Singer (1986), suggests that a higher order of subcellular localisation exists, in addition to the simple nuclear\cytoplasmic division. Analysis of the intracellular distribution of mRNAs for cytoskeletal proteins, demonstrated that actin mRNA concentrations were highest at cell extremities, vimentin mRNA was distributed near the nucleus and tubulin mRNA appeared to be concentrated in the peripheral cytoplasm. Such results demonstrate that cytoplasmic mRNAs are localised in specific non-random cellular patterns, and that localised concentrations of specific proteins, may result from corresponding localisation of their respective mRNAs (Lawrence and Singer, 1986). Another striking example of localised messages can be found among the maternal mRNAs of frogs and flies where localisation has been implicated in the establishment of axial polarity (Gottlieb, 1992).

Other laboratories seeking to define the basic physical structure of the nucleus have provided evidence for a non-chromatin nuclear matrix composed of a network of predominantly proteinaceous fibrils. These laboratories postulate that RNAs are unlikely to diffuse freely through the highly viscous nucleus, but are more likely to be actively transported from their site of synthesis to the nuclear pores across a "solid-phase" nuclear architecture (Lawrence et al., 1989). Spector (1990) also agrees that the nucleus has a defined architecture, and that the components of the machinery, responsible for each step of gene expression are located at defined positions within the nucleus, rather than in a diffuse pattern. Lawrence et al., (1989) and Xing et al., (1993) have gone further to show that mRNA molecules within the nuclear structure are restricted to discrete "tracks". These "tracks" form immediately after transcription and extend from the gene toward the nuclear membrane. Hence, the spatial fate of the mRNA can be correlated with gene location. This is potentially important for antisense activity, as the position of the specific genes may predetermine that the tracks occupied by the complementary RNAs do not coincide with the target RNA. This would rule out any antisense down-regulation of gene expression in the nucleus

thus restricting the usefulness of an antisense RNA molecule to the cytoplasm where it is more likely to be degraded.

This chapter is divided into *cis*-Experiment (a), where the construction, characterisation and effect, of an episomal *cis* construct on α -amylase gene expression is described. This is followed by *cis*-Experiment (b), where the construction, characterisation and effect of an integrated *cis* plasmid are reported. This chimeric sense-antisense gene sequence was cloned into the two different types of expression vector, (i.e. episomal (*cis*-(a)) and integrative (*cis*-(b)) to examine whether the location of the transcriptional unit would alter the outcome. RNA analysis for both *cis*-Experimental systems is then documented, followed by a discussion and future work, which could be undertaken.

***Cis*-Experiment (a)**



■ Sense amylose sequences
■ Antisense amylose sequences

P; Plasmid

Cis-Experiment (a) described here involves the cloning into a single expression unit, part of the α -amylase gene - in antisense orientation - fused to the 3' end of the complete gene. This transcriptional unit, under the control of the *AdHI* promoter and terminator was cloned into a yeast expression vector, to create what will be known as a "*cis*-acting" plasmid. This episome was transformed into the yeast strain DBY746 and the transformants were examined for any change in α -amylase activity levels in comparison to a control construct.

6.1 AN EPISOMAL PLASMID CONTAINING THE CIS-ACTING ANTISENSE α -AMYLASE GENE SEQUENCE

An antisense α -amylase gene sequence (complementary to the 5' end of the sense α -amylase gene) was ligated to the 3' end of a sense α -amylase gene and cloned into the yeast episomal vector pAAH5 to produce the *cis*-acting plasmid, pMAS-H5. An important point to note about this plasmid construction is that the sense α -amylase gene sequence is missing its' transcriptional stop site. The α -amylase sense gene sequence used to construct the *cis*-acting plasmid, pMAS-H5 came from the plasmid pMO2 (see materials section 2.2). Approximately, 243 base pairs of the parental *B. licheniformis* α -amylase gene were deleted, from the 3' end. This deletion disrupted the putative palindromic transcriptional termination site, but left the entire α -amylase gene and its' translational stop site intact (Appendix III). If the α -amylase genes' transcriptional stop site was present this would mean that transcription would terminate before the RNA polymerase would have had a chance to read through to the antisense sequence tagged onto the end of the sense α -amylase gene (Figure 6.1).

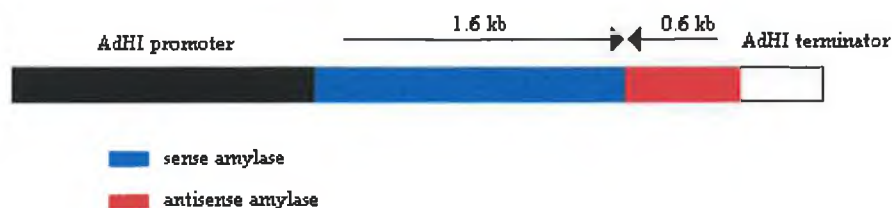


Figure 6.1 Schematic representation of the arrangement of the *cis*-acting sense-antisense gene sequence.

6.1.1 CONSTRUCTION OF THE EPISOMAL *CIS*-ACTING PLASMID, pMAS-H5

The cloning of the sense-antisense α -amylase gene sequences as a single transcriptional unit can be divided into two steps. Firstly, the sense and antisense α -amylase gene sequences were fused together by directional ligation (see Figure 6.1). Secondly, this sense-antisense gene sequence was subcloned into a yeast episomal vector, so that the entire sense-antisense α -amylase gene sequences were under the control of the same promoter.

To construct the *cis*-acting plasmid, pMAS-H5, a functional sense α -amylase gene without its' transcriptional stop site was required, in addition to an α -amylase sequence complementary to the 5' region of the sense α -amylase gene. The functional sense α -amylase gene was removed from the plasmid pMO2 (Olsen, 1995) as a 1.6 kb *Bam*H I - *Hind* III restriction fragment (Appendix III). A 0.6 kb antisense α -amylase gene sequence was also removed as a *Bam*H I - *Hind* III restriction fragment, from the plasmid pAS600. These fragments were ligated together in the presence of *Bam*H I restricted pUC-19, to produce the plasmid pMAS. pUC-19 was chosen as the initial cloning vehicle for the sense-antisense α -amylase gene sequence as it possesses a unique *Bam*H I site for cloning and the *lacZ* phenotype could be exploited for rapid screening of the transformants (a more detailed description of the construction of pMAS is given in the legend to Figure 6.2).

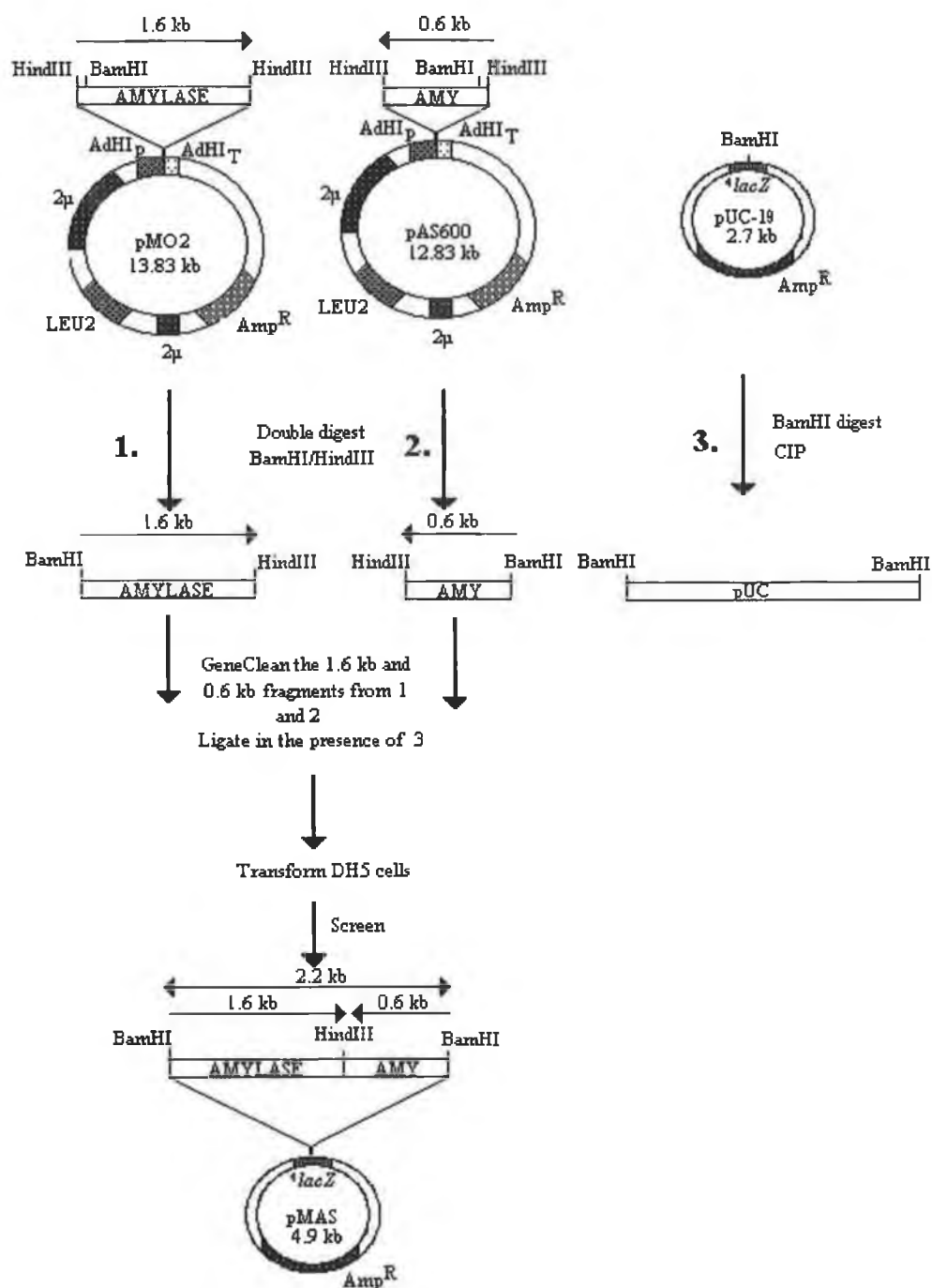


Figure 6.2 Schematic representation of the construction of the plasmid, pMAS.

Plasmid pMO2 (see section 2.2) which contains a truncated version of the α -amylase gene (it lacks the transcriptional stop site, Appendix III) was double-digested with *Bam*HI and *Hind*III

to yield a 1.6 kb fragment. Simultaneously the plasmid pAS600 was double-digested with the same restriction enzymes that resulted in the release of a 0.6 kb 5' α -amylase gene fragment. Both fragments (1.6 and 0.6 kb) were gene cleaned (section 2.6.11.9) from a 0.7% agarose gel and ligated together in the presence of *Bam*H I digested and CIP (section 2.6.11.3) treated pUC-19. The ligation mix was transformed into *E. coli* DH5 α cells. Selection of the transformants involved plating onto LB amp starch (2% Lintners) plates containing X-gal and IPTG, which allows exploitation of the *lacZ* phenotype (colonies with inserts are white, while no insert results in the production of a blue colony). White colonies were then screened for the presence of a 2.2 kb *Bam*H I fragment containing the pMO2 sense α -amylase gene ligated to the 0.6 kb antisense α -amylase fragment. This plasmid was named pMAS and is 4.9 kb in size.

To confirm the successful cloning of the sense-antisense α -amylase gene sequence into pUC-19 to produce the plasmid pMAS, plasmid DNA was extracted from white colonies and analysed by restriction enzyme analysis (Figure 6.3). The diagnostic restriction enzymes chosen were *Bam*H I and *Hind* III. *Bam*H I was chosen to confirm that the insert was the correct size (2.2 kb, lane 2, Figure 6.3) and *Hind* III was chosen to verify that the cloned *Bam*H I fragment contained the desired 1.6 and 0.6 kb fragments. Depending on the overall orientation of the fused *Bam*H I/*Hind* III fragments, a *Hind* III digest could yield either restriction fragments of 1.6 and 3.3kb or 0.6 and 4.3 kb. The former arrangement was observed in this clone (lane 1, Figure 6.3).

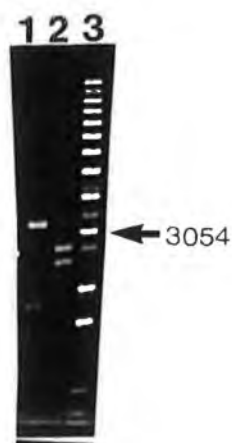
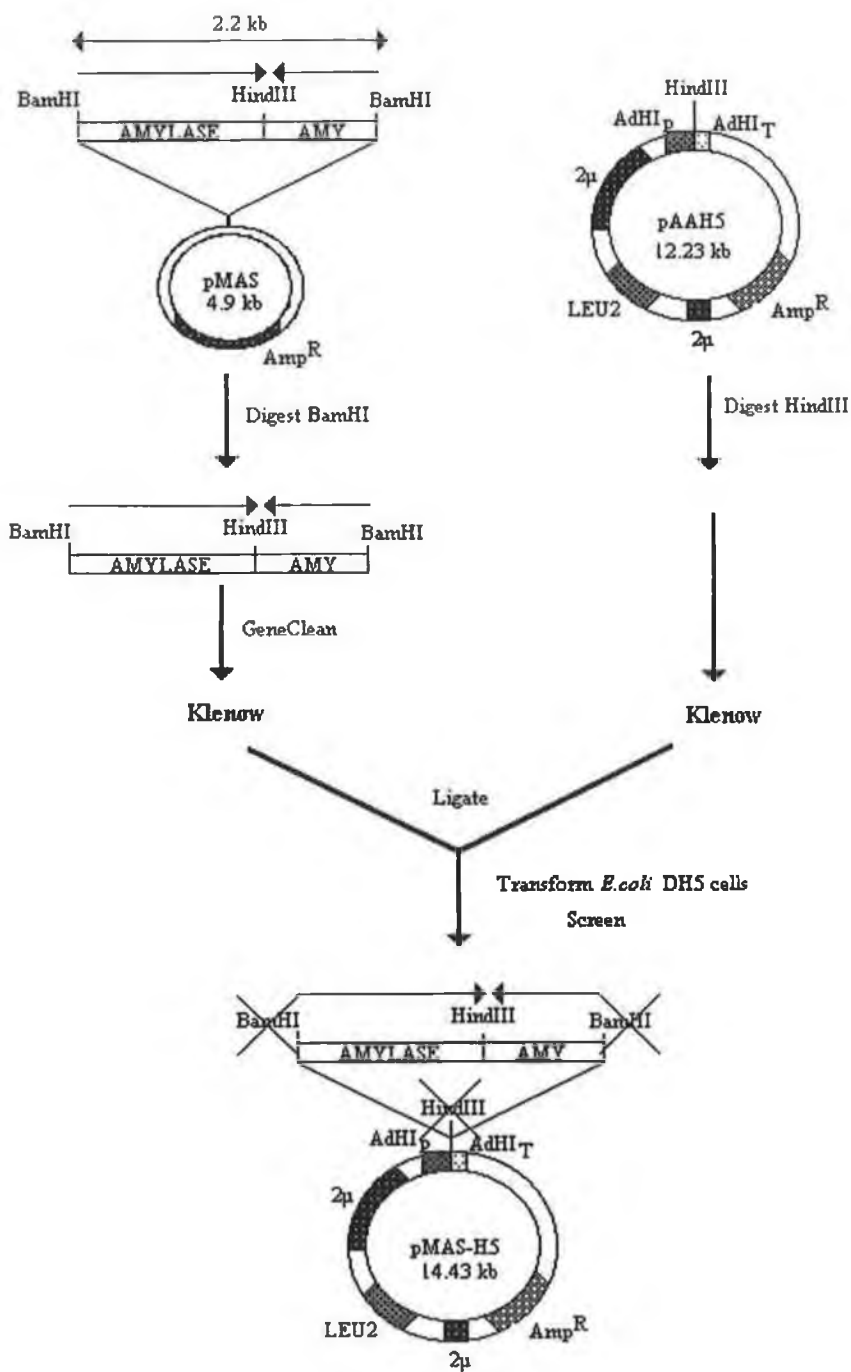


Figure 6.3 Restriction analysis of pMAS.

The appropriate sizes of each of the restriction fragments are shown in kilobases, in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- Lane 1:** pMAS digested with *Hind* III (3.3; 1.6 kb)
Lane 2: pMAS digested with *Bam*H I (2.7; 2.2 kb)
Lane 3: 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb).

The second step in the construction of pMAS-H5 involved restricting the plasmid pMAS with *Bam*H I to release the 2.2 kb fragment containing the sense-antisense α -amylase gene fragment. This fragment was then subcloned into the yeast episomal vector, pAAH5. This vector contains one unique site for cloning, a *Hind* III site, situated between the *AdHI* promoter and terminator. However, this restriction site was unsuitable for the cloning of the sense-antisense α -amylase gene, which was cloned into pUC-19 as a *Bam*H I fragment. To overcome this problem, the plasmid pMAS was restricted with *Bam*H I, the 2.2 kb *Bam*H I fragment was gene cleaned from an agarose gel, and the sticky ends produced after *Bam*H I restriction digestion were filled-in using the Klenow enzyme. Meanwhile the vector pAAH5 was restricted with *Hind* III and the ends produced were once again filled-in using the Klenow enzyme. Both the vector and the fragment were then ligated in a blunt-ended ligation and transformed into DH5 α cells (a more detailed description of the construction of pMAS-H5 is given in the legend to Figure 6.4).



✕ Indicates loss of restriction site

Figure 6.4 Schematic representation of the construction of the plasmid, pMAS-H5.

pAAH5 was restricted with *Hind* III and gene cleaned (section 2.6.11.9) to remove the restriction buffer. In the meantime the 2.2 kb *Bam*HI fragment from pMAS was gene cleaned from a 0.7% agarose gel. Both the *Hind* III digested pAAH5 vector and the 2.2 kb *Bam*HI pMAS fragment were placed in the same microfuge tube and the overhangs produced following restriction digestion were filled-in using the Klenow enzyme (section 2.6.11.2) in the presence of 0.5mM dNTPs for 30 minutes at room temperature. Following this the reaction mix was phenol extracted twice, chloroform extracted once and then ethanol precipitated (section 2.6.11.7). The blunt ends were then ligated with T₄ DNA ligase at 4°C overnight. The ligation mix was then transformed into DH5α cells. Transformants were plated on LB amp starch (2% Lintners) plates. Colonies were then screened for the presence of the 0.6 kb 5' antisense α-amylase fragment tagged onto the 3' UTR of the pMO2 sense α-amylase gene. The correct insert orientation being determined by either a *Kpn* I or a *Hind* III/*Xba* I digest. This plasmid was named pMAS-H5 and is 14.43 kb in size.

