The Regulation of Cellular CD23 Gene Expression by Epstein-Barr Virus Latent Proteins in Immortalised Cells.

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by

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

Signed : Carol Pourr I.D. No. : 92700050

Date : 1/1/96

Dedication

"They are ill-discoverers that think there is no land when they see nothing but sea"

Francis Bacon

This thesis is dedicated to my parents, whose love and support have given me the courage to follow my dreams.

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"Ce qui donne du courage, ce sont les idees"

Georges Clemenceau

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Abstract

Only EBV-infected B lymphocytes which express the B cell activation marker CD23 are capable of establishing immortalised cell lines. Epstein-Barr virus determined nuclear antigen 2 (EBNA-2) is essential for EBV-induced growth transformation (Cohen, *et al*, 1989) and can specifically upregulate CD23 gene expression (Wang, *et al*, 1987). The regulation of CD23 gene expression is likely to be an important factor in EBV-induced B cell growth transformation. The cellular CD23 promoter and EBV TP-1 promoter have previously been shown to be transactivated by EBNA-2 (Wang, *et al*, 1991, Le Roux, *et al*, 1993). Studies have also shown that while EBNA-3A itself has no effect on the TP-1 promoter, it can repress EBNA-2-mediated transactivation in a B cell background (Le Roux, *et al*, 1994). Homologies have been shown between sequences present in the EBV TP-1 EBNA-2 responsive element and other functionally similar sequences which are found in the CD23 promoter region. The purpose of this study was to explore the regulation of CD23 gene expression by the EBV latent proteins EBNA-2 and EBNA-3A.

The most common reporter gene used in transfection studies is CAT (chloramphenicol acetyl transferase), usually assayed using a radioisotopic method. To obviate the need to use a radioassay, an alternative reporter plasmid was constructed and tested in a range of cell backgrounds. This construct consisted of the CD23 promoter cloned upstream of the PAP gene (placental alkaline phosphatase). The assay involves a colorimetric reaction, and is a simpler and cheaper reporter assay (Henthorn, *et al*, 1988).

In order to identify relevant sequence modules in the CD23 promoter, deletion mutants of CD23CAT were constructed for use in co-transfection assays with plasmids encoding EBNA-2/3A. A strategy based on using the nuclease BAL 31 was designed to create nested mutations. Fragments of the CD23 promoter including the EBNA-2 responsive elements were also amplified by PCR for cloning upstream of CAT.

Co-transfections were carried out using plasmids encoding the EBNA-2 and EBNA-3A proteins as well as the CD23 reporter plasmid. No PAP activity was seen in any of the samples containing the pCD23PAP construct. As the pCD23CAT plasmid produced CAT activity in DG75 (an EBV-negative B lymphoma cell line), it appears that the CD23 promoter is active in these cells and that the pCD23PAP construct is not functioning, or gives a low activity beyond the sensitivity of the enzyme assay. In a B cell background, the results indicate that EBNA-2 upregulates the activity of the CD23 promoter, increasing the amount of CAT activity by a factor of 4-5. The EBNA-3A protein represses the EBNA-2-mediated upregulation of the CD23 promoter, with CAT activity returning to basal levels in the presence of both EBV proteins. A construct containing a deleted CD23 promoter gave no basal CAT activity and was not transactivated by EBNA-2. This was as expected, as this construct contains a deletion of approx. 200 bp which should remove the EBNA-2 responsive sequence elements. Restriction analysis of the CD23 promoter sequence led to the identification of a novel Sma I site confirming the presence of a sequence change relative to the published CD23 sequence. These effects are not seen in other cell backgrounds. In conclusion, the results of this study indicate that CD23 gene expression may be regulated by the EBV latent proteins EBNA-2/3A in some cell backgrounds.

1. Introduction

1.1 EBV discovery and classification

Epstein-Barr virus is a ubiquitous human herpes virus that was first identified from studies by Denis Burkitt in the late 1950's on an unusual childhood lymphoma of African children. He showed that the incidence of this lymphoma was determined by geographic factors and corresponded to the malaria belt in Africa, hence indicating an infectious origin (Burkitt, 1962). This prompted the search for possible oncogenic viruses in tissue from what was to become known as Burkitt's lymphoma (BL).

Attempts to infect newborn mice, embryonated hen eggs and test tissue culture systems with BL materials in an effort to isolate virus failed, as did electron microscopy studies. Based on experiments with virus-induced animal tumour cells in culture, a search was made for an inapparent oncogenic virus in BL tissue by establishing long term continuous cell cultures from these materials.

In 1964, the ability to culture BL tumour cells *in vitro* led to the establishment of a cell line (EB1) of continuously growing BL-derived lymphoblasts by the scientists Epstein and Barr. They subsequently identified virus particles using electron microscopy in BL cells of African patients (Epstein, *et al*, 1964). The virus was identified as a unique herpesvirus as it did not react with antisera generated against known herpesvirus, and designated the Epstein-Barr virus. It seemed unusual that a member of the herpes family could be carried as an inapparent infection in a continuous human cell line without destroying the cultures. Ordinary biologic tests failed to identify the isolate so it appears EBV was the first viral agent discovered by electron microscopy alone. An association of EBV with another human cancer, Nasopharyngeal Carcinoma (NPC), was also discovered. This prompted a great deal of investigative work on EBV as a potential model for cell control and cancer studies.

Epstein-Barr virus is classified as a member of the genus *lymphocryptovirus,* which belongs to the subfamily gammaherpesvirinae (for review see (Power, *et al*, 1993)). EBV is a ubiquitous human herpes virus that is transmitted horizontally, infecting over 90% of all human populations. Following primary infection, whether symptomatic or silent,

the virus persists in the healthy host for life by mechanisms that are not fully understood. In the western world EBV is largely associated with infectious mononucleosis (IM) or glandular fever, a debilitating non-malignant lymphoproliferative syndrome (Henle, *et al*, 1979). Infection is usually initiated in the oropharyngeal epithelium which is permissive for virus replication. Infection in the oropharynx is predominately lytic, with complete replication of the virus linked to ordered squamous epithelial differentiation. The continuous shedding of infectious particles in the saliva most likely results in the high rate of virus transmission by mouth to mouth contact.

In appropriate circumstances, EBV appears to contribute to several cancers, namely African endemic Burkitt's Lymphoma (BL) of B cell origin, and anaplastic Nasopharyngeal Carcinoma (NPC) derived from poorly differentiated epithelial cells (Kieff, *et al*, 1990, Magrath, 1990). In recent years there has been a dramatic increase in the reported incidence of EBV-associated non-Hodgkins lymphomas in immunosuppressed individuals (Young, *et al*, 1989). Both transplant and AIDS patients have markedly increased risks of developing EBV-associated lymphomas. EBV is also associated with a fatal X-linked lymphoproliferation which occurs in congenitally immunodeficient patients (Duncans syndrome) (Mayer, *et al*, 1994). Within such a huge natural pool of virus there exists the potential for extensive variation, but the extent of any such variability is not clear. Two strains, or types, of EBV infect humans-serotype A and serotype B. These strains differ in the sequences of the viral genes expressed during latent infection and in their ability to transform B lymphocytes. Studies suggest that both strains are prevalent in the United States and that persons can be coinfected with both strains (Strauss, *et al*, 1993). The current viewpoint is that there do not appear to be disease-specific subtypes of EBV in that the ratio of A type to B type virus in EBV-related tumours reflects the prevalence of infection within the general population.

1.2 General characteristics of herpesviruses.

The herpesviruses represent a very large, clearly defined group of viruses of considerable medical importance and uniqueness. They are double-stranded DNA viruses, ranging in size from 100 kb to 240 kb and are divided into 3 subfamilies :

Family	Characteristics	Site of latency	Example
α-herpesviridae	Rapid replication cycle < 24 hours Wide host range	Nerve ganglions	Herpes Simplex Virus (HSV 1&2)
β-herpesviridae	Long replicative cycle Narrow host range	Epithelial cells and lymphocytes	Cytomegalo-virus (CMV)
γ-herpesviridae	B or T lymphocyte cell tropism	Lymphocytes	Epstein-Barr virus (EBV)

The complex genome is composed of two unique sequence elements (unique short (US) and unique long (UL)), separated by internal repeats (IR) and bordered by terminal repeats (TR). The linear viral DNA is contained in an icosahedral protein capsid which is surrounded by an envelope derived primarily from the nuclear membrane of the infected cell. The virion attaches to the cell surface via specific receptors. Fusion of the viral and cellular membranes allows liberation of the capsid into the cytoplasm. During migration to the nucleus, the capsid is degraded and the nucleoprotein complex penetrates the nucleus. Viral DNA is transcribed by the cellular RNA polymerase II. Primary transcription involves proteins implicated in replication e.g. thymidine kinase or DNA polymerase. In effect, the herpesvirus codes for a large part of its own replication, which follows the rolling circle model - concatamers of genomes linked in tandem are formed and subsequently cleaved at the terminal repeats. Capsid assembly occurs in the nucleus. The virions reach the cell surface via the endoplasmic reticulum.

Herpesvirus infections are very widespread. Transmission occurs via direct contact - by saliva, blood, sexual contact or from mother to child. Primary infection may not be accompanied by clinical signs, but confers lasting immunity. Infection is said to be latent - a small number of genes is expressed and there is no viral production. Under certain conditions e.g. immunosuppression, reactivation can occur.