Plasmid DNA was extracted from colonies after transformation of the ligation mixture into DH5α cells. This plasmids' construction was then confirmed using diagnostic restriction enzyme analysis (Figure 6.5). The enzymes chosen for the determination of the desired orientation of the sense-antisense α-amylase gene sequence insert in pAAH5 were either *Kpn* I or *Hind* III/*Xba* I (see Appendix VIII for details). A *Kpn* I digest of pMAS-H5 with the fragment in the desired sense-antisense orientation relative to the *AdHI* promoter and terminator should give restriction fragments of 7.78, 4.14 and 2.5 kb respectively. If the fragment ligated to pAAH5 in the antisense-sense orientation relative to the *AdHI* promoter and terminator, a *Kpn* I restriction digest should produce fragments of 6.77, 5.15 and 2.5 kb. In the desired sense-antisense orientation a *Hind* III/*Xba* I restriction digest should produce fragments of 10.67 and 3.75 kb whereas in the antisense-sense orientation fragments of 11.68 and 2.74 kb would be visualised.

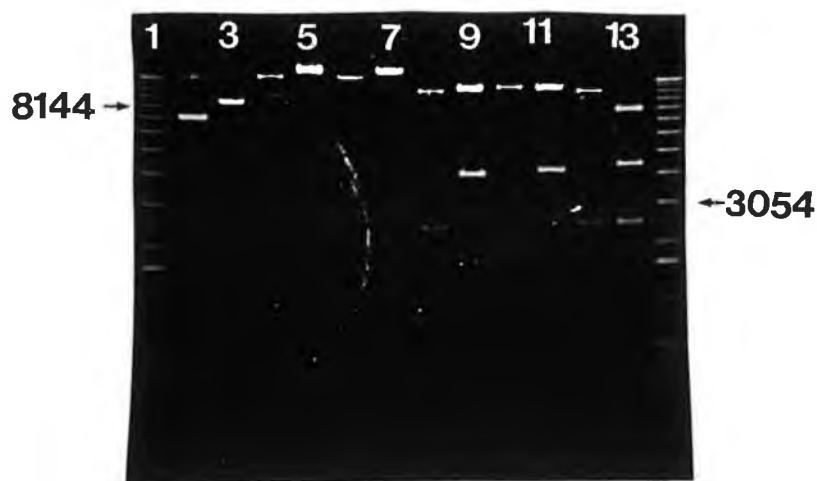


Figure 6.5 Restriction analysis of pMAS-H5.

The appropriate sizes of each of the restriction fragments are shown in kilobases, in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- Lane 1:** 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb).
- Lane 2:** pAAH5 undigested
- Lane 3:** pMAS-H5 undigested
- Lane 4:** pAAH5 digested with *Hind* III (12.23 kb)
- Lane 5:** pMAS-H5 digested with *Hind* III (14.43 kb)
- Lane 6:** pAAH5 digested with *Xba* I (12.23 kb)
- Lane 7:** pMAS-H5 digested with *Xba* I (14.43 kb)
- Lane 8:** pAAH5 digested with *Hind* III \ *Xba* I (10.08; 2.15 kb)
- Lane 9:** pMAS-H5 digested with *Hind* III \ *Xba* I (10.67; 3.75 kb)
- Lane 10:** pAAH5 digested with *Bam*H I (10.23; 2.0 kb)
- Lane 11:** pMAS-H5 digested with *Bam*H I (10.23; 4.2 kb)
- Lane 12:** pAAH5 digested with *Kpn* I (9.73; 2.5 kb)
- Lane 13:** pMAS-H5 digested with *Kpn* I (7.78; 4.14; 2.5 kb)
- Lane 14:** 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb).

Figure 6.5 confirms the construction of pMAS-H5 from the *Hind* III/*Xba* I (lane 9) and *Kpn* I (lane 13) restriction digests. Both these lanes producing the expected band sizes of 10.67 and 3.75 kb in the case of the *Hind* III/*Xba* I restriction, and fragment sizes of 7.78, 4.14 and 2.5 kb when restricted with *Kpn* I. Also the *Hind* III digest of pMAS-H5 (lane 5, Figure 6.5) produced a single high molecular weight band (expected to be 14.43 kb). This band is greater than the 12.23 kb band produced after restriction digestion of pAAH5 with *Hind* III, (lane 4, Figure 6.5), which indicates a successful cloning event. Because, although the unique *Hind* III site for cloning had been removed from pAAH5, there was an internal *Hind* III restriction enzyme site within the cloned sense-antisense α -amylase gene fragment, which when digested with this enzyme, should yield this band of 14.43 kb (lane 5, Figure 6.5). For a detailed restriction map of pMAS-H5 see Figure 6.6.

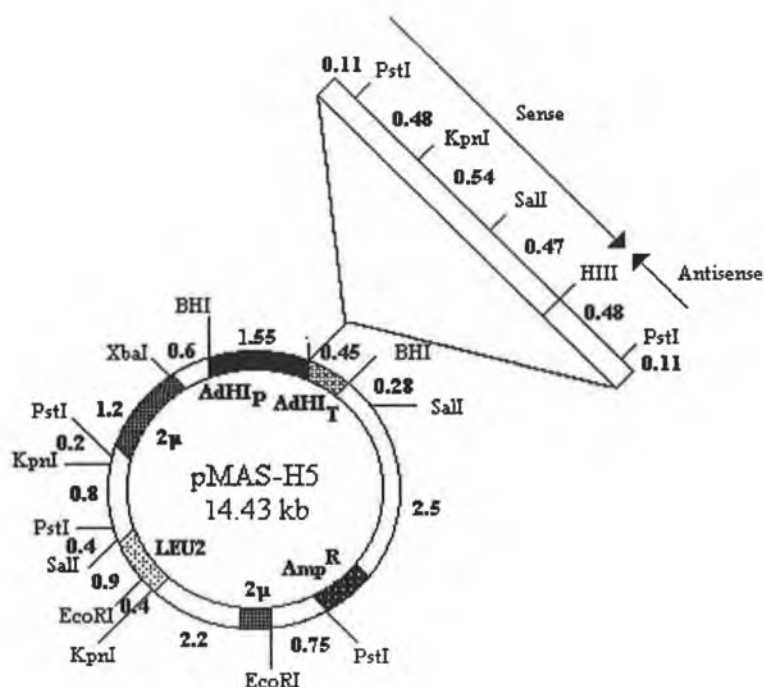


Figure 6.6 Plasmid map of pMAS-H5, (pAAH5 vector with α -amylase *cis* fusion sequences).

BHI represents the restriction enzyme *Bam*H I.

6.2 TRANSFORMATION AND CONFIRMATION OF THE PRESENCE OF THE PLASMID pMAS-H5 IN DBY746

The plasmid pMAS-H5 was transformed into DBY746. Confirmation of the presence of this plasmid in DBY746 was demonstrated initially by its' ability to grow on selective media. The transformation mixture was plated onto minimal media supplemented with uracil only, as the plasmid pMAS-H5 (a derivative of pAAH5, appendix VIII) carries a *LEU2* marker thus a successful transformation event will convert the DBY746 strain to leucine prototrophy. *Leu*⁺ transformants were isolated.

Southern blot analysis was carried out to (i) prove the presence of the plasmid in DBY746 and (ii) to establish the integrity of the plasmid i.e. that no rearrangements or loss/deletions had occurred within the plasmid. Genomic DNA was extracted from DBY746::pMAS-H5 and electrophoresed both undigested and digested with various restriction enzymes on a 0.8% agarose gel, then blotted and probed with a ³²P-labelled sense α -amylase riboprobe (section 4.1.2.4). The restriction enzymes chosen were *Hind* III (which linearises the plasmid to give a band of 14.43 kb, the presence of a band this size on the blot would indicate no deletions from the plasmid) and *Bam*H I (which should cut out the 4.2 kb *AdHI* promoter-sense-antisense α -amylase gene sequence-*AdHI* terminator fragment).

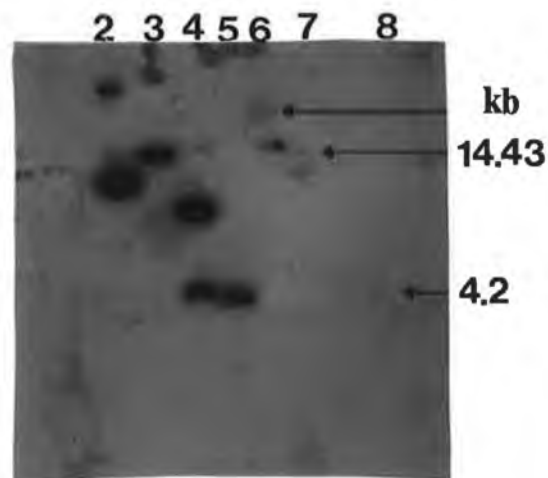


Figure 6.7 Southern blot, probed with a ^{32}P -labelled sense α -amylase riboprobe, to confirm the presence of the plasmid pMAS-H5 in DBY746.

- Lane 1:** 1kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb).
- Lane 2:** pMAS-H5 undigested
- Lane 3:** pMAS-H5 digested with *Hind* III (14.43 kb)
- Lane 4:** pMAS-H5 digested with *Kpn* I (7.78; 4.14 kb)
- Lane 5:** pMAS-H5 digested with *Bam*H I (4.2 kb)
- Lane 6:** DBY746::pMAS-H5 undigested
- Lane 7:** DBY746::pMAS-H5 with digested with *Hind* III
- Lane 8:** DBY746::pMAS-H5 with digested with *Bam*H I

The *Hind* III digest of DBY746::pMAS-H5 (lane 7, Figure 6.7) highlighted 2 bands on the autoradiograph, one of these bands corresponds to undigested DNA, and the second band was equivalent to linearised pMAS-H5. The *Bam*H I digest of DBY746::pMAS-H5 (lane 8, Figure 6.7) digest revealed a 4.2 kb band equivalent to the *AdHI* promoter-sense-antisense α -amylase gene sequence-*AdHI* terminator fragment. From the *Hind* III and *Bam*H I digests one

can conclude the presence of pMAS-H5 in DBY746, also that the plasmid was of the correct size, with the correct insert size of 4.2 kb.

6.3 EFFECT OF THE CIS-ACTING ANTISENSE SEQUENCE ON α -AMYLASE GENE EXPRESSION IN THE YEAST STRAIN DBY746 FROM THE EPISOMAL PLASMID pMAS-H5

When the plasmid pMAS-H5 was transformed into DBY746, the transformation mixture was plated onto minimal media containing 2% Lintners starch. All of the transformants, from visualisation of the plates were unable to degrade the starch, thus demonstrating the ablation of α -amylase activity by the *cis*-acting antisense sequences.

An important point to note here in relation to the chimeric sense-antisense transcript is that there was expression of the α -amylase gene when the plasmid pMAS-H5 was transformed into DH5 α and plated on LB amp plates containing 2% Lintners' starch. This indicates that the α -amylase gene is functional and that the fusion to the antisense sequence and subsequent cloning into the yeast shuttle vector pAAH5 has not knocked-out expression of the gene. Therefore the lack of α -amylase activity observed when transformed into DBY746, is most likely due to the *cis*-acting antisense sequence and not to an anomaly of the cloning. The fact that the sense-antisense construct exhibits α -amylase activity in DH5 α might be explained because transcription and translation are coupled processes in prokaryotes. When a gene is being transcribed ribosomes latch onto nascent transcripts and begin to translate before transcription is complete. Hence the sense sequence would be in the process of being translated before the antisense sequence has even been transcribed. Since most bacterial mRNAs are translated as soon as transcription has created a molecule long enough to be recognised by ribosomes, the degree of inhibition achieved by the antisense RNAs will depend on its rate of interaction with the target. In turn, the rate will depend on the concentration of both RNA species and their reactivity towards one another, assuming that they are in present in the cell.

Approximately 50 colonies, produced after transformation of pMAS-H5 into DBY746, were patched onto minimal media plates containing 2% Lintners starch. No α -amylase activity was observed for any of the transformants (see

Figure 6.8, where one transformant is shown). A DNS assay was performed to confirm this result. The assay was repeated 3 times. The cumulative results of this experiment are represented by the bar chart shown below (Figure 6.9). Results are reported as the % amylase activity relative to the control strain DBY746::pMO2 (100%).



Figure 6.8 Minimal media plate containing 2% Lintners' starch, supplemented with the appropriate amino acid. Section [1] contains the amyolytic strain DBY746::pMO2 and section [2] strain DBY746::pMAS-H5.

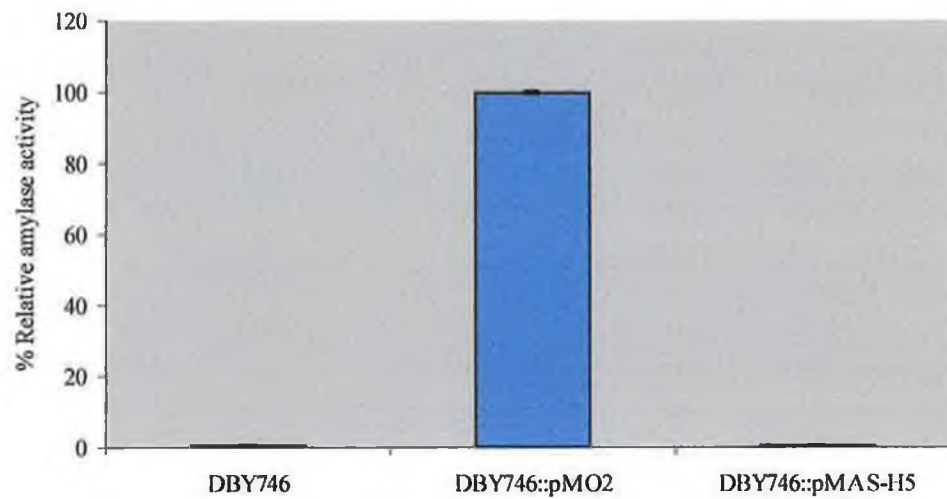
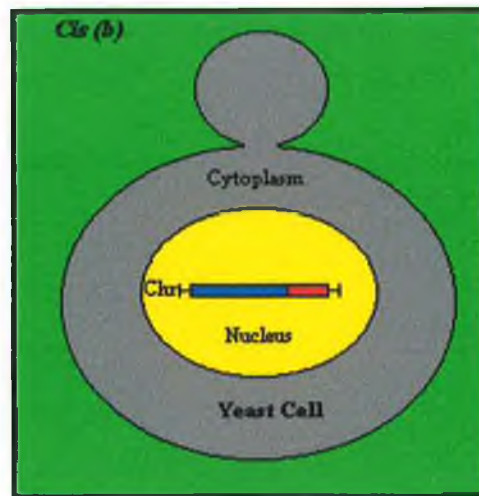


Figure 6.9 Effect of the *cis*-acting antisense sequence on α -amylase gene expression from the yeast episomal plasmid, pMAS-H5.

As can be seen from the barchart (Figure 6.9), the result obtained confirms what was observed after the transformants had been plated onto minimal media starch plates, which was, a complete inhibition of α -amylase activity.

Cis-Experiment (b)



 Sense amylase sequences

 Antisense amylase sequences

Chr, Chromosomal location

In *cis*-Experiment (b), the same sense-antisense α -amylase sequence used in *cis* (a) was subcloned into a yeast-integrating vector. Integration was targeted to the *ura3*-52 locus of DBY746. This was carried out to determine whether the location of the gene was significant.

6.3.1 CONSTRUCTION AND INTEGRATION OF THE “*CIS*-ACTING” INTEGRATING VECTOR, pFL34-MAS

The *AdHI* promoter-sense-antisense α -amylase gene sequence-*AdHI* terminator fragment from pMAS-H5 was cloned into the integration vector pFL34 (section 4.1.1, Appendix I) to produce pFL34-MAS. The plasmid pFL34-MAS is represented schematically in Figure 6.10 and a detailed description of its' construction are included in the legend to this figure.

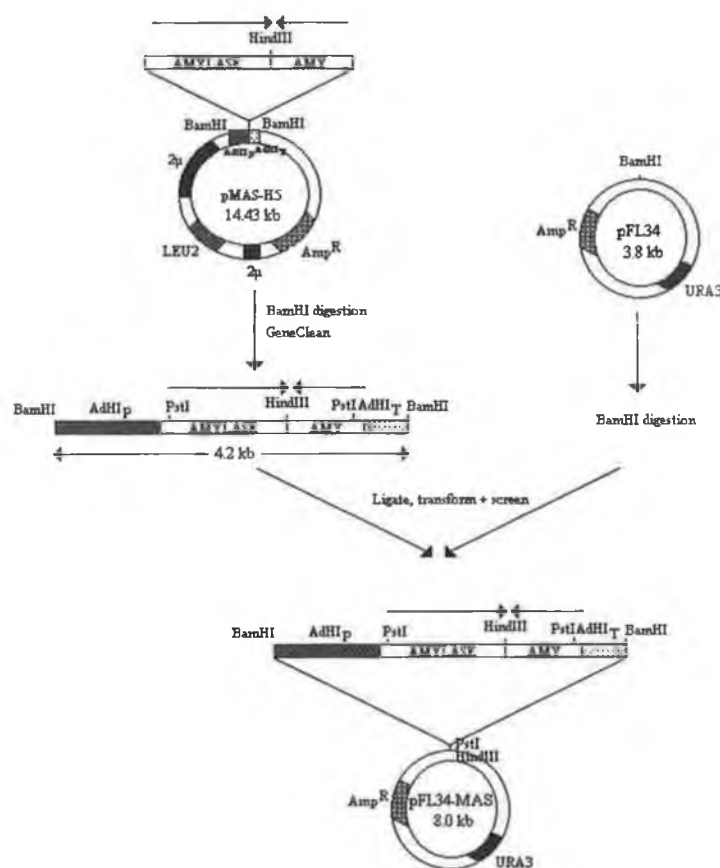


Figure 6.10 Schematic representation of the construction of the integrating *cis*-acting plasmid, pFL34-MAS.

The 4.2 kb *Bam*H I fragment from pMAS-H5 which contains the sense-antisense α -amylase fragment between the *AdHI* promoter and terminator was gene cleaned (section 2.6.11.9) from a 0.7% agarose gel and cloned into the unique *Bam*H I site of the integrating vector pFL34. DH5 α cells were transformed with this ligation mix and screened on LB amp starch (2% Lintners) plates containing X-gal and IPTG, which allows exploitation of the *lacZ* phenotype. With this clone orientation does not matter as the insert is in the correct orientation with respect to the *AdHI* promoter and terminator. This plasmid was named pFL34-MAS and is 8.0 kb in size.

To confirm the successful construction of the plasmid pFL34-MAS, restriction enzyme analysis was performed (Figure 6.11). Plasmid DNA was

extracted from white colonies after transformation of the ligation mix into DH5 α *E. coli* cells. The restriction enzyme *Bam*H I was chosen to prove that the insert was of the correct size (i.e. 4.2 kb). pFL34-MAS was also restricted with *Hind* III and *Pst* I to determine in which orientation was the cassette ligated into pFL34. As mentioned already, orientation does not matter in this case, as the sense-antisense α -amylase gene sequence is in the correct orientation, relative to the *AdHI* promoter and terminator. Also, the restriction fragments obtained after digestion with *Hind* III and *Pst* I are of the expected sizes and are a further proof of the construction of the plasmid pFL34-MAS.