1.3 Diagnosis of EBV infection

Serodiagnosis of infectious mononucleosis (IM) was established a long time before the discovery of its agent. In 1932 Paul and Bunnell described heterophile antibodies, a serological phenomenon associated with IM (Paul, *et al*, 1932). The so-called Paul-Bunnell reaction means an agglutination of erythrocytes from horse, sheep or cattle by the sera of patients with IM. These agglutination tests are now routinely used and are of low cost. Modern versions of this assay utilise purified Paul-Bunnell antigen coated (for example) on latex beads. However, the incidence of heterophile antibodies amongst IM patients is at most 90% and other diseases may provoke a heterophile antibody response. Testing for EBV-specific antibodies is therefore the preferred method for the serological diagnosis of IM.

Routine diagnosis of EBV infection is usually carried out by determining the level and class of antibodies to the various EBV antigens in a patients serum (fig. 1). These standard assays use fixed cells derived from various cell cultures infected with distinct strains of EBV which express a particular viral antigen class (Henle, *et al*, 1971).

Antigen	Description
Viral capsid antigen (VCA)	Complex of protein constituting the viral nucleocapsid
Membrane antigen (MA)	Group of proteins present in or associated with the viral membrane surrounding the nucleocapsid -classified as early or late (EMA/LMA)
Epstein-Barr nuclear antigens (EBNA)	Viral proteins located exclusively in the nucleus of EBV infected cells, of which there are at least six.
Early antigens (EA)- may be classified as diffuse (EA-D) or restricted (EA-R)	Non-structural EBV proteins produced during viral replicative cycle and by EBV undergoing reactivation.
Lymphocyte detected membrane antigens (LYDMA)	Virally encoded non-structural membrane proteins located on the surface of EBV growth-transformed lymphocytes.

Most EBV antigens can be easily determined by direct or indirect immunofluoresence tests (Henle, *et al*, 1974) except the viral nuclear antigens (EBNAs) which can only be detected by the more sensitive anti-complement immunofluoresence method. Serology has played a major part in establishing a relationship between EBV and the major human diseases with which it is now firmly associated.

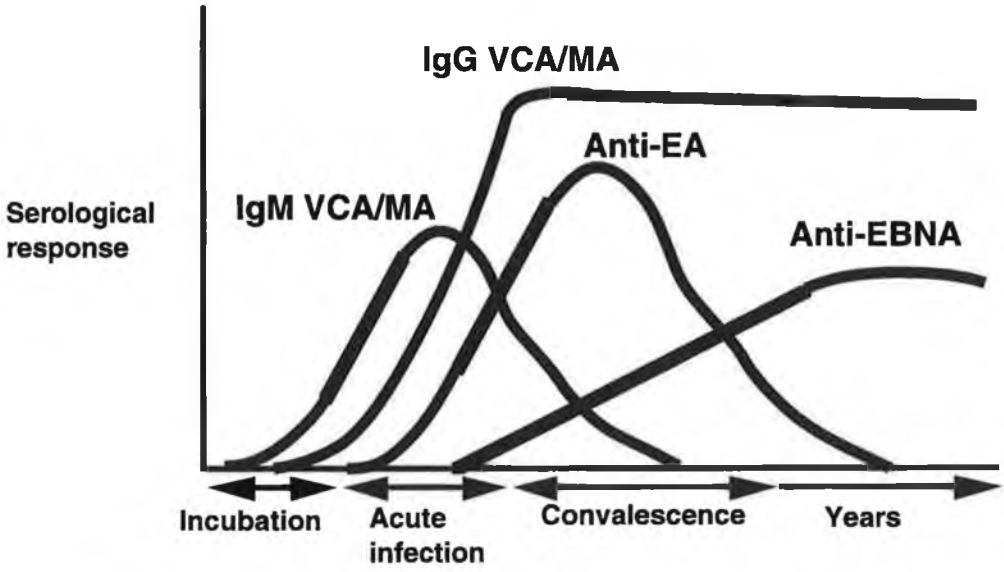


Figure 1. Time course of the serological response to the major viral antigen classes after primary infection with EBV.

Several new techniques have been rapidly applied in recent years to the detection of EBV in clinical material. PCR has greatly improved the ability to detect low copy numbers (PCR will detect 1 or 2 target DNA sequences in ~10^5 cells) (Joske, *et al*, 1993). The greatest limitation of PCR is that it does not identify which cells are giving rise to a positive result. In lymphoma patients the question of whether their tumour cells are EBV DNA-positive is unanswerable by PCR as the signal may have come from other EBV-positive lymphocytes.

However, the PCR technique can be modified to detect "tumour-associated" EBV. In reverse transcription PCR (RT-PCR) the target to be amplified is not EBV DNA, but viral RNA transcripts. Primers for RT-PCR are designed that correspond to the EBV RNA transcripts expected in tumour cells. The resulting cDNA is subsequently amplified by PCR (Joske, *et al*, 1993).

In situ hybridisation has also been used : Two small non-polyadenylated EBV RNAs (EBER 1 and EBER 2) are produced during latent infection. These are transcribed by RNA polymerase III and are present in up to 10^7 copies per cell making them by far the most abundant viral RNA species and a good target sequence for in situ detection (Joske, *et al*, 1993).

1.4 Diseases associated with EBV infection.

Infectious mononucleosis

The clearest causal role for EBV in human disease is in infectious mononucleosis (IM), the symptoms of which represent the course of the immune response to an EBV primary infection that has been delayed into adolescence or adulthood (Vousden, *et al*, 1994). The virus infects B lymphocytes but the mononucleosis is mainly of T lymphocytes which are responding to the EBV infected cells.

Primary EBV infections occurring in early childhood are normally silent or accompanied by such mild symptoms that IM is not usually diagnosed. With advancing age, as is the case with some other common viral illnesses, the severity of EBV primary infection increases. Infection later in puberty can result in the debilitating non malignant lymphoproliferative syndrome known as IM. Clinically apparent IM tends to be a disease of economically advanced countries where a greater proportion of individuals escape infection in childhood, with the peak incidence of mononucleosis occurring in people 15 to 25 years of age. Individuals avoid contacting the virus until late adolescence when they begin dating (hence it is often called kissing disease).

Once a person has contracted IM, virus infected B-cells rapidly proliferate, provoking a characteristic cytotoxic T cell response, and take on an atypical appearance on blood film that is useful in diagnosis. The disease is manifested by enlargement of the lymph nodes and spleen, sore throat, nausea, general weakness and tiredness, and a mild fever that usually peaks in the early evening. The disease lasts for one to six weeks and is usually self-limiting. A persistent active infection can sometimes ensue that continues at some level for many years (Strauss, 1988).

Patients in the acute phase of IM usually have a characteristic EBV- related antibody response. These patients have high titres of IgM and IgG antibodies to VCA and MA (which are structural proteins associated with EBV), they show a transient anti EA-D (diffuse early antigen) response in 85% of cases and no anti-EBNA (Epstein-Barr nuclear antigen) as a rule. After convalescence, antibodies to EA-D disappear, while VCA, MA and EBNA antibodies persist over a lifetime, probably reflecting the persistent or intermittent release of virus at low level. Anti-EBNA

antibodies arise gradually after several weeks or months (Henle, 1982). Since the early 1980's the EBNA's have been shown to consist of a family of at least six proteins. Following IM, antibodies to EBNA-2 appear first and after peaking, decline again in time to lower persistent or non-detectable levels. Antibodies to EBNA-1 have been shown to emerge several weeks or months after anti-EBNA-2 and gradually attain the levels at which they persist. The presence of IgG antibody to VCA indicates past infection and is thus an index of immunity to IM. Current infection is usually established by the presence of anti-VCA antibodies of the IgM type or by showing a rise in antibody titre to one of the several EBV antigen systems. Elevated and persistent anti-EA is likely to reflect an ongoing viral infection (Henle, *et al*, 1981).

EBV and carcinogenesis

There are 2 general strategies whereby viruses are thought to contribute to carcinogenesis. In some cases expression of certain viral genes bypasses the need for one or more of the steps that would normally be involved in multistep carcinogenesis. The other general mechanism seems to involve the virus stabilising and promoting the growth of a cell population in which oncogenic genetic change may then occur (Vousden, *et al*, 1994).

EBV has been closely linked to several cancers, including Burkitt's lymphoma, Nasopharyngeal carcinoma, post transplant B-cell lymphoma, Hodgkins disease and AIDS-related lymphomas.

Burkitt's lymphoma

Burkitt's lymphoma is a poorly-differentiated malignant lymphoma that occurs sporadically throughout the world. There are however, high incidence areas, such as parts of equatorial East Africa and New Guinea (Burkitt, 1962). The malignancy was first described by Denis Burkitt in the late 1950's as a very aggressive tumour of the jaw or ovaries and represents the most common childhood cancer in the tropics (up to 10 cases per 10,000 per year) (Parkin, *et al*, 1984). Burkitt's lymphoma is one of the fastest growing human tumours - it has a doubling time of about 24 hours. Paradoxically the cells are small but with a resting B cell phenotype. The pattern of EBV gene expression in Burkitt's lymphoma is

type I latency : only EBERs (2 small non-polyadenylated RNAs) and EBNA-1 are found (Joske, *et al*, 1993). The tumour cells carry very few activation molecules or viral antigens (EBNA-1 is not a target for cytotoxic T lymphocytes) and hence may evade immune surveillance (Mayer, *et al*, 1994).