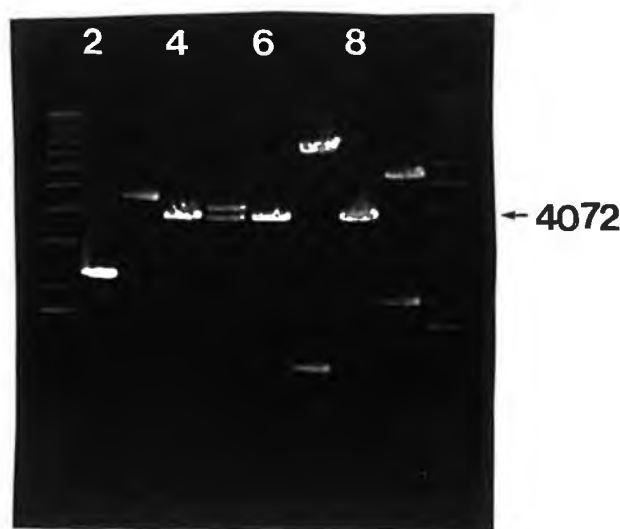


Figure 6.11 Restriction analysis of pFL34-MAS.

The appropriate sizes of each of the restriction fragments are shown in kilobases in brackets below. The relevant sizes of the 1 kb ladder are as indicated.

- | | |
|----------------|---|
| Lane 1: | 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb). |
| Lane 2: | pFL34 undigested |
| Lane 3: | pFL34-MAS undigested |
| Lane 4: | pFL34 digested with <i>Bam</i> H I (3.799 kb) |
| Lane 5: | pFL34-MAS digested with <i>Bam</i> H I (4.2; 3.799 kb) |
| Lane 6: | pFL34 digested with <i>Hind</i> III (3.799 kb) |
| Lane 7: | pFL34-MAS digested with <i>Hind</i> III (6.95; 1.05 kb) |
| Lane 8: | pFL34 digested with <i>Pst</i> I (3.799 kb) |
| Lane 9: | pFL34-MAS digested with <i>Pst</i> I (5.459; 1.97; 0.56 kb) |

Lane 10: 1 kb ladder (12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.506 kb).

From Figure 6.11, the *Bam*H I digest confirms an insert of 4.2 kb into the 3.8 kb vector, pFL34. Also both the *Hind* III and *Pst* I restriction digests which gave fragment sizes of 6.95 and 1.05 kb in the case of the former and 5.459, 1.97 and 0.56 kb in the case of the latter, demonstrate that the cassette ligated in the orientation shown in Figure 6.10.

6.4 INTEGRATION AND CONFIRMATION OF INTEGRATION OF THE PLASMID pFL34-MAS INTO DBY746 TO PRODUCE THE INTEGRANT, SA.

Integration of the plasmid pFL34-MAS was directed towards the *ura3-52* locus of the yeast strain DBY746 by linearisation of this plasmid at the unique *Stu* I site within the *URA3* marker. Integration leads to a duplication of the *URA3* gene and conversion of the strain to uracil prototrophy (Figure 6.12).

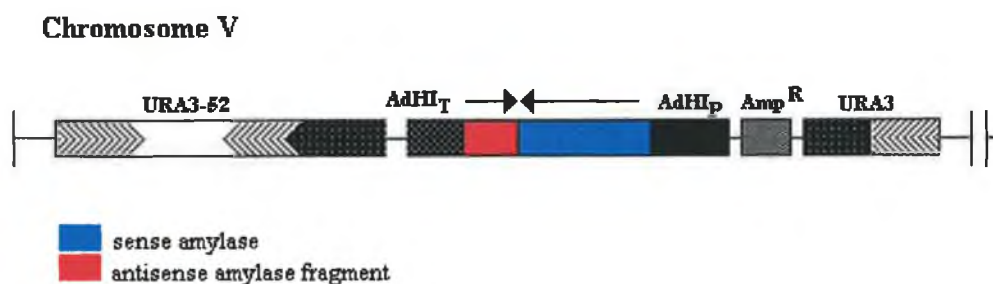


Figure 6.12 Schematic representation of the probable integration event of the plasmid pFL34-MAS into the *ura3-52* locus of DBY746 to produce the integrant, SA.

To show that integration had occurred the transformants were (i) grown on selective media, (ii) the stability of one of the transformants was analysed and (iii) Southern blot analysis was carried out. Only the transformants which

received the *URA3* gene from the plasmid pFL34-MAS were capable of growth on uracil minus minimal media. As the plasmid, pFL34-MAS does not contain an origin of replication, growth of transformants on media lacking uracil would strongly suggest the presence of the integrated plasmid. As DBY746 is auxotrophic for both uracil and leucine, the transformants were plated on minimal media containing only leucine. According to the predicted mechanism of integration, if the *ura* gene is integrated, one would expect the sense-antisense α -amylase gene also to be inserted into the chromosome.

To examine the mitotic stability of the *ura3-52* integrated exogenous DNA sequences the yeast transformants were analysed as described in section 4.1.1.1.2. The results (Figure 6.13) indicated that the *ura3-52* integrated sequences were stably maintained during mitotic growth in rich medium. This high stability of the *ura*⁺ phenotype constitutes additional proof that integration into the yeast genome had occurred.

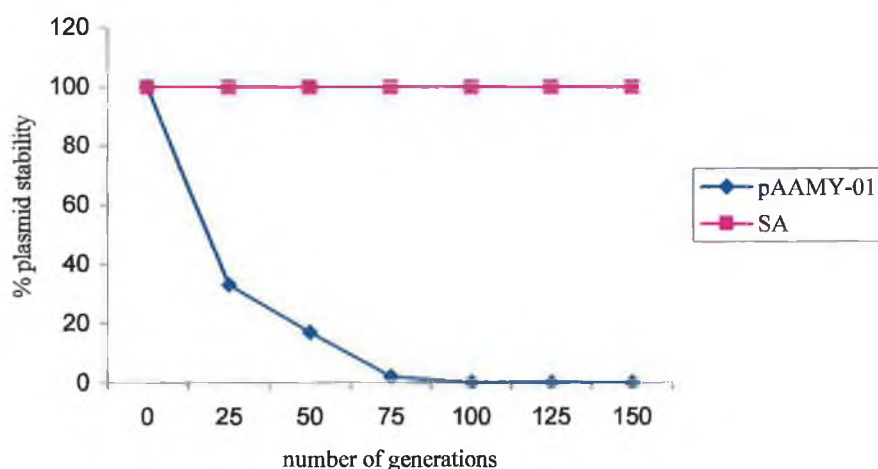


Figure 6.13 Mitotic stability of the *ura3-52* sense-antisense α -amylase integrant, SA.

To prove integration by Southern blot analysis genomic DNA extracted from SA was digested with a restriction endonuclease which cuts outside the plasmid DNA. The enzyme chosen was *Xho* I. This should yield a single band of high molecular weight on the gel when Southern blotted and probed with a ³²P-

labelled α -amylase probe, if integration had been successful. Integration was demonstrated by the presence of a single high molecular weight band (data not shown).

6.5 EFFECT OF THE CIS-ACTING ANTISENSE SEQUENCE ON α -AMYLASE GENE EXPRESSION IN THE INTEGRATED STRAIN SA. FROM THE VECTOR pFL34- MAS.

An inhibitory effect (if any) on the expression of the target gene was analysed initially by plating the transformants of SA onto minimal media plates containing 2% Lintners' starch. After growth at 30°C for 72 hours, the plates were either flooded with iodine vapour or incubated for 12 hours at 4°C, in order to allow the starch to precipitate. The transformants were then checked for haloes. Interestingly, visualisation of these plates, displayed the same result which was found with the DBY746::pMAS-H5 transformants. There were no haloes surrounding any of the colonies thus indicating a complete down-regulation of α -amylase gene activity. A number of colonies from the integrative transformation event were patched onto fresh minimal media plates with an amylolytic strain as a positive control. The same result was obtained, no amylolytic activity was observed (Figure 6.14).

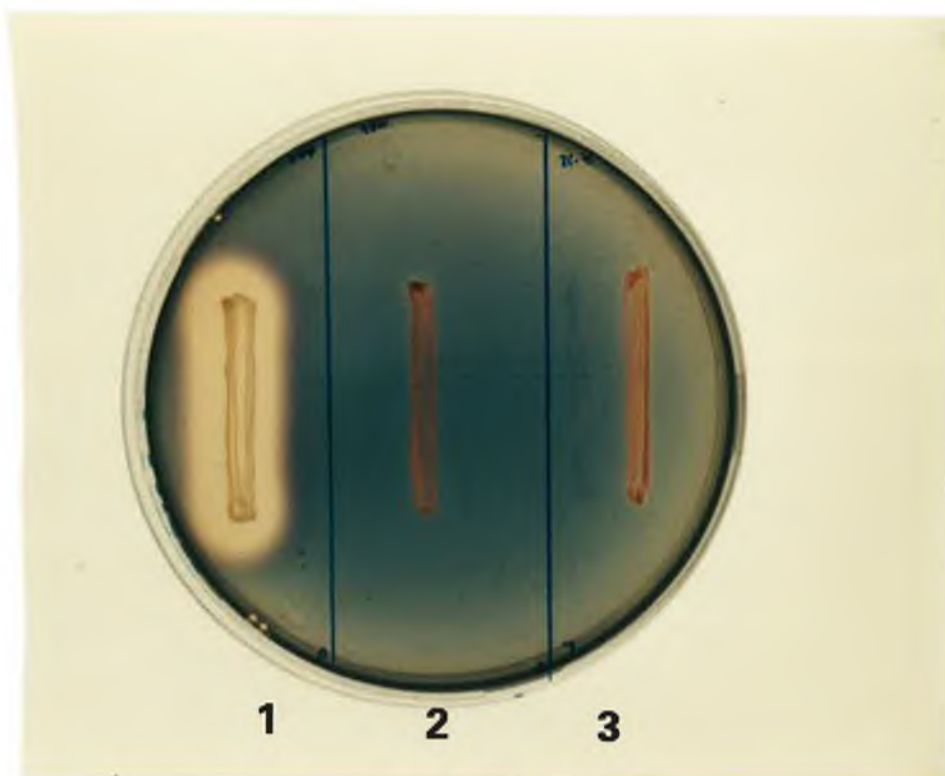


Figure 6.14 Minimal media plate containing 2% Lintners' starch, with each section of the plate supplemented with the appropriate amino acid. Section [1] contains the amylolytic strain DBY746::pMO2, section [2] strain DBY746::pMAS-H5 and [3] SA, the integrant.

A number of the transformants were further analysed by DNS assay to substantiate the findings obtained from observation of the minimal media starch plates. The transformants were grown up in liquid minimal media until the cell growth had reached mid-log phase. The cultures were then spun down at 5,000 rpm for 5 minutes at 4°C and the supernatants were analysed for a change in α -amylase activity in comparison to the control strain SI. The strain DBY746 was used as a negative control in this experiment. All samples were assayed in triplicate and the entire assay was repeated 3 times.

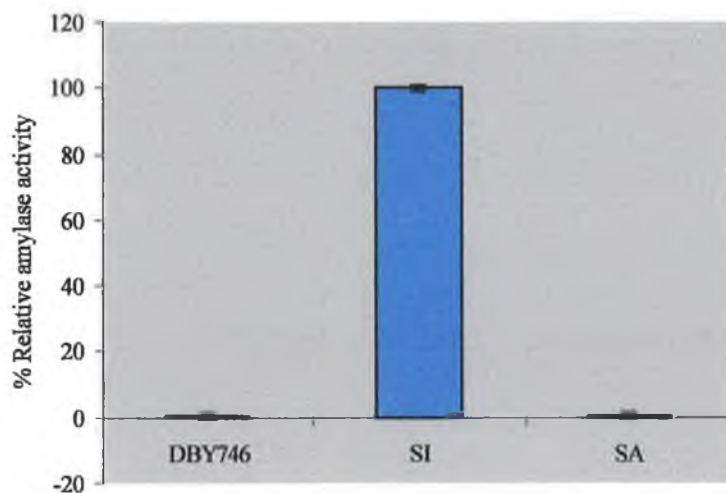


Figure 6.15 Effect of the *cis*-acting antisense sequence on α -amylase gene expression from the integrated yeast strain, SA.

The results of these assays (Figure 6.15) did indeed substantiate what was found when the transformants were grown on minimal media starch plates, that being there was no α -amylase activity detected.

6.6 RNA ANALYSIS

In an attempt to determine the mechanism of suppression of the activity of the α -amylase gene, Northern blot analysis was performed. This analysis involved the extraction of total RNA, under the conditions described in section 2.6.10. The RNA was then separated on a denaturing agarose-formaldehyde gel and bi-directionally transferred to a nitrocellulose membrane. One blot was probed with a ^{32}P -labelled antisense α -amylase riboprobe and the second with a ^{32}P -labelled β -actin riboprobe. The purpose of probing with the latter probe was to demonstrate [1] that the RNA was not degraded and [2] to allow quantification of the levels of RNA produced. The Northern analysis was completed on two

independently prepared RNA samples from a representative transformant for DBY746::pMAS-H5 and SA (data shown for one RNA sample only). DBY746 total RNA was run on the gel as a negative control and DBY746::pMO2 (a plasmid which contains the sense α -amylase gene, minus its' transcription stop site) was run as a positive control.

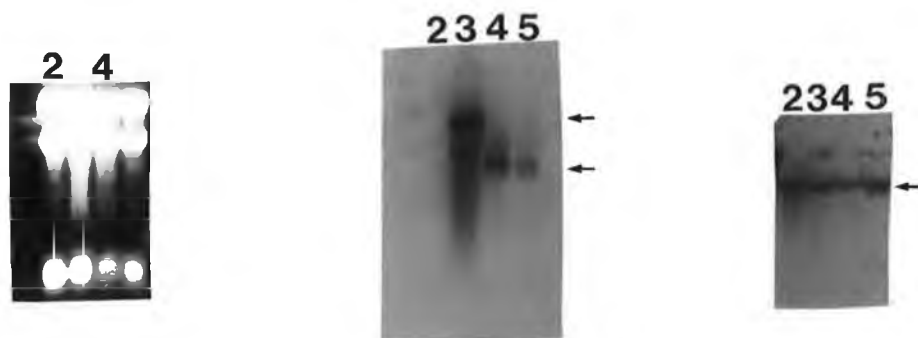


Figure 6.16 [a] Formaldehyde agarose gel of total RNAs. Bi-directional Northern blot of total RNAs probed with 6.16 [b] a ^{32}P -labelled antisense α -amylase riboprobe and with 6.16 [c] a ^{32}P -labelled β -actin riboprobe.

Lane 1:	RNA markers (6,583; 4,981; 3,638; 2,604; 1,908; 1,383; 955; 623; 281 bp)
Lane 2:	DBY746
Lane 3:	DBY746::pMO2
Lane 4:	DBY746::pMAS-H5
Lane 5:	SA

Surprisingly, when lanes 4 and 5 (Figure 6.16 [b]) containing the *cis*-acting episomal plasmid, DBY746::pMAS-H5 and the strain SA containing the *cis*-acting integrated plasmid pFL34-MAS respectively, were probed with the antisense α -amylase riboprobe a 1.4 kb transcript was visualised. A larger transcript of approximately 2.3 kb was expected if transcription of this chimeric sense-antisense transcript was initiated close to the *AdHI* promoter and terminated at the *AdHI* transcript terminator. Hybridisation of the β -actin riboprobe produced a strong discrete band of the expected size of 1.3 kb in the lanes containing DBY746::pMAS-H5 and SA (lanes 4 and 5, Figure 6.16 [c]) which indicates that there was no degradation of the RNA samples loaded.

Studies carried out by Melton (1985) observed a somewhat similar result to what was found here. Melton found that an antisense RNA of 625 base pairs formed an antisense RNA:sense RNA duplex in injected oocytes of ~ 220 base pairs, the same size as the protected antisense:sense RNA duplex observed after hybridisation *in vitro*. The reason why a much shorter duplex was protected is unknown, but the author speculated that this might have been due to the formation of strong intramolecular hairpins. This may be one possible explanation for the existence of the 1.4 kb transcript visualised, as opposed to the expected 2.3 kb transcript. If this is the correct interpretation of the data, this finding possibly indicates a post-transcriptional mechanism of α -amylase gene expression. Intramolecular RNA duplex formation favourable in the *cis* conformation, would stabilise the mRNA by the protection of the molecule from single-stranded nucleases, but still interfere at the translational level by prevention of polysome assembly, thus eliminating α -amylase gene expression.

As previously mentioned antisense transcripts can be rather unstable and are not always easy to detect. Although a random primed probe or a nick translated fragment should detect both sense and antisense RNA, Nellen and Lichtenstein (1997) strongly recommend the use of strand-specific *in vitro* transcripts as hybridization probes. This is because these probes do not only unambiguously allow for distinguishing between the two types of RNA, but they are also more sensitive. They also state that the sense or antisense transcripts may not always correspond in size to the wild-type mRNA or to the length expected from the vector construct respectively; sometimes specific degradation products are observed. The RNA samples in this experiment were probed with an antisense α -amylase riboprobe, which should bind to the sense sequences within the sense-antisense transcript. However, after observing the unexpected size (1.4 kb) of the actual sense-antisense transcript, upon reflection it would have been more informative if the samples were also hybridized with a sense α -amylase riboprobe, as this would detect any specific antisense α -amylase degradation products also.

As expected the negative control strain used in this experiment, DBY746 generated no signal on the autoradiograph when probed with the antisense α -amylase riboprobe (lane 2, Figure 6.16 [b]). However a strong, discrete band of

1.3 kb was visualised when DBY746 was probed with the β -actin riboprobe (lane 2, Figure 6.16 [c]). The positive control, DBY746::pMO2, expressed its' full-length transcript of 1.7 kb when probed with the antisense α -amylase riboprobe (lane 3, Figure 6.16 [b]). Also a strong, discrete band of 1.3 kb was visualised when this sample was probed with the β -actin riboprobe (lane 3, Figure 6.16 [c]).

The intensities of the bands from both blots were analysed on a Pharmacia-Biotech (ImageMaster 1D) Imaging Densitometer. Analysis of the blot probed with the β -actin riboprobe showed a weak hybridisation signal for the lane containing the DBY746::pMAS-H5 RNA due to a lower loading when compared to DBY746::pFL34-MAS or DBY746. However a greater amount of message was being produced by DBY746::pMAS-H5 in contrast to DBY746::pFL34-MAS (2.0:1, respectively) whose β -actin hybridisation signal was strong but when probed with the antisense α -amylase riboprobe the message was weaker. This result was expected as the episomal pMAS-H5 should be present in higher copy number than the integrating plasmid pFL34-MAS and this indeed was what was reflected in the levels of message being produced.

6.7 DISCUSSION

The aims of this experiment were to [1] position the antisense α -amylase gene sequence in the vicinity of the target gene ("location or spatial effect") and [2] to determine if this antisense RNA, synthesized in the same physical space in the cell as the sense RNA, improved upon antisense RNA control. The experiments documented in this chapter succeeded in achieving both of these goals. A chimeric sense-antisense α -amylase gene was cloned into both a yeast episomal vector and a yeast integrating vector, with the result that α -amylase gene expression was inhibited when expressed from either its' episomal or chromosomal position within the cell.