EBV genomes are present in the vast majority of tumours from endemic areas. Some 96% of African cases have been shown to be EBV positive, compared to 30% of sporadic cases in developed countries (Joske, *et al*, 1993). Furthermore, once the disease appears, Burkitt's lymphoma patients have considerably higher (about 10-fold) antibody titres to the EBV VCA and EA-R than do children without the tumour (Lenoir, 1986). The high incidence of BL in the tropics is associated with geographic and climatic features incident with holoendemic malaria. That malaria infection might be a co-factor in the development of BL is supported by the observation that individuals with malaria have reduced T-cell responses to EBV-infected cells (Whittle, *et al*, 1984).

The development of both EBV and non-EBV-related BL always involves the chromosomal translocation of one of the alleles of the *c-myc* oncogene, (which encodes a ubiquitous phosphorylated nuclear protein that functions as a transcriptional regulator controlling cellular proliferation, differentiation and apoptosis (Marcu, *et al*, 1992)). This involves chromosome 8 and one of the 3 chromosomes containing the immunoglobulin loci (chromosome 14 - heavy chain locus, chromosome 2 - κ light chain locus and chromosome 22 - λ light chain locus)(Speck, 1987). The characteristic translocations are : t(2;8) (p12;q24), t(8;14) (q24;q36), t(8;22) (q24;q11). These act to juxtapose *c-myc* to one of the immunoglobulin loci, normally active in B cells, with the result that this oncogene is constitutively active (Klein, 1985). The coding sequences and third exon of *c-myc* are always intact despite the considerable variability of the translocation breakpoint in or around the gene.

In sporadic BL the breakpoint is normally at the switch region, consistent with this type of translocation arising in the lymph node germinal centre where isotype switching occurs. In endemic BLs however, the breakpoint on the immunoglobulin gene is usually at the J region or sometimes in the V or D regions, which would be consistent with the translocation occurring as a result of an error in VDJ joining, which

normally occurs in the bone marrow. So it seems that in the endemic areas in people with chronic malaria, cells with this type of translocation escape immune surveillance and other constraints and can become BL cells. Clonality of the cells and the EBV in them indicates that the virus infects early in the process and may well be in the cell when malignant transformation occurs. Very little is known about EBV in the bone marrow except that it can be transmitted by the bone marrow in a transplant (Gratama, *et al*, 1988). It is generally held that children in endemic areas have an enlarged pool of EBV-immortalised cells susceptible to further genetic change (Joske, *et al*, 1993).

Nasopharyngeal carcinoma

Nasopharyngeal Carcinoma (NPC) is seen worldwide, being rare among European and North American caucasians with age-adjusted incidence rates of less than 1 per 100,000 (Liebowitz, 1994). However, it occurs with a very high incidence in Southern China where it represents the most common tumour in males. NPC is derived from poorly differentiated epithelial cells and arises in the surface epithelium of the posterior pharynx (Parkin, *et al*, 1984). Two striking features of this cancer are the constant detection of EBV genomes in NPC cells and the regular infiltration of the tumour by non-malignant lymphocytes. NPC occurs primarily in adults and is unlikely to be the result of primary EBV infection as it occurs in areas where initial EBV infection occurs during childhood. The etiologic factors identified for NPC include EBV, environmental risk factors (including the ingestion of salt-cured fish), and genetic susceptibility, including a human leukocyte antigen (HLA)-associated risk as well as potential tumour suppressor genes located on chromosome 3 (Liebowitz, 1994).

Patients with NPC have elevated IgG antibodies to VCA and EA-D and this forms the basis of a screening test for the early detection of NPC in high incidence areas of China. A rising titre of IgA type antibodies to EA-D is a good diagnostic indicator of the onset of NPC, probably reflecting local antibody production in the pharynx (Henle, 1982).

The association between EBV and NPC was subsequently confirmed by EBV nucleic acid hybridisation studies on NPC biopsy material and by demonstrating that EBV DNA was present in the NPC tumour cells

(Liebowitz, 1994). Southern blot analysis has shown EBV DNA in NPC is clonal, arising from a single EBV-infected cell. Studies involving immunohistochemistry, western immunoblotting and RT-PCR have shown that EBNA-1 is expressed in nearly all cases examined and that LMP-1 is expressed in approximately 2/3 of cases of EBV positive NPC. LMP-1 (EBV latent membrane protein-1) has profound growth stimulatory effects *in vitro* and may exert similar effects in the nasopharyngeal epithelium. As cells are stimulated to divide, the presence of EBNA-1 ensures the viral genome will replicate and be distributed to progeny cells. LMP-2A and 2B mRNAs are also expressed, with LMP-2A being detected in most cases examined (Brooks, *et al*, 1992). EBNAs other than EBNA-1 have not been detected. They may not be important in EBV-mediated epithelial cell functions. Alternatively, the other EBNAs may be expressed in epithelial cells before their malignant transformation and may be downregulated after this event occurs. Further analysis of EBV gene expression in normal epithelial cells and NPC cells may help resolve this issue. A major problem which has hampered progress towards understanding the relationship between EBV and NPC has been the inability to culture cells from explanted tumours which are of epithelial origin. In contrast with the association of an activated *c-myc* gene and BL, no cellular oncogene involvement with NPC has as yet been identified (Liebowitz, 1994).

EBV contribution to BL and NPC

It might be expected that a contribution of EBV to BL and NPC would be explained by the combination of some of the viral immortalisation genes with oncogenic cell mutations, causing malignant transformation. In fact, most of these genes are not expressed in the tumour cells and the BL cell phenotype (as revealed by B cell surface markers) (Rowe, *et al*, 1987) is quite different from the LCL (lymphoblastoid cell line) cells where the virus is clearly causing the growth transformation. The pattern of only EBNA-1 protein expression (Rowe, *et al*, 1987, Fahraeus, *et al*, 1988) is the minimum necessary to maintain the virus replication in the face of cytotoxic T cell-mediated immune surveillance, directed against epitopes from other immortalisation genes. This is not consistent, however, with complementation of oncogenic cell changes by EBV since no cell gene or process has yet been found to be transregulated by EBNA-1. Perhaps early

in the development of the BL more of the immortalising genes might have been expressed. The tumour cells from EBV-positive BLs always retain the episomal EBV genome, suggesting that it is contributing to cell growth (Vousden, *et al*, 1994).

EBV and other lymphoproliferative neoplasia.

EBV is linked not only to BL and NPC but also to several lymphoproliferative neoplasias in patients with innate or acquired immunodeficiency. A lack of host-effective immunological control mechanisms of EBV replication in primary or persistent infection is considered crucial to the development of these diseases.

In X-linked lymphoproliferative syndrome (Duncans syndrome) in males, a primary EBV infection is followed by a rapidly developing lethal disorder of immunoglobulin production or by a fatally progressing non-Hodgkins lymphoma (Mayer, *et al*, 1994). In recipients of organ transplant who undergo immunosuppressive therapy, most lymphoproliferations have a non-Hodgkins character, characterised by Type III latency where all EBV latent genes are expressed. The tumours proliferate unchecked due to the absence of adequate T cell tumour suppressors. The tumours may be monoclonal or polyclonal. The incidence of EBV-positive lymphoma is approximately 2% after renal transplant, 5% after cardiac transplant, and 1% or less for those receiving bone marrow (Joske, *et al*, 1993).

EBV has also been associated with Hodgkins disease, Type II latency being detected in Reed-Sternberg cells (characterised by the expression of EBER RNAs, EBNA-1, LMP-1 and the absence of EBNA-2) (Joske, *et al*, 1993). Lymphomas are some 60 times more common in AIDS patients than in the general US population (Joske, *et al*, 1993).

The EBV-associated lymphomas found in AIDS patients can be characterised as follows : B cell immunoblastic lymphomas resembling post-transplant lymphomas, Burkitt-like lymphoma, primary central nervous system lymphoma, and Hodgkins lymphoma (Joske, *et al*, 1993).

The patterns of EBV latent gene expression in all of the disorders have the following similarities :

(a) EBERs are expressed

(b) EBNA-1 is expressed

(c) All disorders may also arise without EBV

(d) EBV is present as a mono- or oligo-clonal population (the EBV population has the same number of terminal repeats) i.e. either the virus was present in the cell when malignant conversion occurred or it imposed a malignant phenotype on the cell at a critical phase of differentiation.

1.5 Cell tropism

The host range for EBV is limited to humans and closely related primates and it has a narrow cell tropism, being restricted primarily to B lymphocytes and epithelial cells. Type 2 complement receptors CD21 (CR2, C3dR) function as the main receptor for EBV on B lymphocytes and are carried on up to 10% of all B cells (Strauss, *et al*, 1993). CD21 is a membrane protein of 140 kDa. It is composed of three regions : a 34 amino acid cytoplasmic region, a 24 amino acid transmembrane domain and 15 to 16 extracellular short repeat sequences (short consensus repeats - SCRs). The expression of CD21 diminishes during the differentiation of B cells - the ability of EBV to infect the B cell correlates to the level of expression of its receptor (Nemerow, *et al*, 1990). The major outer envelope glycoprotein is the EBV ligand for CD21. Upon infection, EBV can growth-transform and immortalise human peripheral blood B cells establishing a latent infection. "Latency" is the hallmark of herpesvirus infections and represents the continued presence of virus in infected cells as autonomously replicating episomes without the production of virus particles.

In latent infection, virus penetrates the cell and remains present either as circular episomal DNA (formed through homologous recombination of the terminal repeat (TR) regions) or, less frequently, as linear DNA integrated into the host genome. Episomes, present in low copy number in the host cell nucleus, are copied by host cell DNA polymerase and passed to daughter cells in mitosis. This provides the basis for identification of monoclonal EBV in a cell population as each circularisation event results in a unique number of repeats in the TR

region. Southern blot or PCR analysis of the length of a fused TR region, therefore, can be used to determine clonality of cells derived from a single progenitor (Liebowitz, 1994).

Unlike other agents of B-cell activation, EBV does not induce the cells to terminally differentiate; rather, the infected cells maintain a blastoid state and proliferate permanently (cell immortalisation). These cell lines are termed lymphoblastoid cell lines (LCLs) and express, as a marker, IgM on their surface. LCLs are considered to be immortalised but non-malignant cells as they do not form colonies in soft agar or tumours in nude mice (Mayer, *et al*, 1994). EBV-infected B lymphocytes express a number of B cell activation molecules, including CD23, CD30, CD39, CD40, CD44, and cellular adhesion molecules such as ICAM-1, LFA-1 and LFA-3 (Wang, *et al*, 1990). Infection of B-lymphocytes with EBV is in fact the standard means today of establishing human cell lines from specific donors. There are four main consequences of EBV infection *in vivo*:
(a) persistent antibody titres to viral antigens,
(b) intermittent virus production from the oropharynx,
(c) establishment of viral latency in circulating B lymphocytes, and
(d) T-cell dependent cellular immunity directed to virus-infected B-lymphocytes.
Virus-induced cell-growth transformation appears to be achieved through the action of a limited set of viral proteins, the "latent" EBV gene products.

1.6 Epithelial cell infection

Epithelial cell infection is a critical event in the development of NPC and oral hairy leukoplakia of the tongue, a second EBV-associated epithelial cell proliferation which is seen in immunocompromised patients. Only some primary epithelial cells express CD21, few have been successfully infected with EBV and none has survived the infection. A recent study has provided evidence that antibody to EBV assists entry into certain epithelial cells (Sugden, 1992). Both NPC and oral hairy leukoplakia patients express anti-EBV IgA-type antibodies which are of predictive value in diagnosis.

Most people infected with EBV mount a vigorous humoral immune response to the viral antigens which does not include IgA-type antibodies (Sixbey, *et al*, 1992). Some polarised epithelial cells display receptors for polymeric IgA (pIgA). PIgA can bind this receptor, enter the cell, be modified and released from the opposite face of the cell (fig. 2).

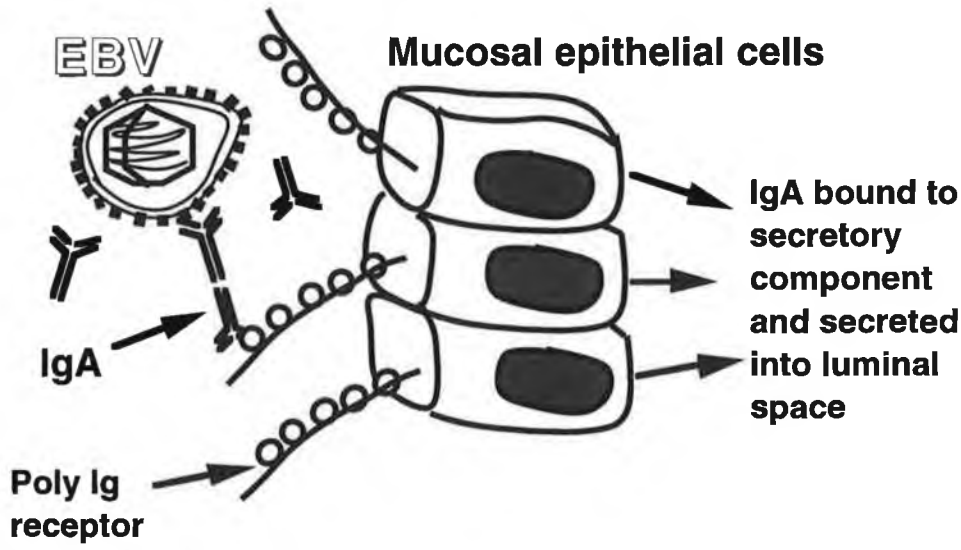


Figure 2. Antibody-assisted entry of EBV.

In cell culture, EBV binding by specific anti-EBV pIgA molecules has been shown to mediate entry of EBV into an established human epithelial cell line that expresses the receptor for pIgA. This is a worrying prospect considering that potential vaccines may induce an IgA response to EBV antigens.

Study of EBV because of the nature of latent infection without ways to produce large quantities of virus particles, and because its target cell is hard to culture, has relied largely on the use of recombinant DNA technology - including cloning and sequencing the genome.

1.7 Molecular biology of EBV

Interest in the molecular biology of EBV has primarily centred around three areas. These are (1) its ability to immortalise human B-lymphocytes in culture, (2) the mechanism of its ability to persist in infected individuals, and (3) as a relatively complex system in which to study gene regulation in human cells. An infectious centre assay for EBV does not exist however, and this has hampered the application of many methods of genetic analysis to this virus.

The EBV genome is a double stranded DNA molecule of approx. 170 kb that encodes over 80 proteins (Speck, 1987) and exists as a linear molecule in the viral capsid. Upon infection, EBV circularises and resides largely as a free episome in the nucleus of infected cells (Speck, 1987). The viral genome is composed of both unique and repetitive sequences. At either end of the genome there are variable numbers of direct terminal repeats involved in virus circularisation. The major internal repeat IR1 towards the left end of the genome is composed of different numbers (strain dependent) of 3.2 kb repeats, with each repeat sequence containing one Bam HI site. Between the repeats lie the unique long (UL) and unique short (US) regions (fig. 3).

Tandemly repeated sequence elements occur within major open reading frames - reflected in many cases as repeated amino acid patterns e.g. a variable number of a 700 bp repeat in EBNA-1 gives rise to the antigenic repeats (Gly,Gly,Ala). In some cases the repeat unit may be spliced out of the mRNA.

The prototype strain of EBV, B95-8, has been completely sequenced (Baer, *et al*, 1984). This is an IM isolate able to enhance the growth of lymphocytes and express nuclear antigen. However, a deletion in B95-8 relative to most strains has been characterised at the DNA sequence level.

During latent infection of B cells in culture only 9 EBV latent proteins are made. These include the six EBV nuclear antigens (EBNAs-1,2,3A,3B,3C,LP), and three latent membrane proteins (LMP-1, LMP-2A (also called TP1) and LMP-2B (or TP2)) (Kieff, *et al*, 1990). The Epstein-Barr encoded RNAs (EBERs) are also produced during latent infection and are by far the most abundant viral RNA species (up to 10^7 copies per cell).

The EBERs are likely to play a role in rescuing infected cells from virus-induced translational arrest, an effect which is known to be interferon-induced (Bhat, *et al*, 1985).

The production of infectious virions requires the expression of many other genes that encode structural components of the virus (VCA and MA) and other proteins which are essential for viral lytic cycle replication and virion assembly. The expression of the EBV BZLF1 immediate early gene - ZEBRA (Z EBV replication activator) acts as the switch that triggers viral replication in latently infected B cells (Strauss, *et al*, 1993). This protein also transactivates expression of other immediate early genes as well as the expression of ZEBRA itself. These genes, in turn, upregulate the expression of early gene products, which include the viral DNA polymerase and thymidine kinase, important for DNA replication. Finally, the late gene products are made, including structural components of the virion such as the VCA, and gp350 which is the major envelope glycoprotein of the virus. Recent studies have led to the identification of an EBV protein that has an extensive homology with human interleukin-10 and it has been shown that both proteins share several properties (Vieira, *et al*, 1992). By inhibiting T cell growth and γ-interferon production, "Epstein-Barr viral interleukin-10" may limit host responses directed at eliminating the virus. In this regard, EBV may have captured a cellular gene that aids its survival.

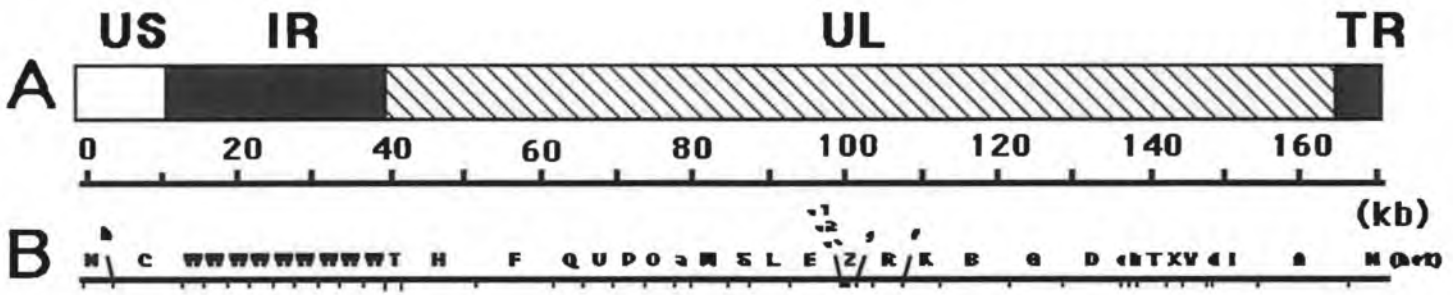


Figure 3 : Schematic representation of the EBV genome.
A : The principal regions of the genome.
US -short unique region
IR -internal repeat (5-12 copies of 3072 bp)
UL -long unique region
TR -terminal repeat (1-20 copies of 500bp)
B : The Bam HI restriction map.

1.8 Genes involved in immortalisation

Much attention has been given to this restricted set of viral proteins known as 'latent proteins' as they are essential for B cell transformation and for the control of latency itself (Sugden, 1989).