Although the results obtained demonstrated a total inhibition of α -amylase gene activity, (from the chimeric *cis*-acting transcript, regardless of its' episomal or chromosomal location), the precise mechanism of the *cis*-effect was not determined. Atkins and Gerlach, (1994) who reported a 90% reduction in

CAT expression, when both target and antisense genes were transcribed in *cis*, found that the steady-state levels of the antisense RNA construct were two to three times higher than that of the CAT RNA. Although, the authors did not determine precisely the mechanism of inhibition by the *cis*-acting RNA, they postulated that in view of the higher steady-state levels of the antisense RNA that this result indicated a post-transcriptional mechanism of CAT inactivation, that is not dependent on antisense-mediated degradation of the mRNA. They propose that an intramolecular duplex formation, favourable in the *cis* conformation, would stabilise the mRNA by the protection of the molecule from single-stranded nucleases, but still interfere at the translational level by preventing polysome assembly, thus reducing CAT expression (Atkins and Gerlach, 1994). A similar translational-level interference has been reported for *cis*-acting antisense RNA regulation of the human α globin cDNA chimeric mRNAs in *in vitro* translation systems (Liebhaber et al., 1992).

Although the results obtained in this chapter, demonstrated a total inhibition of α -amylase gene activity, (from the chimeric *cis*-acting transcript, regardless of its' episomal or chromosomal location), the precise mechanism (similar to Atkins and Gerlach, 1994), of the *cis*-effect was not determined. The RNA analysis carried out in this chapter showed that a 1.4 kb transcript was being made from both the pMAS-H5 (episomal) and pFL34-MAS (chromosomal) plasmids, even though a 2.3 kb chimeric transcript was expected. One possible explanation for the presence of this 1.4 kb transcript may be that the antisense sequence folded-back on the sense and was cleaved in such a way as to produce this 1.4 kb discrete transcript.

Another hypothesis to explain suppression of α -amylase gene expression, may be that the formation of an intramolecular duplex in the nucleus may have prevented nucleo-cytoplasmic transport (Kim and Wold, 1985), hence no translation of the α -amylase gene. Also the fact that the antisense sequence tagged onto the sense α -amylase gene was targeted to the 5' RBS and coding region, may mean that if this transcript did reach the cytoplasm, the fact that the RBS was complexed with the antisense RNA sequence would mean that translation could not proceed. One method to test this particular hypothesis would be the examination of polysome association of the mRNA of interest; if it

is associated with large polysomes (as found in wild-type cells) then there is obviously no effect from the antisense transcripts. If, however, the RNA is found in the monosome peak or with smaller polysomes, it is most likely that there is an antisense-mediated inhibition of translation (Melton, 1985).

Further investigations to determine the precise mechanism of action could include determining what part of the transcript was cleaved (by performing RNA fingerprint analysis). Also, presuming that a specific cleavage occurred, which finally resulted in the 1.4 kb transcript, it would be interesting to determine if the chimeric transcript was degraded from either the 5', 3' or both ends of the transcript. Both 5' → 3' and 3' → 5' exoribonucleases have been detected in yeast. This could be achieved by probing with a probe specific for the CAP binding site or with a poly(T) probe to detect the presence of a poly(A) tail. Also, analysis of the α -amylase gene sequence shows that any transcript of 1.4 kb would not contain the necessary translational start, coding and translational stop sequences, to allow expression of this gene.

It is known that at least 5 factors affect or strongly influence the stability of yeast mRNA; [1] the 5' cap, [2] the 3' poly(A) tail, [3] mRNA length, [4] mRNA stability determinants (sequences within both prokaryotic and eukaryotic mRNAs that strongly influence their half-lives) and [5] the presence or absence of various nucleases in the yeast cell (Brown et al., 1988). Due to the fact that the 1.4 kb transcript appears to be stable one would presume that either the 5' cap or 3' poly-A tail must be present. A recent study carried out by Donahue and Fedor (1997) on the kinetics of hairpin ribozyme cleavage in yeast stated that the impact of cleavage on mRNA abundance is shown to depend directly on intrinsic mRNA stability and surprisingly, the cleavage products were no more labile than uncleaved mRNAs despite the loss of terminal cap structures or poly(A). Hence it is possible that the 1.4 kb transcript visualized could lack either of these structures and still remain relatively stable.

The α -amylase sense gene sequence used to construct the *cis*-acting plasmid contained a deletion which disrupted the putative palindromic transcriptional termination site, but left the entire α -amylase gene and its' translational stop site intact. When designing this experiment it was important to demonstrate that this truncated α -amylase gene from pMO2 was capable (which

it was) of α -amylase activity. In fact, McMahon (1995) and Olsen (1995) both designed experiments to analyse what effect, if any, the disruption of this palindromic sequence would have on the levels of α -amylase produced from both *E. coli* and *S. cerevisiae*. Essentially this palindromic sequence is characteristic of classical prokaryotic terminators, centred some 12 – 24 bases upstream of the termination site (12 bases in this case), immediately followed by a run of 8Ts (7 Ts in the case of this α -amylase gene sequence). There are no obvious terminator signals for RNA polymerase II known in eukaryotes except, in yeast where the 8 base pair consensus TTTTATA has been implicated as a necessary determinant and is provided by the *AdHI* terminator in the pAAH5 vector. Both McMahon (1995) and Olsen (1995) noted that this truncated α -amylase gene gave higher expression than the full gene fragment in both *E. coli* and *S. cerevisiae*.

6.8 FUTURE WORK

From other studies in mammalian systems (Liebhaber et al., 1992) it appears that translation can be blocked if antisense sequences form stable duplexes in the 5' UTR, or in close proximity to the initiation AUG site within the 80S assembly region. It has also been observed that once the 80S ribosome is assembled, and has initiated elongation, it has the ability to displace extensive duplexes and translate substrate mRNA. An interesting experiment in light of the result obtained for the *cis*-acting episomal (pMAS-H5) and integrating (pFL34-MAS) plasmids would be to construct another *cis* plasmid, by inserting an antisense sequence within the 3'UTR of the sense α -amylase gene which, when and if, the antisense sequence folded back on the sense sequence that it would not be complementary to the 5' UTR or RBS, in order to determine if the ribosomes would have the ability to displace the duplex, and thus lead to expression of the gene.

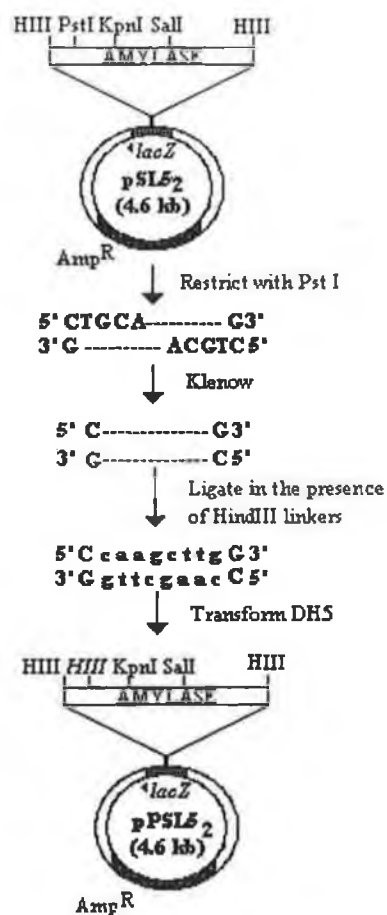
The design of this new *cis* plasmid, pRBS-AS, involved taking a sense α -amylase gene (lacking its' transcriptional terminator) and fusing to its' 3' end an antisense gene sequence complementary to the coding region of the gene, but lacking complementarity to the RBS. The sense α -amylase gene was obtained

from the plasmid pMO2. The antisense gene sequence, lacking complementarity to the RBS was obtained by removing the first 110 base pairs from the sense α -amylase gene. The remaining 1.8 kb *Hind* III α -amylase fragment (minus the 5' UTR and RBS) was gene cleaned and ligated in the presence of the 1.6 kb *Hind* III pMO2 α -amylase fragment and the *Hind* III restricted yeast-shuttle vector, pAAH5. The ligation mix was transformed into DH5 α *E. coli* cells and screened. The clones that were screened did not contain the sense and antisense fragments in the desired orientation (see Figure 6.17) in pAAH5. For a more detailed explanation of how this cloning experiment was designed see the legend to Figure 6.18

Hind III —SENSE—→ *Hind* III ←ANTISENSE— *Hind* III

Figure 6.17 Desired orientation of the α -amylase sense and antisense gene fragment, when cloned into pAAH5.

STEP I



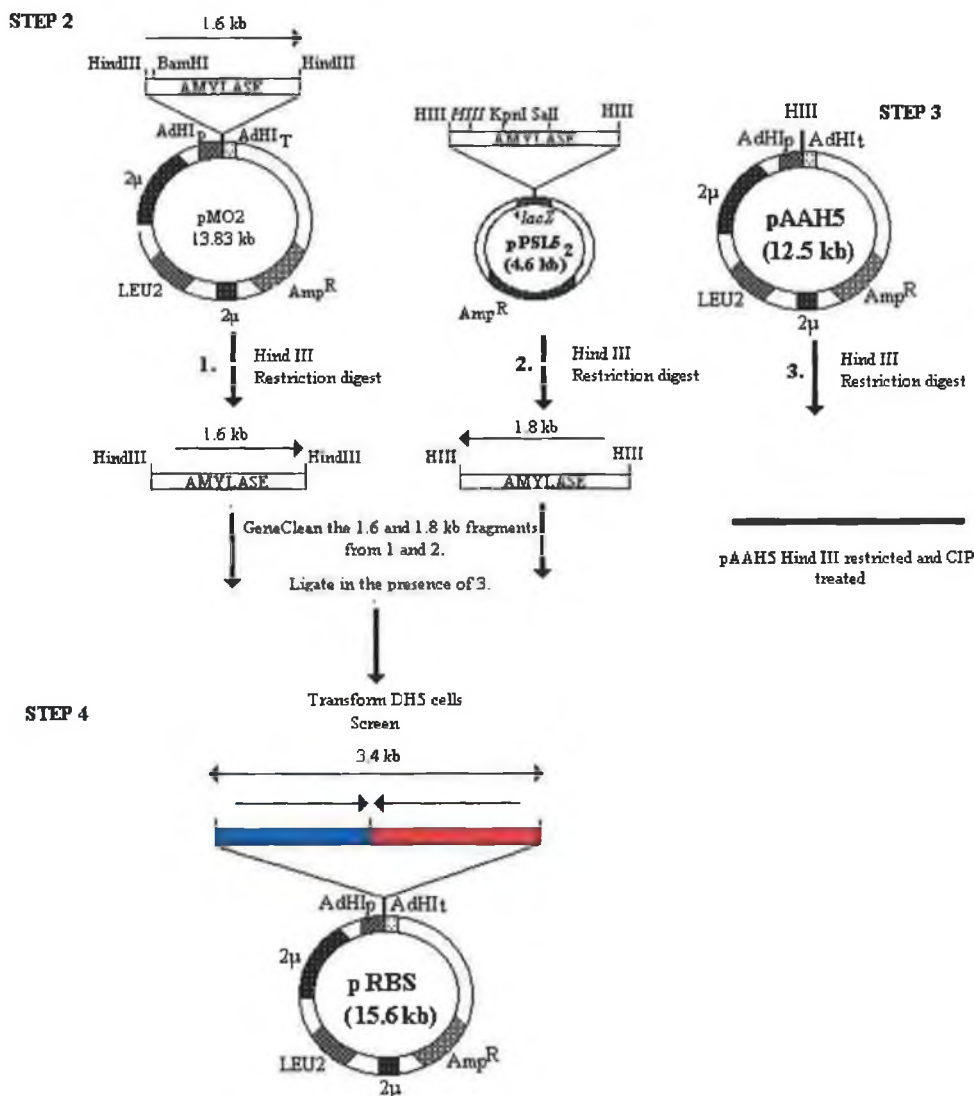


Figure 6.18 Schematic representation of how to construct the *cis*-acting plasmid, pRBS-AS.

Step I: The plasmid pSL5₂ (4.6 kb, McMahon, 1995) which contains the sense α -amylase gene cloned in as a 1.9 kb *Hind* III fragment into the vector pUC8 (2.7 kb, Appendix V) was digested with the restriction enzyme *Pst* I. This site was present approximately 110 base pairs into the 5' coding region of the α -amylase gene, (the pUC8 vector contains no *Pst* I site). After digestion with *Pst* I the 5' overhang was blunt-ended using the Klenow enzymes 3' - 5' exonuclease activity and an 8 mer (5'CAAGCTTG 3') *Hind* III linker was ligated onto the molecule, the plasmid was recircularised using T₄ DNA ligase and the ligation mix was transformed into DH5 α *E. coli* cells. After the Klenow reaction and *Hind* III linker addition the *Pst* I site was destroyed, and this also abolished α -amylase activity. The transformants were screened for the presence of

an additional *Hind* III site, the plasmid now possessing *Hind* III fragments of 2.7 kb, 1.8 kb and 0.1 kb respectively. This plasmid was named pPSL5₂. **Step II:** The plasmids pMO2 and pPSL5₂ were digested with *Hind* III and the 1.6 and 1.8 kb α -amylase gene fragments respectively, were gene cleaned from a 0.7% TAE agarose gel. **Step III:** The yeast shuttle vector pAAH5 which possesses a unique *Hind* III site for cloning situated between the *AdHI* promoter and terminator was linearised with this restriction enzyme to produce a 12.2 kb band which was column cleaned (section 2.6.11.8) to remove any restriction enzyme buffer. It was then dephosphorylated with CIP (section 2.6.11.3). **Step IV:** The linearised pAAH5 was ligated in the presence of the gene-cleaned 1.6 kb and 1.8 kb *Hind* III fragment of pMO2 and pPSL5₂. The ligation mix was transformed into DH5 α *E. coli* cells and the transformants were screened.

One of the clones obtained had the correct inserts but in the wrong orientation. Future work could involve redigesting this clone with *Hind* III, religating, transforming DH5 α and screening for the desired insert. The construct would then be transformed into DBY746 and analysed to determine if there was any effect on α -amylase activity.

CHAPTER 7

Discussion

7. DISCUSSION

In contrast to the situation in plants and animals, there have been few published examples of the use of antisense RNA to regulate gene expression in yeast. This is probably due to the fact that, firstly, homologous recombination is efficient in *S. cerevisiae* and can be used to rapidly and routinely replace, disrupt, or alter the sequence of any gene, thereby facilitating analysis (Rothstein, 1991). With such an effective approach available in yeast for analyzing genes, there was less need to develop alternative strategies, such as antisense. Secondly, the widespread development and successful application of antisense RNA as a gene therapy strategy in mammalian cells (Ho and Parkinson, 1997; Putnam, 1996) and as a way of altering traits in plants (Mol et al., 1990; Kuipers et al., 1995; Bendahmane and Gronenborn, 1997) had lessened the need to examine the parameters governing antisense function in simple organisms such as yeast, when the desired outcome can be successfully achieved in the organism of interest. Thirdly, when genetic modifications of yeast strains are required for commercial purposes such as in the brewing or baking industries, strain modification is usually accomplished using traditional techniques. Yeast strains produced by conventional techniques prevent potential concerns regarding the release of strains containing recombinant DNA (Atkins et al., 1994).

Nevertheless, it is now apparent that some antisense strategies work effectively, while others fail completely or result in only partial suppression. The precise reason(s) for this variability in effectiveness are not well understood, thus reliable application of antisense RNA mediated gene suppression is not always possible. These observations therefore, created the need for rapid and simple *in vivo* model systems to evaluate the parameters governing antisense function. Lower eukaryotes, and in particular fungi, are good candidates for such systems based on a number of features (Arndt and Atkins, 1996).

Although antisense RNA technology has many advantages, a major drawback is that success is rather unpredictable as gene silencing can vary depending on the fragments used to generate antisense transcripts. Additionally, there can be variability among different clones and some genes have completely

resisted antisense-mediated gene silencing (Nellen and Sczakiel, 1996). Also the stability or instability of the RNA targeted may influence the outcome of an antisense experiment (Inouye, 1988). According to Bricca (1995) most successful attempts to inhibit mRNA translation have been directed towards transiently expressed genes, with the target mRNA being at a low level when the antisense molecule is introduced. It was hoped that by using *Saccharomyces cerevisiae* as a model system that more could be learned about the design, the use and the critical parameters of artificial antisense RNA technology.

After completing experimental work on 4 different *trans*-Experimental systems in *Saccharomyces cerevisiae*, it appears however, that this organism is somewhat refractory to antisense RNA regulation, in particular when the two components of the system reside episomally and chromosomally. This conclusion has been confirmed by many other studies in *S. cerevisiae* where sense and antisense genes have been tested in a *trans* configuration, (Arndt, 1993; Arndt et al., 1994; Atkins and Gerlach, 1994; Olsson et al., 1997). A question that arises is whether the yeast *S. cerevisiae* differs in some specific way from other eukaryotic cells, to occasion an intracellular environment incompatible with antisense RNA regulation. If this is the case, the reasons for failure of the *majority* of *trans*-acting RNAs, may be a reflection of certain unique features inherent in the biology of this yeast.

The success of *cis*-Experimental systems in *S. cerevisiae*, where the transcription of the sense and antisense genes were linked to maximize RNA-RNA interaction suggests that it is the spatial coincidence of the interacting genes that is critical to the success of the experiments. Although, this is an obvious statement it may be more crucial in this organism that spatial coincidence is optimized due to the fact that the majority of genes in *S. cerevisiae* lack introns (Goffeau et al., 1996), as does the reporter gene used in this study. This lack of introns suggests that most mRNAs in this organism are less dependent on splicing as a means of post-transcriptional regulation. The lack of a splicing step may permit more rapid nuclear transport, than for typical spliced RNAs.

Also, the third stage in mRNA trafficking (after movement of the message from its' site of transcription to the nuclear pore and translocation of the mRNA through the pore) where the mRNA is distributed in the cytoplasm in the

appropriate context for translation appears to be done in a specific non-random manner (Amberg et al., 1992). Therefore if the interacting RNAs are not coincident in the nucleus, it is far less likely that they will be coincident in the much larger area of the cytoplasm. Additionally, this rapid nuclear export would mean that any potential points of regulation in the nucleus, such as transcription and RNA processing, would be bypassed (Murray and Crockett, 1992).

Another factor to support the fact that the failure of antisense RNAs in *trans* may be due to *S. cerevisiae*' cell biology is the identification of a yeast splicing protein (Lin and Rossi, 1996). This protein interacted *in vivo* with a *cis*-ribozyme by specifically recognising the ribozyme structure. The binding of this protein to the ribozyme can account for the inability of ribozymes to efficiently cleave in yeast. More recently, Castanotto et al., (1998) found that when yeast extracts were added to *in vitro trans* cleavage reactions, the cleavage ability of the ribozyme was hampered, whereas the addition of mammalian extracts yielded an enhancement of the reactions. These results confirm the presence of factor(s) that can block this type of antisense function in the yeast intracellular environment.

An alternative or additional possibility for the failure of antisense systems to interfere with gene expression in *trans*, is that *S. cerevisiae* may lack a factor(s) required for antisense RNA function. Antisense RNA is predicted to function by forming a specific RNA duplex with target mRNA (Erickson and Izant, 1992). The effectiveness of this form of regulation, as we know is thus dependent on efficient RNA-RNA interaction and the fate of the resulting RNA duplex. It has been suggested that both of these features are influenced by cellular factors (Nellen and Lichtenstein, 1993). One candidate enzyme is the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 which has RNA annealing activity. Other host-encoded activities that have been implicated in the modification of RNA duplexes include double-stranded RNA adenosine deaminase (dsRAD), double-stranded RNases and RNA helicases. *S. cerevisiae* has been shown to lack an hnRNP A1-like renaturation activity, however sequencing of a 43.5 kb segment of chromosome XIV has revealed the presence of a putative adenosine deaminase gene, which should lead to an enhancement of antisense activity. This sequence presents significant similarity to adenosine deaminase from different organisms, such as *E. coli*, mouse and human (Mallet

et al., (1995). The enzyme which catalyses the deamination of adenosine to inosine, was purified and characterized by Marmocchi et al., (1987) in *S. cerevisiae*.

This enzyme requires a double-stranded RNA substrate and it acts to convert adenosine (A) residues within its substrate to inosine (I) residues. Due to the fact that IU base pairs are less stable than AU base pairs, the double-stranded RNA substrate becomes increasingly single-stranded during the reaction. Also, as inosines are read as guanosines during translation, this modification would be expected to change the coding capacity of the DNA drastically, this in turn should lead to the production of a non-functional protein, with a concomitant enhancement of an antisense RNA effect (Bass, 1992). However, although this enzyme is present in *S. cerevisiae*, there are obviously other factors needed for the successful application of antisense in a *trans* configuration.

When this work began, the *trans*-Experimental systems studied in this thesis were designed so as to investigate many of the parameters deemed to be important for the successful application of antisense RNA technology. Some of these parameters are shown in Table 7-1, (Arndt and Atkins, 1996).

Table 7-1 Parameters with the potential to influence antisense RNA mediated suppression of gene expression *in vivo*.

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1. Relative position of the antisense gene and the target gene in the host genome
 2. Differential timing of expression of the target gene and the antisense gene
 3. Rate of turnover of target mRNA and antisense RNA
 4. Intracellular location and stability of the target mRNA:antisense RNA duplex
 5. Secondary and tertiary structure of the target mRNA and antisense RNA
 6. Are there cellular enzymes either present or absent in the system under investigation, which could enhance or repress antisense RNA inhibition?
 7. By what mechanism will the antisense RNA inhibit target gene expression?
 8. What is the optimum ratio of antisense:target RNA?

Upon reading the literature and in light of the findings presented in this thesis, there does not seem to be a single defined set of parameters governing antisense regulation.

Trans-Experiment I (a) and (c) were designed in a way, which have been shown to be successful in mammalian and plant systems, where the gene to be silenced is present on a chromosome and the antisense sequence is introduced either episomally or as an oligonucleotide. The overall picture from the most recent literature on antisense data suggests that antisense oligonucleotides are more effective inhibitors of gene expression than artificial antisense genes. However antisense genes were chosen for this study in preference to oligonucleotides, as once an antisense gene is proved to be effective, gene inhibition would become a stable property of the transformed cells or organisms and it would not be necessary to provide a continuous supply of antisense molecules. The reasons for oligonucleotides being more successful than antisense genes may include; [1] the relatively low number of antisense transcripts that can be synthesised by the transformed cell with respect to the much greater number of antisense oligonucleotides able to penetrate the cell and [2] antisense genes are generally constructed in such a way that the antisense RNA is as long as the mRNA molecule. The formation of the antisense RNA/mRNA hybrids could then be easily prevented by the formation of intramolecular secondary structure(s). One future possibility to bridge oligonucleotide and antisense gene technology would be to construct antisense genes by shuffling only those sequence domains that are effective targets in the inhibition by antisense oligonucleotide sequences.

Trans-Experiment I (a) and *trans*-Experiment II utilized the full-length α -amylase gene for the construction of the antisense molecule on the basis that full-length sequences had proven successful in other systems (Knecht, 1989; Pear et al., 1993; Kuipers et al., 1995). Also studies of the kinetics of RNA-RNA interactions among the naturally occurring antisense RNAs found in bacteria, have led to the hypothesis where longer antisense RNAs, such as the antisense RNA used in this system, may have a larger number of nucleation sites and thus greater potential for RNA-RNA annealing. Knecht (1989) states that in relation to the size of an antisense transcript that the larger the antisense molecule, the faster and more stable the duplex formed. However, Knecht also states that results have been so variable that no absolute size limit can be stated, but it seems reasonable that the larger fragments will work better. On the other hand, in

contrast to these hypotheses, other authors (Olsson et al., 1997) have suggested that large mRNAs may provide more opportunity for secondary structure formation and therefore they may not bind with enough efficiency to their sense RNAs to prevent translation.

The success of an interaction between a sense and antisense RNA will be dependent on the accessibility of the target RNA. The number of accessible sites on an RNA molecule is determined by a combination of the inherent structure of the RNA and its collection of bound proteins at any one moment (Branch, 1998). Computer-aided secondary structure predictions which could help to approach part of this problem are according to Nellen and Lichtenstein (1993) 'surprisingly unpredictable'. They cite the example of the 3' UTR of the transferrin receptor mRNA which can fold into two very different structures of similar free energy. While one of them reflects the regulatory iron responsive elements, the other one is most likely not formed *in vivo*.

Another study fairly recently carried out (Milner et al., 1997) emphasized the extent to which the native RNA structure can restrict the binding of an antisense molecule. Milner et al., (1997) examined the ability of 1,938 oligonucleotides (ranging in size from monomers to 17-mers) to bind to a 122 nucleotide RNA representing the 5' end of β -globin mRNA. They found that very few oligonucleotides bound stably to the mRNA and concluded that binding is probably restricted to those regions in the RNA which provide accessible substructure. Similarly Chen and co-workers (1997) demonstrated that cellular proteins and ribonucleoprotein complexes, such as ribosomes could prevent ribozyme-mediated cleavage. They showed that a reporter gene was ribozyme-insensitive in wild-type *E. coli* but was ribozyme-sensitive in a 'slow-ribosome' mutant. As can be seen from these examples it is very difficult to predict what regions of an RNA molecule will be accessible *in vivo* hence effective antisense molecules must be found empirically by screening large numbers of candidates.

Two of the potential secondary structures that could be formed by the full-length antisense α -amylase gene were obtained using the Mfold prediction algorithm (Appendix X). Unfortunately, as demonstrated by the examples given by Nellen and Lichtenstein (1993) and Milner et al., (1997) one cannot trust the output of these programs to even approximately represent the true *in vivo*

structure. Reasons for this are firstly, an RNA molecule can start to fold in a 5'→3' direction as it is being synthesized, therefore there may be a decisive kinetic element that in part determines which complementary regions will in fact pair and which will not. Secondly, the initial folded structure may change its conformation by local rearrangements of the base pairs, thus relaxing toward lower energy states. Thirdly, tertiary interactions between unpaired base pairs in loops or at the ends of a molecule may alter the structure. Finally, most RNAs are strongly bound to other RNAs or to proteins when in their functional state. The secondary structure prediction methods currently in use are far from capturing all the essentials of this complex folding process.

Once again, as with the length of an antisense molecule, depending on what gene is being targeted and in what organism, antisense genes targeting the 5', 3' untranslated and coding regions have been shown to be effective. *Trans*-Experiment I (c) involved targeting different regions of the *Bacillus licheniformis* α-amylase gene, such as the 5' and 3' untranslated and coding regions. However none of the antisense genes were successful, this result in light of the outcomes of *trans* II and the *cis*, suggests that spatial coincidence was not ensured.

The ratio of antisense RNA to sense RNA is one of the parameters governing antisense RNA function. However as with the length and regions which the antisense RNA targets, the optimum appears to be different for different genes and cell types. For example an antisense RNA directed against the creatine-kinase B gene was capable of inhibiting translation at a ratio of 1 to 2 copies of antisense RNA per copy of native RNA (Cirullo et al., 1992). Expression of the mhc A protein was dramatically inhibited with a ratio of antisense over sense transcripts of 10:1 (Knecht, 1989). Whereas a 100 – 200 fold excess of antisense to sense RNA was necessary to reduce the expression of the thymidine kinase gene by 4 – 5 fold.

The majority of successful applications of antisense technology in *S. cerevisiae* have been achieved through a *cis* configuration (Atkins and Gerlach, 1994; Ferbeyre et al., 1995). Ferbeyre et al., (1995) state that hammerhead ribozymes can be catalytically active in yeast and can reduce gene expression only when the target is in *cis* and very close as evidenced by the comparisons of the effects of 5' *cis* (close to the target site), 3' *cis* (far from the target site) and

trans ribozymes. Following on from this finding Ferbeyre et al., (1996) treated yeast cells expressing hammerhead ribozyme RNA with the α -factor mating pheromone, with the result that the levels of the targeted mRNA, *ADE1* was reduced by 50%. The rationale for this study was to evaluate the hypothesis that yeast metabolism is simply too rapid to allow for a ribozyme-based cleavage activity and that the success of *trans*-activity in mammalian cells may owe its feasibility to a markedly slower cell cycle and corresponding slower RNA metabolism.

The *cis*-Experiments described in this thesis produced the desired inhibitory effect on the gene of interest and further reinforces the importance of spatial coincidence, especially in the light of the findings of Ferbeyre et al., (1996). *Trans*-Experiment II also produced a partial suppression of α -amylase gene expression, even though the ratio of antisense to sense RNA was lower in one co-transformant (2:1) than what was achieved in *trans*-Experiment I (a) and (c), 4, 3.5 and 3.5:1 respectively. The major difference between these experimental systems was the fact that in *trans*-Experiment II both sense and antisense genes were transcribed episomally and possibly were more spatially coincident than the *trans* configurations in I (a) and (c). The majority of papers that have shown the failure of antisense to inhibit gene expression in *trans* in *S. cerevisiae* (Atkins and Gerlach, 1994; Olsson et al., 1997) have designed their *trans*-Experimental systems similar to *trans*-Experiment I, where the sense and antisense genes were expressed from different locations within the cell, i.e. either chromosomally or episomally. In retrospect, the fact that the antisense and sense genes which were both expressed from episomes in *trans*-Experiment II, lead to a 12% reduction in α -amylase activity suggests that the spatial coincidence was more favourable in this experimental system, than *trans* I and possibly, this particular type of *trans* configuration is worthy of further investigation.

Although *S. cerevisiae* has not been successfully utilized as a host for the analysis of antisense-mediated gene regulation, it has raised a number of basic questions about gene expression, in particular with respect to RNA metabolism. Central to this discussion is to be able to understand more fully what key features influence RNA diffusion, RNA transport and RNA compartmentalisation *in vivo*? An insight into these aspects of RNA metabolism may provide a better

framework within which to re-examine and further assess the theory of location effect/spatial coincidence.

Particular factors/proteins (for example hnRNP A1) have been identified which influence gene control by an antisense mechanism. May be such factors also play a role in yeast cells, but have yet to be identified. Possible future work, which could be undertaken for the development of *S. cerevisiae* as an experimental host for studying antisense function, could include mutagenesis of the yeast strain, which may result in a modified strain in which antisense function is enhanced. An alternative, but complementary approach would be to introduce into yeast cells some of the genes which have been shown to influence antisense activity in other systems, and test whether they enhance antisense activity. A further approach could involve the introduction of a gene library from another eukaryote and then selecting for genes which complement antisense RNA function.

CHAPTER 8

References

8. References

Amberg, D.C., Goldstein, A.L. and Charles, N.C. (1992). **Isolation and characterisation of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA.** *Genes and Development*. 6: 1173 – 1189.

Ammerer, G. (1983). **Expression of genes in yeast using the *AdHI* promoter.** In *Methods in Enzymology*. Academic press, New York. 101: 192 - 201.

Armstrong, K. A., Som, T., Volkert, F. C., Rose, A. and Broach, J. R. (1989). **Propagation and expression of genes in yeast using 2 μ circle vectors.** In Barr, P. J., Brake, A. J. and Valenzuela, P. (Eds.). *Yeast Genetic Engineering*. Butterworth Publishers, Stoneham, 165 - 192.

Arndt, G.M., Atkins, D., Patrikakis, M. and Izant, J.G. (1995). **Gene regulation by antisense RNA in the fission yeast *Schizosaccharomyces pombe*.** *Mol. Gen. Genet.* 248: 293 - 300.

Arndt, G.M. (1993). **Antisense RNA expression in the yeast *Saccharomyces cerevisiae*.** PhD. Thesis, University of Saskatchewan, Canada.

Arndt, G.M. and Atkins, D. (1996). **RNA catalysis in fungi.** In Eckstein, F. and Lilley, D.M.J. (Eds.) *Nucleic Acids and Molecular Biology*. 10: 343 – 366.

Arndt, G.M., Xiao, W. and Rank, G.H. (1994). **Antisense RNA regulation of the *ILV2* gene in yeast: a correction.** *Curr. Genet.* 25: 289.

Atkins, D and Gerlach, W.L. (1994). **Artificial ribozyme and antisense gene expression in *Saccharomyces cerevisiae*.** *Antisense Research and Development*, 4: 109 -117.

Atkins, D., Arndt, G.M. and Izant, J.G. 1994. **Antisense gene expression in yeast.** Biol Chem Hoppe-Seyler. 375: 721 -729.

Atkins, D., Patrikakis, M. and Izant, J.G. (1995). **The *ade6* gene of the fission yeast as a target for antisense and ribozyme RNA-mediated suppression.** Antisense Research and Development. 5: 295 - 305.

Bachellerie, J-P., Michot, B., Nicoloso, M., Balakin, A., Jingwei, N. and Fournier, M.J. (1995). **Antisense snoRNAs: a family of nucleolar RNAs with long complementarities to rRNA.** Trends. Biochem. Sci. 20: 261 - 264.

Bass, B. L. (1992). **The double-stranded RNA unwinding/modifying activity.** In Murray, J. A. H. (Ed.). *Antisense RNA and DNA.* Wiley - Liss, New York; pp 159 - 174.

Bendahmane, M. and Gronenborn, B. (1997). **Engineering Resistance Against Tomato Yellow Leaf Curl Virus (TYLCV) using Antisense RNA.** Plant Molecular Biology. 33: 351 - 357.

Birnboim, H.C. and Doly, J. (1979). **A rapid alkaline extraction procedure for screening recombinant plasmid DNA.** Nucleic Acids Research, 7: 1513 - 1523.

Boles, E. and Zimmermann, F.K. (1994). **Open reading frames in the antisense strands of genes coding for glycolytic enzymes in *Saccharomyces cerevisiae*.** Mol. Gen. Genet. 243: 363 - 368.

Bonneaud, N., Ozier-Kalogeropoulos, O., Guoya, L., Labouesse, M., Miniviel-Sebastia, L. and Lacroute, F. (1991). **A family of low and high copy replicative, integrative and single-stranded *S.cerevisiae* / *E.coli* shuttle vectors.** Yeast ,7: 609 - 615.

Botstein, D. and Fink, G.R. (1988). **Yeast: An Experimental Organism for Modern Biology.** Science. 240: 1439 – 1443.

Bourque, J.E. and Folk, W.R. (1992). **Suppression of gene expression in plant cells utilising antisense sequences transcribed by RNA polymerase III.** Plant Mol. Biol. 19: 641.

Branch, A.,D. (1998). **A good antisense molecule is hard to find.** Trends in BioScience. 23: 45 - 50.

Brantl, S. and Wagner, E.G. (1994). **Antisense RNA-mediated transcriptional attenuation occurs faster than stable antisense/target RNA pairing: an *in vitro* study of plasmid pIP501.** EMBO J. 13: 3599 - 3607.

Bricca, G. (1995). **Sense, antisense, nonsense: where's the right way?** J. Mol. Med. 73: 417 – 419.

Brown, A.J.P., Purvis, I.J., Chinnappan, T.S., Bettany, A.J.E., Loughlin, L. and Moore, J. (1988). **Messenger RNA degradation in *Saccharomyces cerevisiae*.** Gene. 72: 151 – 160.

Canavan, P. (1994). **Studies on the plasmid stability, plasmid copy number and endo (1,3)(1,4) β -glucanase production by free and alginate immobilised recombinant *Saccharomyces cerevisiae* cells.** PhD. Thesis, Dublin City University.

Cantwell, B.A., Brazil, G., Murphy, N. and McConnell, D.J. (1986). **Comparison of expression of the endo- β -1,3-1,4-glucanase gene from *Bacillus subtilis* in *Saccharomyces cerevisiae* from the CYC1 and ADH1 promoters.** Curr. Genet. 11: 65 – 70.

Carle, G.F. and Olson, M.V. (1985). **An electrophoretic karyotype for yeast.** Proc. Natl. Acad. Sci. USA. 82: 3756 - 3760.

- Castanotto, D., Haitang, Li, Warren, C., Rossi, J.J. and Deshler, J.O. (1998). **Structural similarities between hammerhead ribozymes and the spliceosomal RNAs could be responsible for lack of ribozyme cleavage in yeast.** *Antisense and Nucleic Acid Drug Development*. 8: 1 – 13.
- Cesareni, G. and Banner, D.W. (1985). **Regulation of plasmid copy number by complementary RNAs.** *TIBS*. 303 -306.
- Chen, H., Febeyre, G. and Cedergren, R. (1997). **Efficient hammerhead ribozyme and antisense RNA targeting in a slow ribosome *Escherichia coli* mutant.** *Nat. Biotechnol.* 15: 432 – 435.
- Cirullo, R.E., Whitaker, K.B. and Holmes, E.W. (1992). **Use of antisense RNA to study post-transcriptional regulatory processes.** In Murray, J. A. H. (Ed.). *Antisense RNA and DNA*. Wiley - Liss, New York. 175 – 182.
- Clarke, M.L., Patrikakis, M. and Atkins, D. (2000). **Comparative analysis of artificial antisense RNA regulation in fission yeast and human cells.** *Biochem. Biophys. Res.* 268: 8 – 13.
- Coleman, J., Green, P.J. and Inouye, M. (1984). **The use of RNAs complementary to specific mRNAs to regulate the expression of individual bacterial genes.** *Cell* 37: 429 - 436.
- Cryer, D. R., Eccleshal, R. and Marmur, J. (1975). **Isolation of yeast DNA.** *Meth. Cell. Biol.* 12: 39.
- Csiszar, K., Houmard, J., Damerval, T. and Tandeau de Marsac, N. (1987). **Transcriptional analysis of the cyanobacterial *gvpABC* operon in differentiated cells: occurrence of an antisense RNA complementary to three overlapping transcripts.** *Gene* 60: 29 - 37.
- Daugherty, B.L., Hotta, K., Kumar, C., Ahn, Y.H., Zhu, J. and Pestka, S. (1989). **Antisense RNA: effect of ribosome binding sites, target location, size and**

concentration on the translation of specific mRNA molecules. Gene. Anal. Techn. 6: 1 -16.

del Solar, G., Acebo, P. and Espinosa, M. (1997). **Replication control of plasmid pLS1: the antisense RNA II and the compact *rnaII* region are involved in translational regulation of the initiator RepB synthesis.** Mol. Microbiology 23: 95 -108.