EBNA-1

The EBNAs play important roles in the modulation of viral and cellular gene expression. EBNA-1 is required both for latent replication of the EBV genome and as a regulator of viral gene transcription (Speck, 1987, Sugden, 1989). EBNA-1 is encoded in a 2kb open reading frame mapping to the left end of the viral Bam HI K fragment and contains the IR3 repeat. This gives rise to a polypeptide of about 70 kDa (varies among strains), approx. 200 residues of which are composed of an irregular co-polymer of glycine and alanine, the remainder being rich in arginine and proline (Speck, 1987).

EBNA-1 is present in all lymphoblastoid and BL cell lines examined (Speck, 1987). A cloned region of the Bam HI C fragment functions as the replication origin for maintenance of viral genomes (ori-P) in latently infected cells only when EBNA-1 is present. This function appears similar to the SV40 large T antigen (Speck, 1987). The state of latent infection is maintained by the EBNA-1 protein; it binds to ori-P allowing the viral genome to be maintained in the nucleus of the B cell (Sugden, 1989, Strauss, *et al*, 1993).

EBNA-2

EBNA-2 is encoded by a 1.5 kb open reading frame (BYRF-1) spanning the junction between Bam HI Y and H fragments. There are 2 serologically distinct forms of EBNA-2 due to divergent genomic sequences in BYRF-1 (EBNA-2A and EBNA-2B) whose antigenic properties allow typing of the virus into Type A and Type B. The naturally occuring mutant virus P3HR-1 which cannot transform cord blood lymphocytes contains a deletion spanning the EBNA-2 coding region. This deletion is responsible for the mutant phenotype of P3HR-1 (Allday, *et al*, 1994). Superinfection of the EBV positive BL cell line Raji with P3HR-1 virus results in recovery of recombinant immortalising viruses

Two of the EBV latent genes encode proteins that transactivate other viral genes. While EBNA-1 transactivates the EBNA genes, EBNA-2 transactivates the expression of 2 EBV latent membrane proteins, LMP-1 and LMP2. EBNA-2 is essential (but not sufficient) for cellular immortalisation to occur (Hammerschmidt, *et al*, 1989) and is involved in regulating the activities of both cellular and viral genes. Cellular genes whose expression is upregulated by EBNA-2 include the two B cell surface antigens CD23 and CD21 (Suter, *et al*, 1987, Wang, *et al*, 1987, Cordier, *et al*, 1990, Aman, *et al*, 1990) and also the *c-fgr* proto-oncogene (Wang, *et al*, 1990, Knutson, 1990) (the EBNA-2A protein transactivates more efficiently than EBNA-2B (Cordier, *et al*, 1990)). EBNA-2 has an acidic transactivating domain at its C terminus (amino acids 426 to 483) which is functionally similar to that of another herpesvirus acidic transactivator VP16 (Tong, *et al*, 1995). Expression of EBNA-2 in Rat-1 cells affects the serum requirements of the cells and induces increased levels of CD23 cell surface antigen expression in an EBV negative human B cell line (Wang, *et al*, 1987). CD23 is the human Fc ε receptor IIa and expression is increased by exposure to IL-4. Some antibodies to CD23/FcεRII stimulate a proliferation response in uninfected B cells (Gordon, 1989). The CD23 molecule has itself been shown to be intimately involved in a pathway of autocrine growth stimulation (the functions of this molecule are reviewed in greater detail later in this text). The secreted form of CD23 may be a B cell growth factor, but the full length molecule may be a receptor, thus providing autocrine stimulation of EBV infected B cells (Strauss, *et al*, 1993). Hence EBV can induce B cells to accumulate an IL-4 responsive surface antigen that is involved in cellular proliferation. EBNA-2 has also been shown to activate the genes which encode the latent membrane proteins (Wang, *et al*, 1990, Zimber-Strobl, *et al*, 1991). The expression of a reporter gene under the control of the HIV LTR (long terminal repeat) has also been shown to be increased by EBNA-2 (Scala, *et al*, 1993). Transfection of an EBV-negative cell line with a plasmid encoding EBNA-2 confers resistance to the anti-proliferative effect of α-interferon (IFN-α) (Aman, *et al*, 1990).

EBNA-3A/3B/3C.

EBNA-3A and EBNA-3B are encoded by genes which have been mapped to the Bam HI-E region of the EBV genome. The EBNA3 transcripts have been shown to be rightward in direction, with each containing short and long open reading frames joined together by splicing to form the long open reading frames encoding each protein (Kerdiles, *et al*, 1990). These proteins are similar in structure, sequence and size. They are composed respectively of 944, 938 and 992 amino acids, with molecular weights from 145 kDa, to 165 kDa and are rich in proline residues. Antigenic variations of the EBNA-3 proteins between viral strains are not as marked as for EBNA-2 (comparison of sequences of each of the EBNA3 proteins from strains B95-8 and AG876 show homologies from 72% to 90%). These variations are probably not important for immortalisation or for cell growth (Sample, *et al*, 1990).

No precise functions have as yet been described for EBNA-3A/B/C. Structural similarities of these proteins suggest that they may have one or more identical functions. Stable transfection of EBNA-3C into the EBV positive cell line Raji (where EBNA-3C is not functional) results in increased expression of LMP-1 and CD21 (Allday, *et al*, 1994, Wang, *et al*, 1990). Recently it has been shown that the stable expression of EBNA-3B in a cell line derived from the EBV negative B cell line DG75 stimulates the expression of the B cell activation marker CD40 and represses the expression of the differentiation antigen CD77/BLA (Silins, *et al*, 1994). Since EBNA-3 proteins exhibit DNA-binding capability in crude protein extracts from EBV-transformed cell lines, and EBNA-3C contains sequences homologous to a basic leucine zipper motif found in one class of mammalian transcription factors, it is likely EBNA-3C functions as a transcription activator (Sample, *et al*, 1994). The EBNA-3 proteins have recently been shown to repress the EBNA-2 mediated transactivation of viral and cellular genes *in vitro* (Le Roux, *et al*, 1994). Hence this group of proteins is implicated in the regulation of gene expression of EBV-infected B lymphocytes. Construction of EBV recombinants has shown the proteins do not have the same importance in the immortalisation process : EBNA-3A and EBNA-3C are necessary, while EBNA-3B can be deleted without any effect to the immortalisation power of the virus (Tomkinson, *et al*, 1993).

LMP-1

The EBV latent membrane proteins appear to play an important role in cellular transformation. LMP-1 is encoded by the open reading frame BNLF1. It is composed of 386 amino acids and its molecular weight varies from 58 and 63 kDa depending on the viral strain. It has been shown to act as a direct oncogene in transformation assays (Baichail, *et al*, 1989). It is also a potent stimulator of phenotypic changes in B lymphocytes (Wang, *et al*, 1990, Sugden, 1992). These latter effects include stimulation of the expression of several leukocyte intercellular adhesion molecules (LFA-1, LFA-3 and ICAM-1) and the transferrin receptor, all of which are known to contribute to B cell growth regulation (Peng, *et al*, 1993). Expression of LMP-1 in epithelial cells transforms them morphologically, and in B lymphoma cells LMP-1 prevents programmed cell death or apoptosis. Genetic analyses using viral mutants indicate that EBNA-2 and LMP-1 are essential for transformation.

By associating with the intermediate filament protein vimentin, LMP-1 is likely to be involved in calcium channelling, an activity which leads to the transduction of signals with relevance to cellular proliferation (Hammerschmidt, *et al*, 1989). Recent evidence suggests that LMP-1 promotes the survival of its host B cell by a mechanism which allows it to gain direct access to the long-lived memory B-cell pool.

LMP-2A and LMP-2B

LMP-2A and LMP-2B are likely to interact with the LMP-1 as all three proteins are known to co-localise in the lymphocyte plasma membrane (Longnecker, *et al*, 1990). They are composed of 9 exons - one from the BamHIA fragment of the genome, and 8 from the Bam HI-Nhet fragment. The genome must be circular for expression of these proteins as some of the exons are in part located in the region containing the TR. Evidence suggests an important role for LMP-2A/2B in EBV-related malignancies : the LMP-2A gene has been shown to be expressed in uncultured Burkitts lymphoma cells (Parkin, *et al*, 1984) and LMP transcripts have also been recently described in a majority of NPC specimens (Busson, *et al*, 1992).

Thus transformation of B cells is a complex process that involves the function of several EBV gene products to maintain the viral genome in the cell, transactivate viral and cellular gene products important for B cell growth, and prevent apoptosis.

1.9 Viral transcription in latent infection

Producer cell lines e.g. B95-8 have almost the entire viral genome represented at some level in cytoplasmic poly A+ mRNA, whereas non-producer strains e.g. Raji, show only 5% of the genome represented as polysomal mRNA. In either case the most abundant transcripts are 2 small RNA Pol-III products encoded at the left end of the Bam-HI C fragment (Epstein-Barr encoded RNAs - EBERs). These have been located in the nuclei of infected cells by in situ hybridisation (Speck, 1987). The transcription of all EBV genes except EBERs is mediated by the host cellular RNA polymerase II with most EBV promoters functioning *in vitro* with an RNA polymerase II system and displaying no unusual functions.

All of the viral messages except the transcripts encoding the two forms of the latent membrane protein LMP-2A/B are transcribed from left to right (Speck, 1987). Transcription of LMP-1 is driven by a specific promoter (Fennewald, *et al*, 1984) located in a 3 kb region at the extreme right of the viral genome. This promoter is bidirectional, also driving transcription of LMP-2A/B (Laux, *et al*, 1989). The circularisation of the viral genome is necessary for the synthesis of the LMP-2A/B proteins, as they contain exons situated in the TR region.

The EBNAs are all translated from a group of related mRNAs which are formed by the alternate splicing and 3' processing of a primary transcript that is >100 kb long. These mRNAs all share long 5' leaders that are assembled by multiple splicing events and joined to 3' coding regions that are unique to each EBNA. The leader sequences are composed of multiple repeating units of two small exons which originate from within the viral long internal repeat sequence (IR1) (Walls, *et al,* 1991).

Transcription of the EBNAs is directed by two promoters : Wp (BW-R1) (Sample, *et al*, 1986) and Cp (BC-R2) (Bodescot, *et al*, 1987). Wp is located in the IR1 region of the genome - hence there are multiple copies of this promoter. It is more active than Cp - activity is stimulated

by binding of EBNA-1 to OriP (Sugden, *et al*, 1989). Immediately after infection, Wp directs synthesis of the EBNAs, followed by a switch to Cp in favour of Wp under the influence of EBNA-2 (Woisetschlaeger, *et al*, 1989).

In the case of Type I or II latency where EBNA-1 is the only protein expressed, transcription is driven by a different promoter : Fp, which is situated in the BamHI-F region of the genome (Sample, *et al*, 1991). In this case EBNA-1 has a negative regulatory effect on the activity of this promoter (Sample, *et al*, 1992).

1.10 Immunological considerations

Normal or malignant B lymphocytes which have not fully differentiated to plasma cells are susceptible to virus penetration, adsorption and establishment of latency. The population of infected B cells produces an EBV specified surface antigen LMA (latent membrane antigen) which is recognised by the cellular immune system. Immediately after primary infection, EBV-carrying B cells multiply rapidly. The initial advantage of host cell expansion may become a disadvantage to the virus as the processed derivatives of some EBV latent proteins have been shown to serve as targets for cytotoxic T cells (CTLs). All healthy EBV-carrying individuals are known to possess potent virus-specific CTLs which will control the expanding virus-infected B cell population (Rickinson, 1990, Tosato, 1987).

Activated immunoblasts may also respond to non-immune feedback controls that protect the host from over-expansion of stimulated B cell clones (Klein, 1989). Immune suppressed individuals will occasionally be unable to contain the population of infected B cells and will go on to develop lymphomas. It seems likely the reactivation of latent virus is related to the development of some of the EBV-associated lymphoproliferative disorders seen in AIDS patients.

It has now come to light that such T cell responses are rarely if ever directed against epitopes derived from EBNA-1, but are preferentially directed against the other latent proteins (Murray, *et al*, 1990). The restricted expression of EBV latent genes together with the associated eclipsing of cellular adhesion molecules enables such virus-infected cells

to evade T-cell immunosurveillance. However, in B-cell lymphomas of immunosuppressed patients, the tumour cells express a full set of EBV latent proteins as well as the cellular adhesion molecules that bind T cells.

These cells probably grow in an uncontrolled fashion because the immune system is defective. In BL, however, a host-cell-dependent difference in viral gene regulation means that EBNA-1 is the only latent protein to be expressed due to a difference in the state of viral DNA methylation (Cedar, 1988). EBNA-1 is the only protein which is expressed independently of host cell phenotype, presumably due to it's essential role in replicating the genome. It now seems that EBV is maintained in a type of small resting B cell that only expresses EBNA-1 as the sole viral protein and does not express the cellular molecules needed for interactions with cytotoxic cells.

1.11 Avoiding apoptosis

Protection from apoptosis is conferred through expression of a single EBV latent protein LMP-1 by up-regulating expression of the cellular oncogene *bcl-2* (Henderson, *et al*, 1991). *Bcl-2* is a strong repressor of programmed cell death in lymphocytes. Its dominant role as an apoptosis regulating gene is reflected in the wide distribution of expression of *bcl-2* in different cellular systems as well as different species (Delia, *et al*, 1992, Garcia, *et al*, 1992). The *bcl-2* proto-oncogene is a highly expressed gene located on chromosome 18 next to the breakpoint of the t(14;18) translocation of follicular lymphomas (Finke, *et al*, 1994). Although not tumorigenic by itself, *bcl-2* collaborates with other oncogenes such as *myc* by preventing apoptosis (Fanidi, *et al*, 1992).

Recent evidence suggests that persistence is based on the long term survival of individual B cells within the recirculating memory B cell pool (Yao, *et al*, 1989). However, entry into memory is normally restricted to a very small proportion of the total numbers of B cells generated *in vivo* and depends on positive selection in germinal centres, where all B cells are programmed to die by apoptosis unless rescued into the long lived pool by virtue of high affinity antigen binding (Liu, *et al*, 1989). The majority of germinal centre B cells are *bcl-2*-negative and recent evidence

suggests that memory cell selection involves upregulation of *bcl-2* (Pezzella, *et al*, 1990). Hence virus-infected B cells have the potential to bypass physiological selection *in vivo* and gain direct access to the long lived memory B cell pool by *bcl-2* upregulation by the expression of LMP-1 (Henderson, *et al*, 1991).

EBV-immortalised cells undergo several phenotypic changes that facilitate immortalisation. One important change is the establishment of an autocrine loop where the EBV infected B cells produce growth factors that stimulate their proliferation (Strauss, *et al*, 1993). Early in the course of acute IM, activated cytotoxic T cells are found in the peripheral blood. A minority of virus-carrying cells may avoid immune restraints by downregulation of EBNA-2 and associated eclipse of the activation markers. BL cells expressing only CALLA, BLA and EBNA-1 but not EBNAs 3A/3B/3C/LP or any of the immunoblast markers represent the neoplastic counterpart of latently infected normal B cells with a corresponding phenotype. As long as they remain in G_O such cells would escape rejection by the immune system. The small heavy lymphocytes of normal healthy individuals that can grow directly into cell lines are consistent with the expected phenotype. The LCLs represent the most likely counterpart of the EBV-transformed B blasts that proliferate in infectious mononucleosis, whereas BL cells are more like resting cells although they are not in G_O. LCLs are also more sensitive than BL cells to CD8+CTLs generated by repeated stimulation with autologous EBV-transformed LCLs. Such T cells kill LCLs in an MHC class I restricted fashion. BL cells derived from the same donor are usually resistant as long as they maintain the BL phenotype, but tend to become sensitive as they change to a more immunoblastic LCL-like cell (Khanna, *et al*, 1995).

Three phenotypic traits are associated with the resistance of BL cells to CTLs. First BL cells do not express the potentially immunogenic EBNA 3A/3B/3C/LP and LMP products. Second, both EBV-carrying and EBV-negative BL cells downregulate certain HLA class I polymorphic determinants relative to the corresponding LCLs and hence are less sensitive to ordinary allospecific CTLs directed against MHC class I antigens. Thirdly, BL cells express certain cell adhesion molecules at a lower level than the corresponding LCLs. The consistence of the downregulation of the 3 traits mentioned makes it likely that they

represent phenotypic vestiges of the normal precursor cell. The cell phenotype-dependent downregulation of EBV in BL cells would then reflect the adaptation of the virus to persist in a latent immunologically non-recognisable form in a normal cell that tends to escape immune rejection for other reasons (Khanna, *et al*, 1995).

Previously BL, LCL and NPC cells were believed to express the same set of virally encoded "growth transformation associated proteins". Recent studies have shown differential gene expression in the three cell types (Klein, 1989). EBNA-1 is the only protein which is expressed independently of host cell phenotype, presumably due to it's essential role in replicating the viral genome. EBNA-2 is expressed in LCL cells but not in BL or NPC. Downregulation of gene expression is associated with DNA methylation. There are a small minority of BL EBNA-2 defective viruses but most carry intact EBNA-2 genes that are not expressed. Evidence suggests downregulation of EBNA 3A,3B,3C and LP similarly to EBNA-2 in BL and probably in NPC as well. LMP is expressed in LCL and downregulated in type 1 BL like EBNA-2. NPC biopsies express LMP in about 50% of tumours (Liebowitz, 1994).

1.12 Where is the reservoir of EBV ?

Following primary infection the virus persists in the healthy host for life. Since virus replication destroys epithelial cells, and yet virus excretion continues for years, EBV infection of cells in the oropharynx would have to be low grade, coursing asynchronously from cell to cell with time for uninfected epithelial cells to regenerate and differentiate. The detection of EBV genomes in oropharyngeal and salivary epithelial cells has supported this view. However, most recent studies favour the concept of virus carriage in the lymphoid compartment via the long term survival of non-productively infected B cells (Gratama, *et al*, 1988, Yao, *et al*, 1989). New findings with bone marrow transplant recipients support this view. Bone marrow transplantation has been shown to eradicate the resident EBV population if the marrow donor is seronegative. If the donor is seropositive, the donors virus can replace that of the recipient. The eradication of the resident virus suggests the latent virus is harboured in a tissue compartment destroyed by the treatment and replaced by the graft, indicating that the viral reservoir responsible for lifelong EBV

persistence is the hemopoietic compartment (Klein, 1989). Evidence suggests that long lived resting B cells in G_0 may carry at least part of the latent viral genomes in healthy EBV seropositives (Lewin, *et al*, 1990).