Delauney, A.J.,Tabaeizadeh, Z. and Verma, D.P.S. (1988). **A stable bi-functional antisense transcript inhibiting gene expression in transgenic plants.** Proc. Natl. Acad. Sci. U.S.A. 85: 4300 - 4304.

Delihias, N. (1995). **Regulation of gene expression by *trans*-encoded antisense RNAs.** Mol. Microbiology. 15: 411 - 414.

Deshler, J. O., Haitang, L., Rossi, J.J. and Castanotto, D. (1995). **Ribozymes expressed within the loop of a natural antisense RNA form functional transcription terminators.** Gene. 155: 35 - 43.

Donahue, C.P. and Fedor, M.J. (1997). **Kinetics of hairpin ribozyme cleavage in yeast.** RNA. 3: 961 – 973.

Ecker, D. J. and Freier, S. M. (1998). **PNA, antisense, and antimicrobials.** Nature Biotechnology. 16: 332.

Erikson, R.P. and Izant, J.G. (1992). **Gene Regulation: biology of antisense RNA and DNA.** Raven Press, New York.

Ferbeyre, G., Bratty, J., Chen, H. and Cedergren, R. (1995). **A hammerhead ribozyme inhibits *ADE1* gene expression in yeast.** Gene. 155: 45 – 50.

Feinberg, A.P and Vogelstein, B. (1983). **A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity.** Anal. Biochem. 132: 6 - 13.

Ferbeyre, G., Bratty, J., Chen, H. and Cedergren, R. (1996). **Cell cycle arrest promotes *trans*-hammerhead ribozyme action in yeast.** J. Biol. Chem. 271: 19318 – 19323.

Fujii, T., Kondo, K., Shimizu, F., Sone, H., Tanaka, J.-I. and Inoue, T. 1990. **Application of a ribosomal DNA integration vector in the construction of a brewer's yeast having α -acetolactate decarboxylase activity.** Applied and Environmental Microbiology 50: 997 - 1003.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Phillipsen, P., Tettelin, H. and Oliver, S.G. (1996). **Life with 6000 genes.** Science. 274: 546 – 567.

Good, L. and Nielsen, P. E. (1998). **Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA.** Nature Biotechnology. 16: 355 - 358.

Gottlieb, E. (1992). **The 3' untranslated region of localised maternal messages contains a conserved motif involved in mRNA localisation.** Proc. Natl. Acad. Sci. USA. 89: 7164 - 7168.

Green, P. J., Pines, O. and Inouye, M. (1986). **The role of antisense RNA in gene regulation.** Annu. Rev. Biochem. 55: 569 - 597.

Hamilton, A.J., Lycett, G.W. and Grierson, D. (1990). **Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants.** Nature. 346: 284 - 287.

Hasan, N., Somasekhar, G. and Szybalski, W. (1988). **Antisense RNA does not significantly affect expression of the *galK* gene of *Escherichia coli* or the *N* gene of coliphage lambda.** Gene 72: 247 – 252

- Haseloff, J. and Gerlach, W.L. (1988). **Simple RNA enzymes with new and highly specific endoribonuclease activities.** *Nature* 334: 585 - 591.
- Highlander, S.K. and Novick, R.P. (1990). **Mutational and physiological analyses of plasmid pT181 functions expressing incompatibility.** *Plasmid*. 23: 1 - 15.
- Hjalt, T.A.H. and Wagner, E.G.H. (1995). **Bulged-out nucleotides protect an antisense RNA from RNase III cleavage.** *Nucleic Acids Research* 23: 571 - 579.
- Ho, P.T.C. and Parkinson, D.R. (1997). **Antisense Oligonucleotides as Therapeutics for Malignant Diseases.** *Seminars in Oncology*, 24: 187 -202.
- Holmes, D.S. and Quigley, M. (1981). **A rapid boiling method for the preparation of bacterial plasmids.** *Anal. Biochem.* 114: 193 - 197.
- Hunter, S. (1991). **Heterologous gene expression in *Saccharomyces cerevisiae*: Analysis of *Bacillus subtilis* β -glucanase and *Escherichia coli* β -glucuronidase.** PhD. Thesis, Dublin City University.
- Inouye, M. (1988). **Antisense RNA: its functions and applications in gene regulation - a review.** *Gene* 72: 25 - 34
- Izant, J.G. and Weintraub, H. (1984). **Inhibition of thymidine kinase gene expression by antisense RNA: a molecular approach to genetic analysis.** *Cell* 36: 1007 - 1015.
- Johston, M. and Davies, R.W. (1984). **Sequences that regulate the divergent *GAL1* - *GAL10* promoter in *Saccharomyces cerevisiae*.** *Mol. Cell. Biol.* 4: 1440 - 1448.

Katsuki, M., Sato, M., Kimura, Yokoyama, M., Kobayashi, K. and Nomura, T. (1988). **Conversion of normal behaviour to shiverer by myelin basic protein antisense cDNA in transgenic mice.** Science. 241: 593 – 595.

Kerr, S.M., Stark, G.R. and Kerr, L.M. (1988). **Excess antisense RNA from infectious recombinant SV40 fails to inhibit expression of a transfected interferon-inducible gene.** Eur. J. Biochem. 175: 65 - 73.

Kim, J. H., Kim, S. G., Chung, D. K., Bor, Y-C. and Batt, C. A. (1992). **Use of antisense RNA to confer bacteriophage resistance in dairy starter cultures.** Journal of Industrial Microbiology. 10: 71 - 78.

Kim, S.K. and Wold, B.J. (1985). **Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA.** Cell 42: 129 - 138.

Knecht, D.A. (1989). **Application of Antisense RNA to the Study of the Cytoskeleton: Background, Principles, and a Summary of Results Obtained with Myosin Heavy Chain.** Cell Motility and the Cytoskeleton. 14: 92 – 102.

Koraimann, G., Terferle, K., Markolin, G., Woger, W. and Hogenauer, G. (1996). **The FinOP repressor system of plasmid R1: analysis of the antisense RNA control of *traJ* expression and conjugative DNA transfer.** Mol. Microbiology. 21: 811 - 821.

Korpela, K., Buchert, P. and Soderland, H. (1987). **Determination of plasmid copy number with nucleic acid sandwich hybridisation.** J. Biotechnol. 5: 267 – 277.

Krinke, L. and Wuff, D.L. (1988). ***Oop* RNA , produced from multicopy plasmids, inhibits λ cII gene expression through an RNase III-dependent mechanism.** Genes Dev. 1: 1005 – 1013.

Kuipers, A.G.J., Soppe, W.J.J., Jacobson, E. and Visser, R.G.F. (1995). **Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression.** Mol. Gen. Genet. 246: 745 - 755.

Kumar, M. and Carmichael, G.G. (1997). **Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts.** Proc. Natl. Acad. Sci. USA. 94: 3542 - 3547.

Law, R.H.P. and Devenish, R.J. (1988). **Expression in yeast of antisense RNA to *ADE1* mRNA.** Biochem. Int. 17: 673 - 679.

Lawrence, J.B. and Singer, R.H. (1986). **Intracellular localisation of messenger RNAs for cytoskeletal proteins.** Cell. 45: 407 - 415.

Lawrence, J.B., Singer, R.H. and Marselle, L.M. (1989). **Highly localised tracks of specific transcripts within interphase nuclei visualised by *in situ* hybridisation.** Cell. 57: 493 -502.

Liebhaber, S.A., Russell, J.E., Cash, F.E. and Eshleman, S.S. (1992). **Inhibition of mRNA translation by antisense sequences.** In Murray, J.A.H. (Ed.) *Antisense RNA and DNA*. Wiley-Liss, New York; 163 - 174.

Lin, J. and Rossi, J.J. (1996). **Identification and characterisation of yeast mutants that overcome an experimentally introduced block to splicing at the 3' splice site.** RNA. 2: 835 - 848.

Liu, T., Williams, J.G. and Clarke, M. (1992). **Inducible expression of calmodulin antisense RNA in *Dictyostelium* cells inhibits the completion of cytokinesis.** Mol. Biol. Cell 3: 1403 - 1413.

Lonnberg, H. and Vuorio, E. (1996). **Towards genomic drug therapy with antisense oligonucleotides.** Ann. Med. 28: 511 - 522.

- Lopes, T.S., de Wijs, I.J., Steenhauer, S.I., Verbakel, J and Planta, R.J. (1996). **Factors affecting the mitotic stability of high-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*.** *Yeast*, 12: 467 – 477
- Lopes, T.S., Hakkaart, G-J. A.J., Koerts, B.I., Raue, H.A. and Planta, R.J. (1991). **Mechanism of high-copy-number integration of pMIRY-type vectors into the ribosomal DNA of *Saccharomyces cerevisiae*.** *Gene*, 105: 83 – 90
- Lopes, T.S., Klootwijk, J., Veenstra, A.E., van der Aar, P.C., van Heerikhuizen, H., Raue, H.A. and Planta, R.J. (1989). **High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*: a new vector for high level expression.** *Gene*, 79: 199 -206.
- Malavasic, M.J. and Elder, R.T. (1990). **Complementary transcripts from two genes necessary for normal meiosis in the yeast *Saccharomyces cerevisiae*.** *Mol. Cell. Biol.* 10: 2809 - 2819.
- Mallet, L., Bussereau, F. and Jacquet, M. (1995). **A 43.5 kb Segment of Yeast Chromosome XIV, which contains *MFA2*, *MEP2*, *CAP/SRV2*, *NAM9*, *FKB/FPR1/RBP1*, *MOM22* and *CPT1*, Predicts an Adenosine Deaminase Gene and 14 New Open Reading Frames.** *Yeast*. 11: 1195 – 1209.
- Mandel, M. and Higa, A. (1970). **Calcium-dependent bacteriophage DNA infection.** *J. Mol. Biol.* 53: 159 - 162.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). **Molecular Cloning: A Laboratory Manual.** Cold Spring Harbour Laboratory Press, Cold Spring Harbour.
- Marathe, S.V. and McEwen, J.E. (1999). **Expression of the divergent transcription unit containing the yeast *PET122* and *OXAI* genes.** *Biochem. Mol. Biol. Int.* 47: 971 – 977.

- Marmocchi, F., Lupidi, G., Vernardi, G. and Riva, F. (1987). **Adenosine deaminase from *Saccharomyces cerevisiae*: purification and characterisation.** Biochemistry International. 14: 569 – 580.
- Martin, C. E. and Scheinbach, S. (1989). **Expression of proteins encoded by foreign genes in *Saccharomyces cerevisiae*.** Biotech. Adv. 7: 155 - 185.
- McMahon, H. (1995). **Cloning and expression of the *Bacillus licheniformis* α -amylase gene in *Saccharomyces cerevisiae*.** PhD. Thesis, Dublin City University.
- Melton, D.A. (1985). **Injected anti-sense RNAs specifically block messenger RNA translation *in vivo*.** Proc. Natl. Acad. Sci. USA 89: 144 – 148.
- Miller, G.L., Blum, R., Glennon, W.E. and Burton, A.L. (1960). **Measurement of carboxymethylcellulase activity.** Anal. Biochem. 1: 127 - 132.
- Milner, N., Mir, K.U. and Southern, E.M. (1997). **Selecting effective antisense reagents on combinatorial oligonucleotide arrays.** Nat. Biotechnol. 15: 537 – 541.
- Mitsuhashi, M. (1997). **Strategy for designing specific antisense oligonucleotide sequences.** J. Gastroenterol. 32: 282 - 287.
- Mizuno, T., Chou, M.Y. and Inouye, M. (1984). **A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA).** Proc. Natl. Acad. Sci. USA 81: 1966 - 1970.
- Mochizuki, D., Miyahara, K., Hirata, D., Matsuzakai, H., Hatano, T., Fukui, S. and Miyakawa, T. (1994). **Overexpression and secretion of cellulolytic enzymes by δ -sequence mediated multicopy integration of heterologous DNA sequences into the chromosomes of *Saccharomyces cerevisiae*.** Journal of Fermentation and Bioengineering. 77: 468 - 473.

Mol, J., van Blokland, R. and Kooter, J. (1991). **More about co-suppression.** Trends. Biotech. 9: 182 - 183.

Mol, J.N.M., van der Krol, A.R., van Tunen, A.J., van Blokland, R., de Lange, P. and Stuitje, A.R. (1990). **Regulation of plant gene expression by antisense RNA.** FEBS Lett. 268: 427 - 430.

Morfeldt, E., Taylor, D., von Gabain, A. and Arvidson, S. (1995). **Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNA III.** EMBO J. 14: 4569 - 4577.

Murray, J. A. H. and Crockett, N. (1992). **Antisense techniques: An overview.** In Murray, J.A.H. (Ed.) *Antisense RNA and DNA*. Wiley-Liss, New York; 1- 49.

Nasr, F., Becam, A-M., Brown, S.C., DeNay, D., Slonimski, P.P. and Herbert, C.J. (1995). **Artificial antisense RNA regulation of *YBR1012* (*YBR136w*), an essential gene from *Saccharomyces cerevisiae* which is important for progression through G1/S.** Mol. Gen. Genet. 249: 51 -57.

Nasr, F., Becam, A-M., Slonimski, P.P. and Herbert, C.J. (1994). ***YBR1012* an essential gene from *S. cerevisiae*: construction of an antisense conditional allele and isolation of a multicopy suppressor.** C. R. Acad. Sci. Paris. 317: 607 - 613.

Neckers, L. and Whitesell, L. (1993). **Antisense Technology: biological utility and practical considerations.** Am. J. Physiol. 265: L1 - L12.

Nellen, W. and Lichtenstein, C. (1993). **What makes an mRNA anti-sensitive?** TIBS. 18: 419 – 423.

Nellen, W. and Lichtenstein, C. (1997). **Evaluation of Antisense Effects.** In Lichtenstein, C. and Nellen, W. (Eds.) *Antisense Technology: A Practical Approach*. IRL Press, Oxford, New York, Tokyo; p 25 – 38.

Nellen, W. and Sczakiel, G. (1996). **In vitro and in vivo action of antisense RNA.** Molecular Biotechnology. 6: 7 – 15.

Ng, R. and Abelson, J. (1980). **Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*.** Proc. Natl. Acad. Sci. USA. 77: 3912 - 3916.

Nielsen, P.E. (1999). **Peptide nucleic acids as therapeutic agents.** Curr. Opinion. In Structural Biology. 9: 353 – 357.

Nishikura, K. and Murray, M. (1987). **Antisense RNA of proto-oncogene *c-fos* blocks renewed growth of quiescent 3T3 cells.** Mol. Cell. Biol. 7: 639 –649.

Ohmen, J.D., Burke, K.A. and McEwan, J.E. (1990). **Divergent overlapping transcripts at the *PET122* locus in *Saccharomyces cerevisiae*.** Mol. Cell. Biol. 10: 3027 - 3035.

Olsen, M. (1995). **Heterologous gene expression in yeast: Cloning and expression of α -amylase and cathepsin L protease in *Saccharomyces cerevisiae*.** M.Sc. Thesis, Dublin City University.

Olsson, L., Larsen, M. L., Ronnow, B., Mikkelesen, J. D. and Nielsen, J. (1997). **Silencing *MIG1* in *Saccharomyces cerevisiae*: Effects of antisense *MIG1* expression and *MIG1* gene disruption.** Applied and Environmental Microbiology. 63: 2366 - 2371.

Orr-Weaver, T.L. and Szostak, J.W. 1983. **Multiple, tandem plasmid integration in *Saccharomyces cerevisiae*.** Molecular and Cellular Biology, 3: 747 - 749.

Ortlepp, S.A., Ollington, J.F. and McConnell, D.J. (1983). **Molecular cloning in *Bacillus subtilis* of a *Bacillus licheniformis* gene encoding a thermostable α -amylase gene.** Gene. 23: 267 - 276.

- Pear, J.R., Sanders, R.A., Summerfelt, K.R., Martineau, B. and Hiatt, W.R. (1993). **Simultaneous Inhibition of Two Tomato Fruit Cell Wall Hydrolases, Pectinmethylesterase and Polygalacturonase, with Antisense Gene Constructs.** Antisense Research and Development. 3: 181 - 190.
- Pendick, D. (1992). **Better Than the Real Thing.** Science News. 142: 376 - 377.
- Pepe, C.M., Maslesa-Galic, S. and Simons, R.W. (1994). **Decay of the IS10 antisense RNA by 3' exoribonucleases: evidence that RNase II stabilises RNA-OUT against PNPase attack.** Mol. Microbiology. 13: 1133 - 1142.
- Pestka, S., Daugherty, B. L., Jung, V., Hotta, K. and Pestka, R. K. (1984). **Anti-mRNA: Specific inhibition of translation of single mRNA molecules.** Proc. Natl. Acad. Sci. USA. 81: 7525 - 7528.
- Peterson, J. A. and Myers, A. M. (1993). **Functional analysis of mRNA 3' end formation signals in the convergent and overlapping transcription units of the *S. cerevisiae* genes *RHO1* and *MRP2*.** Nucleic. Acids. Res. 21: 5500 - 5508.
- Philippson, P., Stotz, A. and Scherf, C. (1991). **DNA of *Saccharomyces cerevisiae*.** In Methods in Enzymology. Academic press, New York. 194: 169 - 182.
- Pines, O. and Inouye, M. (1986). **Antisense RNA regulation in prokaryotes.** Trends. Genet. 2: 284 - 287
- Pratt, L.A., Hsing, W., Gibson, K.E. and Silhavy, J. (1996). **From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*.** Mol. Microbiology. 20: 911 - 917.
- Putnam, D.,A. (1996). **Antisense strategies and therapeutic applications.** Am J Health-Syst Pharm. 53: 151 - 160.

- Rebagliati, M.R. and Melton, D.A. (1987). **Antisense RNA injections in fertilised frog eggs reveal an RNA duplex unwinding activity.** *Cell* 48: 599 - 605.
- Riesner, D. (1997). **Nucleic Acid Structures.** In Lichtenstein, C. and Nellen, W. (Eds.) *Antisense Technology: A Practical Approach*. IRL Press, Oxford, New York, Tokyo; p 1 – 24.
- Rittner, K., Burmester, C. and Sczakiel, G. (1993). *Nucleic Acids Res.* 21: 2809.
- Rogers, J.C. (1988). **RNA complementary to α -amylase mRNA in barley.** *Plant Molecular Biology*. 11: 125 - 138.
- Rose, M. and Winston, F. (1984). **Identification of a Ty insertion within the coding sequence of the *S.cerevisiae* URA3 gene.** *Mol. Gen. Genet.* 193: 557 - 560.
- Rossi, J.J. and Sarver, N. (1990). **RNA enzymes (ribozymes) as antiviral therapeutic agents.** *Trends. Biotech.* 8: 179 - 183.
- Rothstein, R. (1991). **Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast.** *Methods Enzymol.* 194: 281 - 301.
- Sakai, A., Ozawa, F., Higashizaki, T., Shimizu, Y. and Hishinuma, F. (1991). **Enhanced secretion of human nerve growth factor from *Saccharomyces cerevisiae* using an advanced δ -integration system.** *Bio/Technology*. 9: 1382 - 1385.
- Salmons, B., Groner, B., Friis, R., Muellener, D. and Jaggi, R. (1986). **Expression of antisense mRNA in *H-ras* transfected NIH/3T3 cells does not suppress the transformed phenotype.** *Gene*. 45: 215 - 220.