1.13 Vaccines and therapy

The magnitude and variety of clinical problems posed by EBV-linked neoplasia justify the efforts aimed at developing an effective vaccine. At present there is no EBV vaccine available for use in humans. Because a live EBV vaccine, however attenuated, could retain it's oncogenic potential, research is being concentrated on a subunit vaccine or an inactivated virus vaccine. The goal has been to develop vaccines that confer high levels of specific virus-neutralising protective antibodies and thereby prevent EBV from attaching to susceptible cells. Components of the EBV MA are known to have potential as candidate immunogens (Rickinson, 1990). Research has concentrated on use of the EBV gp350 envelope glycoprotein, which binds to the known virus receptor on B cells. Gp350 elicits a cellular immune response and CD4-positive T cell clones from infected humans recognise epitopes on gp350. The best results so far have been achieved in a primate model using purified gp350 incorporated into immunostimulatory complexes and administered with an immunoadjuvant. After EBV challenge, a protection against the development of lymphomas was observed in vaccinated animals (Morgan, *et al*, 1988). Despite the progress made in this area there remain several obstacles to vaccine production. Concern exists that the vaccine-induced immunity would wane and merely delay the age of primary infection, hence making infection worse, or that the vaccine would not protect against infection by a natural route. Serum from humans infected with EBV contains antibodies to gp350 antigens. In addition most people have salivary IgA antibodies to gp350, suggesting such antibodies are unlikely to be the basis of long-term immunity in healthy seropositive individuals. It seems unlikely that a vaccine based solely on gp350 will be completely effective in preventing infection; however it may have the potential to significantly reduce the load of infectious virus and thus limit the long-lived EBV-positive B cell pool (Khanna, *et al*, 1995). There is still a long way to go before routine vaccination becomes a way to guard against EBV infections.

Reactivation of EBV after bone marrow transplantation leads in many cases to lymphoproliferative disease that responds poorly to standard therapy and is usually fatal. To prevent or control this complication, recently EBV-specific cytotoxic T lymphocyte (CTL) lines have been prepared from donor leukocytes and infused into allograft recipients (Rooney, *et al*, 1995). In the three patients with EBV reactivation, EBV DNA concentrations (measured by semi-quantitative PCR), which had increased 1000-fold or more, returned to the control range within 3-4 weeks of immunotherapy. It appears that EBV-specific donor-type T cell lines may offer a safe and effective therapy for the control of EBV-associated lymphoproliferation.

1.14 Regulation of CD23 gene expression by EBV.

The B cell activation marker CD23 has been closely associated with EBV infection, being highly induced in EBV infected B lymphocytes. However, it is also expressed after B cell activation by antigen, mitogen, or IL-4 stimulation. CD23 is closely associated with an EBV immortalising function, since infection with the EBV non-transforming virus P3HR-1 does not induce CD23, and only EBV infected B lymphocytes which express CD23 are capable of establishing immortalised cell lines. EBNA-2 is essential for EBV-induced growth transformation (Cohen, *et al*, 1989) and can specifically upregulate CD23 gene expression (Wang, *et al*, 1987). CD23 induction may therefore be an important pathway for EBNA-2 induction and EBV infection.

CD23 is synthesised as a 45 kDa precursor and is expressed on the surface of B cells early after activation (Thorley-Lawson, *et al*, 1985), before being shed into the culture medium in a 25 to 33 kDa soluble form (sCD23). Several functions have been described for sCD23 including the control of IgE synthesis and cytokine-like properties such as fever induction, migration inhibiting factor activity, B cell growth and differentiation and an accessory role in T cell proliferation (Gordon, 1991). Higher levels of sCD23 have also been seen in HIV-infected patients as compared to healthy individuals (Hober, *et al*, 1993).

CD23 has been shown to be the low affinity receptor for the Fc fragment of immunoglobulin E. CD23 (FcεRII) is found on the surface of a

variety of haematopoietic cells including lymphocytes, monocytes, eosinophils, and platelets (Gonzales-Molina, *et al*, 1977, Delespesse, *et al*, 1986, Spiegelberg, *et al*, 1980, Capron, *et al*, 1981, Joseph, *et al*, 1986). The molecule was first discovered when Spiegelberg and colleagues showed low affinity binding of IgE to human B lymphocytes. With an association constant of $10^7\ M^{-1}$, the receptor responsible was clearly distinct from the high affinity IgE receptor of mast cells and basophils. The 45 kDa receptor is a glycoprotein without a signal sequence (Suter, *et al*, 1987). The extracellular portion of FcεRII is cleaved proteolytically (Nakajima, *et al*, 1987) and fragments are released which retain their ability to bind IgE.

CD23 - expressed only weakly on a minority of freshly isolated, resting B cells from peripheral blood or tonsil - is both dramatically and rapidly upregulated on the B cell surface following activation by stimuli such as EBV, phorbol esters and interleukin 4 (IL-4). IL-4 can induce the expression of low affinity receptors for IgE (CD23) and class II MHC antigen on resting B lymphocytes (Defrance, *et al*, 1987, Rousset, *et al*, 1988). Stimulation of B cells with insolubilised anti-IgM antibody increases the number of cells expressing CD23 upon culturing with IL-4 and enhances the level of CD23 expression on these cells. CD23 induction is specific for IL-4 since IL-1α, IL-2, IFN-γ, B cell-derived B cell growth factor (BCGF), and a low molecular weight BCGF were ineffective. None of these lymphokines acted synergistically with IL-4. IFN-γ strongly inhibited the induction of CD23 and class II MHC antigen (Defrance, *et al*, 1987, Rousset, *et al*, 1988). Both constitutive and induced CD23 expression is a feature restricted to primary B cells bearing IgM/IgD. All EBV-immortalised B cell lines are CD23-positive.

On appropriate activation into cycle or following transformation by EBV, B cells release soluble cleavage products of the 45 kDa membrane molecule that contain autocrine growth-promoting activity. In addition, $CD23^-$ cells, normally poor responders to polyclonal activators, become responsive in the presence of soluble CD23 preparations particularly when co-cultured with fixed or irradiated $CD23^+$ cells. The growth-promoting actions of IL-4 also relate closely to the release of soluble CD23 in activated B cell cultures. Antigen-specific B-cell blasts generated by immunisation with tetanus toxoid were found to elicit enhanced IgE

antibody production *in vitro* in the presence of medium conditioned by EBV-transformed lymphoblastoid cells. The activity responsible could be removed and neutralised specifically by CD23 antibodies. This data suggests that CD23 is involved in general B cell regulatory pathways.

Sarfati and colleagues were the first to isolate an IgE-binding factor equivalent to soluble CD23 from the supernatants of EBV-immortalised B cell lines and they showed that it could modulate IgE production in primary B cell cultures. An antibody to CD23 was found to ablate IL-4 promoted IgE synthesis from peripheral blood mononuclear cells. IgE reacts with and influences the behaviour of membrane-bound CD23, upregulating the receptor due to stabilisation by prevention of cleavage (Suter, *et al*, 1989).

Two species of FcεRII ($FcεRII_a$/$FcεRII_b$) have been identified. They differ by six amino acids at the cytoplasmic N-terminus. $FcεRII_a$ is expressed in normal B cells and in certain B cell lines, whereas $FcεRII_b$ is detectable in several leukocytic cell types such as T cells, eosinophils and monocytes. Although $FcεRII_b$ is not found on normal B lymphocytes and monocytes, it can be induced by IL-4. IL-4 causes a two- to four-fold upregulation of the $FcεRII_a$ promoter activity (Suter, *et al*, 1989). The DNA element responsive to IL-4 maps to the first 250 bp of the 5' flanking region.

Infection of B cells with EBV *in vitro* results in their polyclonal activation and immortalisation into lymphoblastoid cell lines. Although all mature B lymphocytes will bind and internalise virus particles, only a small percentage of cells actually become activated and/or immortalised after infection (Tosato, 1987). The CD23 antigen has been used to separate immortalised and non-immortalised cells after EBV infection (Azim, *et al*, 1988). The vast majority of cells expressing cytoplasmic immunoglobulin are negative for CD23 and for EBNA, and are non-immortalised. Conversely, the CD23 positive, immortalised population are positive for EBNA and negative for cytoplasmic immunoglobulin. This indicates a diversity in the response of B lymphocytes to EBV infection and suggests separate pathways for terminal differentiation and immortalisation.

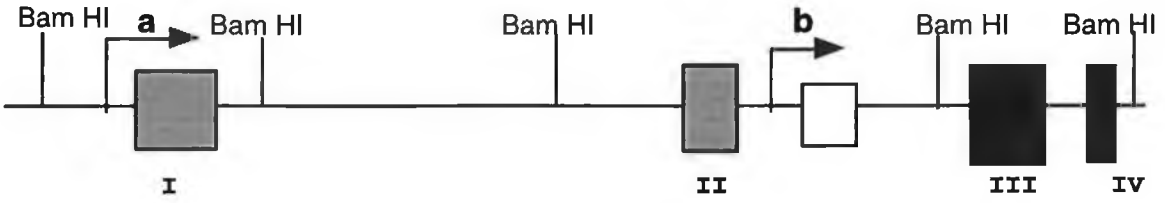
CR2(CD21) and CD23 (FcεRII) are normal B cell molecules expressed

early during B cell activation and are implicated in the regulation of cell cycle. They are also regularly expressed on LCL cells. This supports the suggestion that EBV might activate the normal B cell proliferation pathway and contribute to the establishment of an autocrine secretion loop of B cell growth factors during B cell immortalisation. A set of B cell activation molecules, including the EBV receptor CR2 (CD21) and the B cell activation antigen CD23, is turned on by infecting EBV-negative B lymphoma cell lines with immortalising strains of the virus B95-8. The P3HR1 non-immortalising strain of the virus, which is deleted for the entire EBNA-2 protein open reading frame, is incapable of inducing the expression of CR2 and CD23, suggesting a crucial role for EBNA-2 in activating these molecules (Cordier, *et al*, 1990).