Scherzinger, C.A. and Knecht, D.A. (1993). **Co-Suppression of *Dictyostelium discoideum* Myosin II Heavy-Chain Gene Expression by a Sense Orientation Transcript.** *Antisense Res. Dev.* 3: 207

Schuch, W. (1991). **Using Antisense RNA to Study Gene Function.** *Symp. Soc. Exp. Biol.* 45: 117 - 127.

Schultz, L.D., Markus, H.Z., Hofmann, K.J., Montgomery, D.L., Dunwiddie, C.T., Kniskern, P.J., Freedman, R.B., Ellis, R.W. and Tuite, M.F. (1994). **Using Molecular Genetics to Improve the Production of Recombinant Proteins by the Yeast *Saccharomyces cerevisiae*.** *Ann. N.Y. Acad. Sci.* 721: 148 – 157.

Sherman, F., Fink, G.R. and Hicks, J.B. (1986). **Methods in Yeast Genetics.** . Cold Spring Harbour Laboratory Press, Cold Spring Harbour.

Simons, R.W. (1988). **Naturally occurring antisense RNA control - a brief review.** *Gene* 72: 25 - 34.

Simons, R.W. and Kleckner, N. (1983). **Translational control of *IS10* transposition.** (1983). *Cell.* 34: 683 - 691.

Simons, R.W. and Kleckner, N. (1988). **Biological regulation by antisense RNA in prokaryotes.** *Annu. Rev. Genet.* 22: 567 - 600.

Simons, R.W., Hoopes, B.C., McClure, W.R. and Kleckner, N. (1983). **Three promoters near the termini of *IS10*: pIN, pOUT and pIII.** *Cell.* 34: 673 - 682.

Smith, C.J.S., Watson, C.E., Ray, J., Bird, C.R., Morris, P.C., Schuch, W. and Grierson, D. (1988). **Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes.** *Nature* 334: 734 - 736.

Southern, E.M. (1975). **Detection of specific sequences among DNA fragments separated by gel electrophoresis.** *J. Mol. Biol.* 98: 503 - 517.

Spector, D.L. (1990). **Higher order nuclear organisation: Three-dimensional distribution of small nuclear ribonucleoprotein particles.** Proc. Natl. Acad. Sci. USA. 87: 147 - 151.

Sullenger, B.A. and Cech, T.R. (1993). **Tethering ribozymes to a retroviral packaging signal for destruction of viral RNA.** Science. 262: 1566 – 1569.

Szafranski, P., Mello, C. M., Sano, T., Smith, C. L., Kaplan, D. L. and Cantor, C. R. (1997). **A new approach for containment of microorganisms: Dual control of streptavidin expression by antisense RNA and the T7 transcription system.** Proc. Natl. Acad. Sci. USA. 94: 1059 - 1063.

Takacs, A. and Banerjee, A. (1997). **Inhibition of vesicular stomatitis virus in cells constitutively expressing an antisense RNA targeted against the virus RNA polymerase gene.** Journal of General Virology. 78: 125 - 130.

Takayama, K.M. and Inouye, M. (1990). **Antisense RNA.** Crit. Rev. Biochem. Mol. Biol. 25: 155 - 184.

Thomas, C. M. (1992). **Regulation of gene expression and function by antisense RNA in bacteria.** In Murray, J.A.H. (Ed.) *Antisense RNA and DNA*. Wiley-Liss, New York; 51 -76.

Thompson-Jager, S. and Domdey, H. (1990). **The intron of the yeast actin gene contains the promoter for an antisense RNA.** Curr. Genet. 17: 269 - 273.

Tieman, D.M., Harriman, R.W., Ramamohan, G. and Handa, A.K. (1992). **An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit.** Plant Cell 4: 667 - 679.

Uhlmann, E. and Peymann, A. (1990). **Antisense oligonucleotides: A new therapeutic principle.** Chemical Reviews. 90: 544 - 584.

van der Krol, A.R., Lenting, P.E., Veenstra, J., van der Meer, I.M., Koes, R.E., Gerats, A.G.M., Mol, J.N.M. and Stuitje, A.R. (1988b). **An antisense chalcone synthase gene in transgenic plants inhibits flower pigmentation.** *Nature*. 333: 866 - 869.

van der Krol, A.R., Mol, J.N.M. and Stuitje, A.R. (1988a). **Modulation of eukaryotic gene expression by complementary RNA or DNA sequences.** *BioTechniques*. 6: 958 -976.

van der Krol, A.R., Mur, L.A., de Lange, P., Mol, J.N.M. and Stuitje, A.R. (1990). **Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effects.** *Plant Mol Biol*. 14: 457 - 466.

van Duin, M., van den Tol, J., Hoeijmker, J.H.J., Bootsma, D., Rupp, L.P., Reynolds, P., Prakash, L. and Prakash, S. (1989). **Conserved pattern of antisense overlapping transcription in the homologous human *ERCC-1* and yeast *RAD10* DNA repair gene regions.** *Mol. Cell. Biol*. 9: 1794 - 1798.

Vogelstein, B. and Gillespie, D. (1979). **Preparative and analytical purification of DNA from agarose.** *Proc. Natl. Acad. Sci. U.S.A.* 76: 615 - 619.

Weintraub, H., Izant, J.G. and Harland, R.M. (1985). **Anti-sense RNA as a molecular tool for genetic analysis.** *Trends Genet*. 1: 22 - 25.

Williams, T. and Fried, M. (1986). **A mouse locus at which transcription from both DNA strands produces mRNAs complementary at their 3' ends.** *Nature*. 322: 275 - 278.

Wilson, I.W., Siemering, K.R., Praszler, J. and Pittard, A.J. (1997). **Importance of structural differences between complementary RNA molecules to control of replication of an IncB plasmid.** *J. Bact*. 179: 742 -753.

Wormington, W. M. (1986). **Stable repression of ribosomal protein L1 synthesis in *Xenopus* oocytes by microinjection of antisense RNA.** Proc. Natl. Acad. Sci. USA. 83: 8639 - 8643.

Xiao, W. and Rank, G.H. (1988). **Generation of an *ilv* bradytrophic phenocopy in yeast by antisense RNA.** Curr. Genet. 13: 283 - 289.

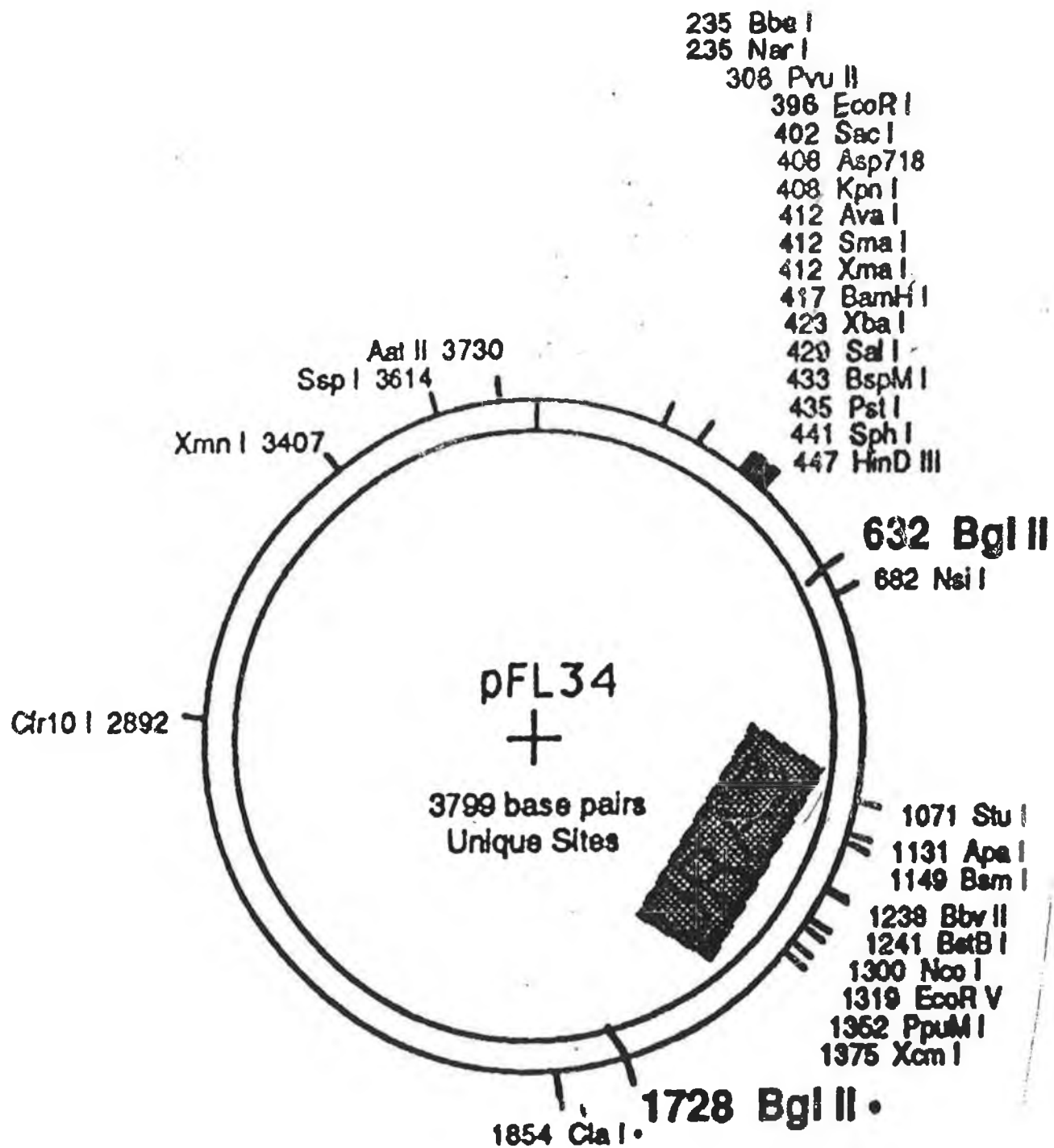
Xing, Y., Johnson, C.V., Dobner, P.R. and Lawrence, J.B. (1993). **Higher level organisation of individual gene transcription and RNA splicing.** Science. 259: 1326 - 1330.

Yukki, T., Nomura, T., Tezuka, H., Tsuboi, A., Yamagata, H., Tsukagoshi, N. and Udaka, S. (1985). **Complete nucleotide sequence of a gene coding for a heat and pH stable α -amylase of *Bacillus licheniformis*: comparison of the amino acid sequences of 3 bacterial liquefying α -amylases deduced from the cDNA sequences.** J. Bacteriology. 98: 1147 - 1156.

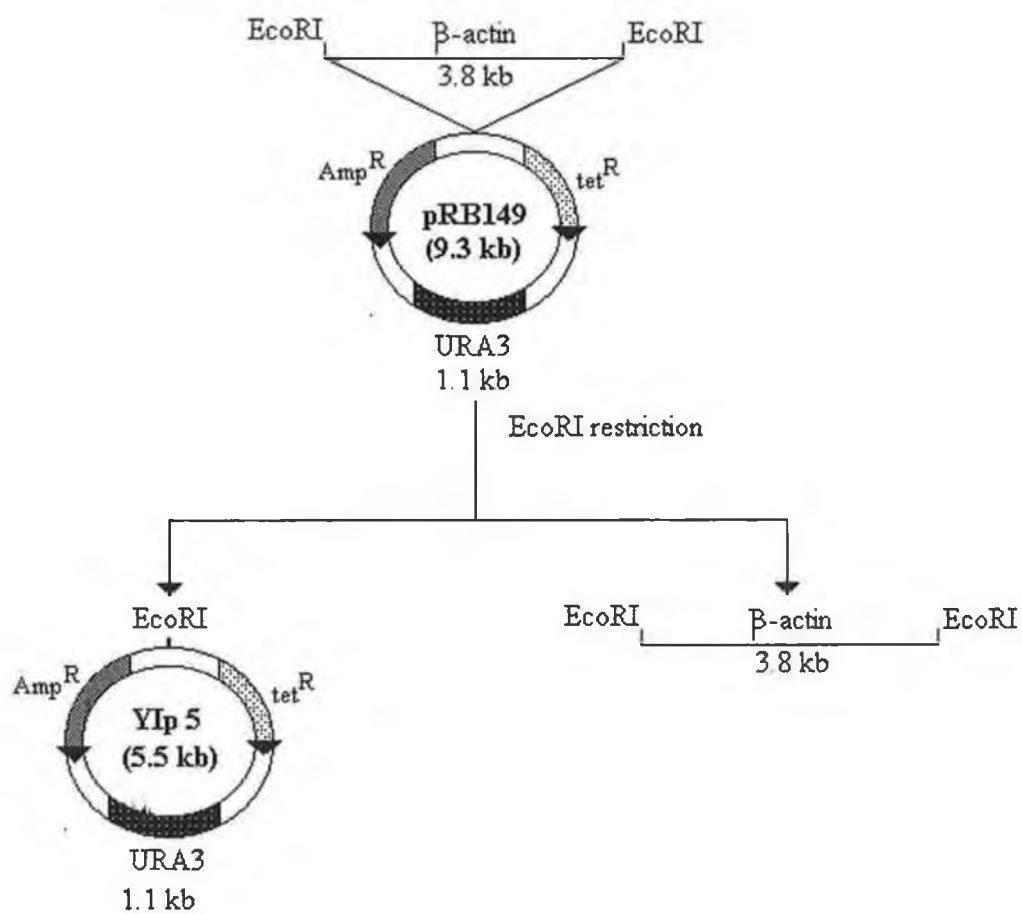
Zamenik, P.C. and Stephenson, M.L. (1978). **Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide.** Proc. Natl. Acad. Sci. USA. 75: 280 - 284.

Appendices

Appendix I - Plasmid map of pFL34.



Appendix II - Schematic representation of the plasmid pRB149 (Canavan, 1994).



Appendix III - α -amylase gene sequence.

[illegible]

SD - POSSIBLE RBS

RESTRICTION
ENZYME
SITES

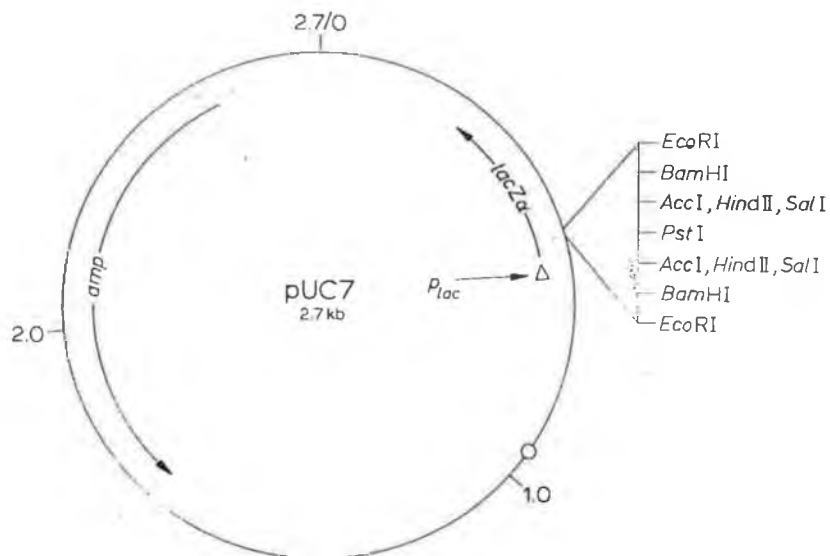
TRANSLATION STOP
CODON
PALINDROMIC SEQUENCES

Appendix IV – The α -amylase gene sequence (minus its transcription stop site)
cloned into pAAH5 in antisense orientation to produce, pMO3.

HincIII 5600 30 TRANSLATION START
 TACCTTACCTGATGTTTACATTGAAAGGGGAGGAGAAATCATGAAACAACAAAAACGGCTTTACGCC 70
 TTCGAACCTAGGCATACAAAGTGTAACCTTCCCCCTCTTCTAGTACTTTGTTGTTTTGCGGAAATGCGG
 CGATTGCTGACGCTGTTATTGCGCTCATCTTCTTGCTGCCTCATTGAGCAGCGCGGCAAAATCTTA 140
 GCTAACGACTGCGACAATAAACCGGAGTAGAAGAACGACGGAGTAAGACGTCGTCGCGCGCTTAGAAT
 ATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGACGGCCAACTTGGAAAGCGCTTGCAAAA 210
 TACCTGCGACTACGTCAATAAACTTACCATGTACGGGTACTGCGCGTGTAAACCTTCGCGAACGTTTT
 CGACTCGGCATATTTGGCTGAACACGGTATTACTGCGCTCTGGATTCCCCCGGCATATAAGGGAACGAGC 280
 GCTGAGCCGTATAAACCGACTTGTGCCATAATGACGGCAGACCTAAGGGGGCCGTATATTCCCTTGCTCG
 CAAGCGGATGTGGGCTACGCTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGACGCTTC 350
 GTTCGCTACACCCGATGCCACGAATGCTGGAATACTAAATCCCCTCAAAGTAGTTTTTCCCTGCCAAG
 GGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAGTCTTCATTCCGCGCATTAACGT 420
 CCTGTTTCATGCGGTGTTTTCTCTCGACGTTAGACGCTAGTTTTTCAGAAGTAAGGGCGCTGTAATTGCA
 TTACGGGGATGTGGTCAACCAACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTGAAGTC 490
 AATGCCCTACACCAAGTAGTTGGTGTTCCTCGCGGCACTACGCTGGCTTCTACATTGGCGCACTTCAG
 GATCCCGCTGACCGCAACCGCGTAATTTCAAGGAGAACACCGAATTAAGCCTGGACACATTTTCATTTTC 560
 CTAGGCGTACGCTGGCTGGCGCATTAAGTCTCTTGTGGCTTAATTCGGACCTGTGTAAGGATAAGG
 CGGGGCGCGGCGACACATACAGCGATTTTAAATGGCATTGAGGCTTTTACCGGAACCGATTGGGACGA 630
 GCGCGCGCGCTGCTGATGTGCTAAATTTACCGTAACCATGGTAAAGTGCCTTACCTGCTAACCTGCT
 GTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTTGGGAAGTTTCAATGAAAC 700
 CAGGGCTTTCGACTTGGCGTAGATATTCAAAGTCTCTTCCGAACCCCTAACCCCTCAAAGGTTACTTTTG
 GGCAACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTGCGAGCAGAAATTAAGA 770
 CCGTTGATACTAATAAACTACATACGGCTGTAGCTAATACTGGTAGGACTACAGCGTCTCTTAATTCT
 GATGGGCACTTGGTATGCCAATGAAGTCAATTTGACGGTTTCCGTCTTGATGCTGTCAACACATTAA 840
 CTACCCCGTGAACCATACGGTTACTTGACGTTAACCTGCCAAAGGCAGAACTACGACAGTTTGTGTAAT
 ATTTTCTTTTTCGCGGATTTGGGTTAATCATGTGTCAGGGAAGGAAAGGAAATGTTTACGGTAGCT 910
 TAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAG
 GAATATTTGGCAGAACTGACTTGGGCGCGCTGAAAGCAATTTGAACAAACAAATTTTAACTATTAGTGT 980
 CTTATAACCGTCTTACTGAACCCGCGCGACCTTTTGATAAACTTGTTTTGTGTTAAATTTAGTAAGTCACA
 TTGACGTGCGCTTCATTATCAGTTCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTT 1050
 AACTGCACGGCGAAGTAATAGTCAAGGTACGACGTAGCTGTGTCCTCCGCCGATACCTATCTCTTAA
 CCTGAACAGTACGGTCTTTTCCAAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAG 1120
 CGACTTGTCTATGCCAGCAAGGTTTCGTAGGCAACTTTCGCCAATGTAAACAGCTATTGGTACTATGTGTC
 CCGGGGCAATCGCTTGAAGTCCAAACATGGTTTAAAGCGGCTTGTACGCTTTTATTCTCACAA 1190
 GGCCCCGTTAGCGAACTCAGCTGACAGGTTTGTACCAAAATTCGGCGAAGCAATGCGAAAATAAGAGTGT
 GGAATCTGGATACCCCTCAGGTTTTCTACGGGATATGTACGGGACGAAAGGAGACTCCGAGCGCGAAAT 1260
 CCCCTAGACCTATGGGAGTCCAAAAGATGCCCTATACATGCCCTGCTTTCTCTGAGGGTTCGCGCTTTA
 TCCTGCCTTGAACACAAAATTTGAACCGATCTTAAAGCGAGAAAACAGTATGCGTACGGAGCACAGCAT 1330
 AGGACGGAACTTTGTGTTTTAACTTGGCTAGAATTTTCGCTCTTTGTATACCGCATGCCTCGTGTGTA
 GATTATTTGACACCATGACATTTGTCGGCTGGACAAAGGAAAGGCGACAGCTCGGTTGCAAAATTCAGGTT 1400
 CTAATAAAGCTGGTGGTACTGTAAACAGCCGACCTGTTCCCTTCGCTGTGAGCCAACTTTAAGTCCAA
 TGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAGCGAATGTATGTGCGCGGCAAAACGCGCGTGA 1470
 ACCGCCGTAATTATTGCTGCTGGCTGGGCCACCCGTTTCGCTTACATACAGCCGCGCTTTGGCGGCACT
 GACATGGCATGACATTACCGGAAACCGTTTCGGAGCCGTTTGTCTCAATTCGGAAGGCTGGGGAGAGTTT 1540
 CTGTACCGTATGTAATGGCCTTGGCAAGCCTCGGCCAACAGTAGTTAAGCCTTCGACCCCTCTCAAA
 CACGTAAACGGCGGCTCGGTTTCAATTTATGTTCAAGATGACAGAGCAGAGAGGACGGATTTCCTGAAG 1610
 GTGCATTTGCCGCCCCAGCCAAAGTTAAATACAGTTTCTATCTCTGCTCTCCTGCTTAAAGGACTTC
 CAAA
 CTTT

RESTRICTION ENZYME SITES
 TRANSLATION
 STOP CODON
 PALINDROMIC SEQUENCES.
 50 - POSSIBLE RBS

Appendix V – Plasmid map of pUC8.



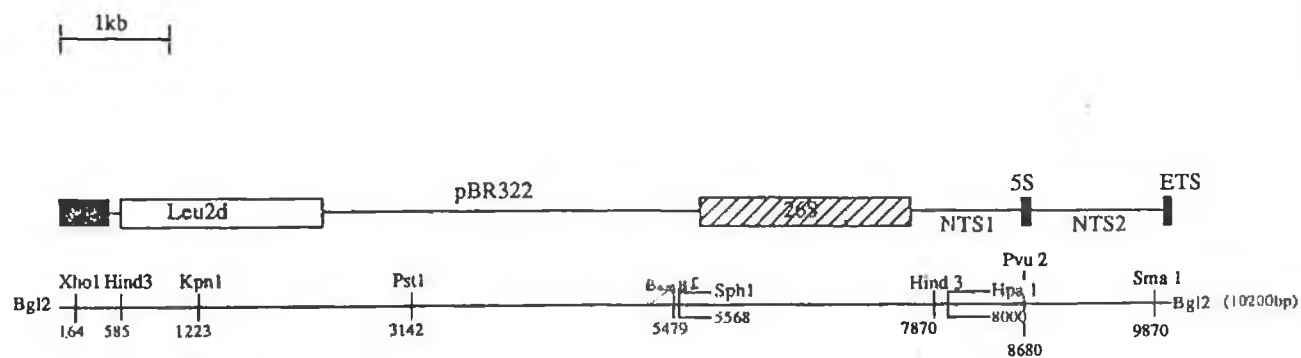
Replicon	: pMB1 ²	Amplifiable	: yes
Size	: 2.7 kb	Self-transmissible	: no
Selectable phenotype	: Amp ^R	Mobilisation genes	: no
Genetic markers	: <i>lacZα</i> ⁺	Mob site	: yes
Cloning sites for gene-inactivation	: (<i>AccI</i>), (<i>Bam</i> HI), (<i>Eco</i> RI), (<i>Hind</i> II), (<i>Pst</i> I), (<i>Sal</i> I)		
Other cloning sites	: not given		

Plasmid pUC7 can be used for cloning DNA sequences in the *lacZα* region providing a detection of transformants containing recombinant plasmids on the basis of their Lac⁻ phenotype. Bacteria harboring plasmids without inserts make blue colonies on indicator plates (plates with X-gal) whilst those harboring recombinant plasmids make white colonies (ref. 18). Plasmid pUC7 contains several unique restriction sites in the *lacZα* region, downstream of the *lacZ*-initiation codon; the sequence with the cloning sites in the *lacZα* region is the same as in M13mp7 (I-A-v-1). The terminal sequence of any DNA cloned in the plasmid can be characterized using the universal M13 primers.

Plasmid pUC7 consists of the *Eco*RI-*Pvu*II fragment of pBR322 (I-A-iv-1) containing the *amp* gene and a 391 bp *Hae*II fragment containing the *E. coli-lacZα* sequence cloned into a *Hae*II site. The 391 bp fragment contains the *lac* promoter-operator region and 59 codons of the beginning of the *lacZ* gene. This part is sufficient to complement the M15 deletion of the *lacZ* gene (ref. 70). The *Acc*I, *Hind*II and *Pst*I restriction sites in the pBR322 part of the plasmid were removed by mutation.

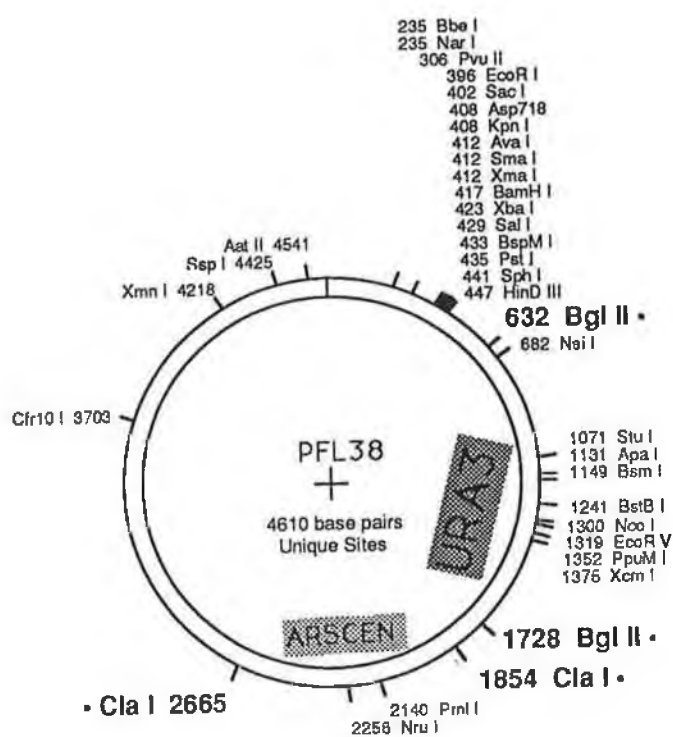
Plasmids pUC8 (2.7 kb; ref. 67) and pUC9 (2.7 kb; ref. 67) have the same properties as pUC7 but contain other sequences in the *lacZα* region useful for cloning DNA sequences in different reading frames with respect to the initiation codon. Plasmids pUC8 and pUC9 contain the same cloning sites in the *lacZα* region as M13mp8 (I-A-v-1) and M13mp9 (I-A-v-1), respectively. Plasmids pUC8 and pUC9 allow one to clone double-digested restriction fragments separately in both orientation with respect to the *lac* promoter.

Appendix VI – Plasmid map of pMIRY.

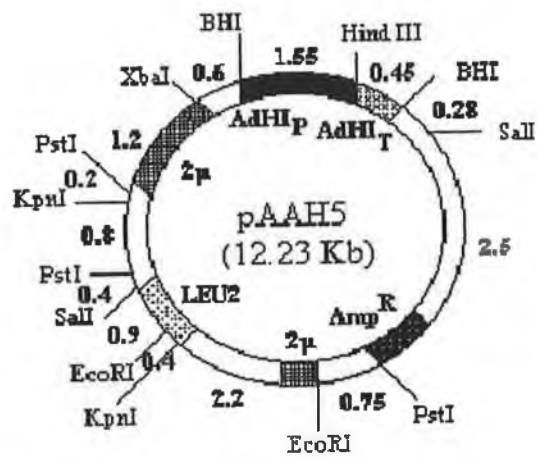


Cla1-122/1163
EcoR1-573/1643/2390/6857/7224/7815/10120
Hha1-86
Hinc2-1908/2846/5479/5770/6380
Hpa2-228/403/511/7895/8520/9245/9870
Pvu1-818
Stu1-5883/7568

Appendix VII - Plasmid map of pFL38

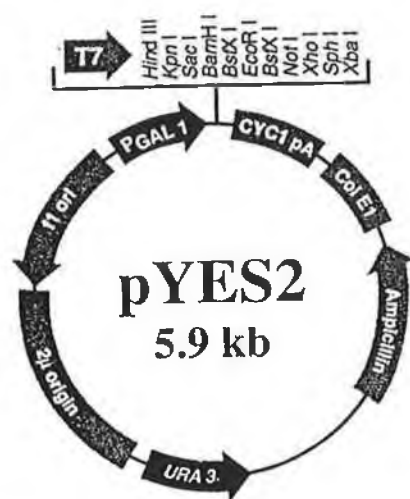


Appendix VIII- Plasmid Map of pAAH5.

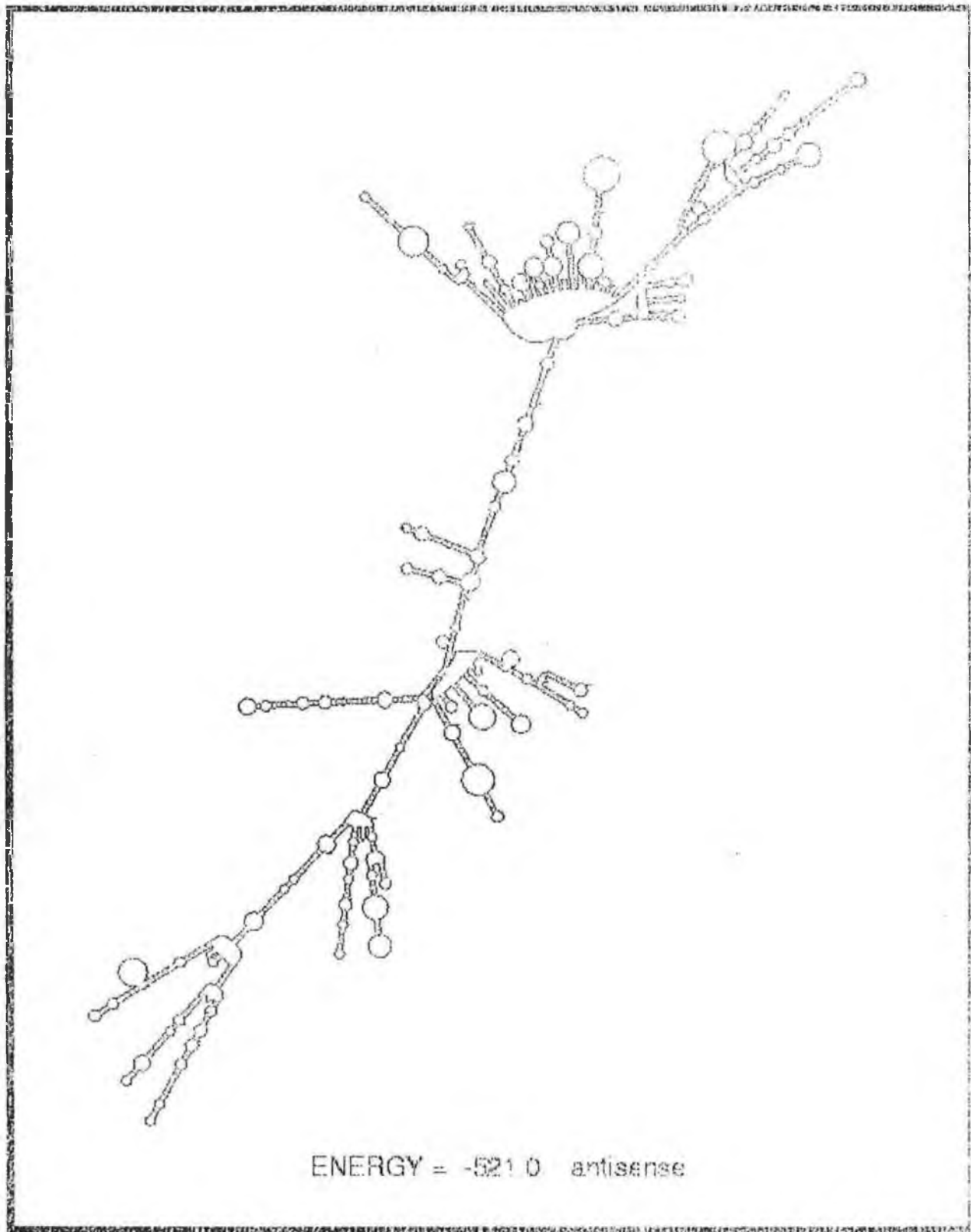


Note: BHI, *Bam*H I.

Appendix IX – pYES2.

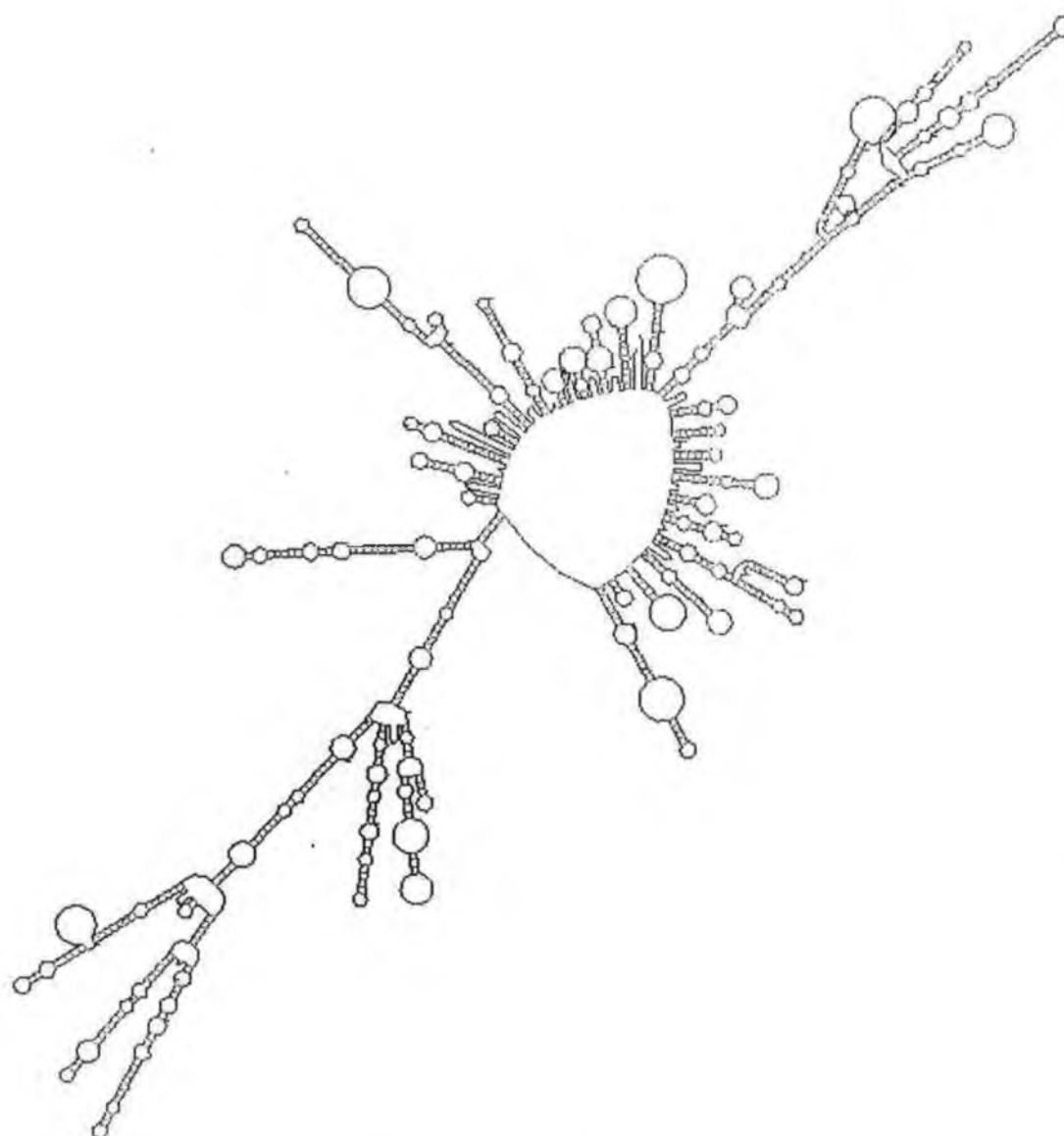


Appendix X – Mfold structure (1) of the antisense α -amylase gene sequence.



Structure 1

Appendix X – Mfold structure (2) of the antisense α -amylase gene sequence.



ENERGY = -520.4 antisense

Structure 2