B lymphocytes require co-operation with other cell lineages in order to initiate, augment, or maintain their activation to clonal expansion and expression of their effector function. Complement components, interleukin-1 (IL-1) and T cell derived factors represent soluble mediators which contribute to these processes (Gordon, *et al*, 1986). CD23, one of the first lineage-restricted antigens to appear on the activation of human B cells, conveys growth information which is identical to that elicited by a T cell derived, low molecular weight BCGF (B cell growth factor).

CD23 is involved in triggering the progression of activated B lymphocytes through the G1 phase of the cell cycle (Gordon, *et al*, 1986). It has been suggested that CD23 might have a dual role in B cell proliferation : the surface molecule might act as a growth factor receptor, and the processed molecule itself acts as a growth factor. Activation of CD23 expression could be implicated in the establishment of an autocrine loop of growth factors. EBV-transformed B cells expressing FcεRII release IgE-BFs into culture supernatants, which enhanced IgE synthesis, suppressed IgG synthesis but had no significant effect on IgA and IgM synthesis (Sarfati, *et al*, 1984).

The human CD23 gene promoter region:



The 4.5 kb genomic fragment encoding the 5' portion of the CD23 gene is shown with the type a and type b promoters (↱),the unique type a first and second exons (▯), the unique type b first exon (□), and the third and fourth exons which are common to both mRNA types (▮). Bam HI sites are indicated.

(Suter, *et al*, 1987, Yokota, *et al*, 1988).

Organisation of the human CD23 gene promoter region is complex. Transcription occurs from 2 independent promoters giving rise, through alternative splicing, to three RNA species which are translated in proteins called CD23 type a and type b, diverging in their intracytoplasmic domains (Cordier-Bussat, *et al*, 1993). Epstein-Barr virus nuclear antigen 2 (EBNA-2) is an essential gene for EBV induced B lymphocyte growth transformation (Cohen, *et al*, 1991). Single gene transformation experiments demonstrated that EBNA-2 specifically upregulates EBV latent membrane protein 1 (LMP-1), and cellular CD21, CD23 and *c-fgr* gene expression (Abbot, *et al*, 1990, Knutson, 1990, Wang, *et al*, 1987). EBNA-2 gene transactivation is an essential occurrence for EBV-induced growth transformation and immortalisation (Cohen, *et al*, 1991).

All EBV-transformed lymphocytes express high CD23 levels, and EBV immortalised B cells arise from the subpopulation of cells expressing CD23 soon after infection. Even though CD23 expression in B lymphocytes is normally downregulated after immunoglobulin isotype switch, EBV-transformed B cells which have switched to immunoglobulin G or A expression continue to express high CD23 levels (Kikutani, *et al*, 1986). B lymphoma cell infection with non-transforming EBV (P3HR-1) induces little CD23, whereas transforming EBV infection induces high CD23 levels (Calendar, *et al*, 1987). Recombination of EBNA-2 into the P3HR-1 genome results in the restoration of transforming ability and CD23 induction.

Differences in the growth phenotype of type 1 and type 2 EBV-transformed B lymphocytes correlate with differences in CD23 expression levels. As well as transforming B cells less efficiently, type 2 EBVs also induce less CD23 than type 1 after *in vitro* infection of EBV negative Burkitt's Lymphoma cells (Calendar, *et al*, 1987). These EBV type-specific effects are due specifically to EBNA-2 (Cohen, *et al*, 1989). An important role for CD23 in EBV induced growth transformation is suggested by the observation that EBNA-2 and LMP-1 act synergistically to induce CD23 (Wang, *et al*, 1990). EBV infection of B cells also interferes with corticosteroid-mediated regulation of CD23 (Paterson, *et al*, 1994).

Latent EBV infection and growth transformation of B lymphocytes is characterised by EBV nuclear and membrane protein expression (EBNA and LMP respectively). LMP1 preferentially increases FcεRIIb CD23 mRNA, while EBNA-2 expression increases FcεRIIa CD23 mRNA. However, co-expression of EBNA-2 and LMP-1 increase total CD23 mRNA without increasing the high relative abundance of FcεRIIb to FcεRIIa CD23 mRNA induced by LMP-1. Thus, LMP-1 likely activates the FcεRIIb CD23 promoter, while EBNA-2 more likely transactivates a regulatory element common to both the FcεRIIa and FcεRIIb promoters (Wang, *et al*, 1990).

EBNA-2 transactivates transcription of the CD23 promoter through a specific *cis*-acting DNA element (Wang, *et al*, 1991b). The minimal *cis*-acting CD23 DNA element delineated so far is a 186 bp -275/-89 type a CD23 promoter DNA fragment. This is contained within a type a promoter fragment (-335/+80) which is clearly EBNA-2 responsive (Wang, *et al*, 1991b). EBNA-2 type specific differences in growth transformation correlate with their effects on CD23 expression rather than their effects on LMP-1 gene expression (Wang, *et al*, 1991b). EBNA-2 transactivation of the LMP-1 and the BAM HI-C (BCR-2) promoters by type 1 versus type 2 EBV has also been seen by infection with type 1 and type 2 EBV (Calendar, *et al*, 1987), infection with recombinant EBV containing type 1 or type 2 EBNA genes (Cohen, *et al*, 1991), and stable transfection with type 1 and type 2 EBNA-2 in EBV-negative B-lymphoma cells (Wang, *et al*, 1990). In contrast, equal or stronger type 2 EBNA-2 transactivation of LMP-1 has been demonstrated by similar LMP-1 levels in B cells transformed with recombinant EBVs containing type 1 or type 2 EBNA-2 genes (Cohen, *et al*, 1991), by transfection of type 1 or type 2 EBNA-2 into B lymphoma cells

infected with EBNA-2-deleted EBV e.g. P3HR-1 (Wang, *et al*, 1990), and by EBNA-2 co-transfection with LMP-1 genomic DNA. The EBNA-2 type-specific response of the CD23 *cis*-acting element is qualitatively different to that of an LMP-1 *cis*-acting element to type 1 and type 2 EBNA-2. This suggests that EBNA-2 transactivation of cell genes rather than viral genes is the basis for more efficient transforming properties of type 1 EBV (Wang, *et al*, 1991b).

The joint interaction of EBV LMP-1 and EBNA-2 in inducing CD23 distinguishes CD23 from other B cell genes whose expression is upregulated by a single EBV protein (Wang, *et al*, 1991a), and indicates that CD23 induction may be central to EBV-induced B lymphocyte growth transformation. EBNA-2 transactivates CD23 transcription through a DNA element which is EBNA-2 responsive. In addition, EBNA-2 transactivates LMP-1 (Wang, *et al*, 1991a) which activates the type b CD23 promoter (Wang, *et al*, 1990). In LMP-1-expressing cells EBNA-2 synergises with LMP-1 to induce high level type a and type b CD23 expression (Wang, *et al*, 1990).

The CD23 *cis*-acting DNA element is similar in effects and location to an IL-4-responsive element mapped to the CD23 type a promoter region (-253/-43) (Suter, *et al*, 1989), suggesting that EBNA-2 might usurp normal growth regulatory pathways. IL-4 induces low level type b CD23 mRNA expression from normally undetectable levels in B lymphoma cells but also has a threefold effect on the type a CD23 mRNA (Yokota, *et al*, 1988). Thus IL-4 appears to have effects on CD23 expression similar to EBNA-2 rather than LMP-1, which preferentially induces type b CD23 mRNA (Wang, *et al*, 1990, Peng, *et al*, 1993). Constitutive EBNA-2 transactivation of an IL-4 responsive element may represent one signal contributing to EBV induced B cell immortalisation (Wang, *et al*, 1991b).

EBNA-2 transactivation of the CD23 promoter is dependent on interaction with a cellular DNA-binding protein, CBF-1 (Cp binding factor-1), which is essential for promoter targeting (Sauder, *et al*, 1994). This interaction was first seen in EBNA-2 transactivation of the viral BCR-2 (Cp) promoter (Ling, *et al*, 1994). A protein thought to be CBF-1 binds to a 191 bp mapped EBNA-2 responsive region located at position -85 to -277 bp upstream of the CD23 promoter. The identity of this protein was confirmed by showing that the bound complex was competed for by the

CBF-1 binding site from BCR-2, that the bound protein could be supershifted with a bacterially expressed fusion protein containing amino acids 252 to 425 of EBNA-2 but was unable to interact with a non-CBF-1-binding EBNA-2 mutant, and that in UV crosslinking experiments the BCR-2 CBF-1 binding site and the CD23 probe bound proteins of the same size (Ling, *et al*, 1994). A central core sequence, GTGGGAA, common to all known EBNA-2 responsive elements, is crucial for CBF-1 binding. Mutation of this core sequence abolishes CBF-1 binding, and the mutated sequence is unable to mediate EBNA-2 transactivation (Ling, *et al*, 1993, Yalamanchili, *et al*, 1994). Flanking sequences on either side of this core influence the affinity for CBF-1. These differences in affinity can be directly correlated with changes in the flanking sequences (Ling, *et al*, 1994). A similar core sequence has been identified as a high affinity binding site for the human recombination signal sequence binding protein RBP-Jκ. CBF-1 has been purified and found to be identical to RBP-Jκ. This protein binds to specific sequences in EBNA-2-responsive elements and recruits EBNA-2 to its cognate DNA sequences *in vitro* suggesting RBP-Jκ may mediate EBNA-2 transactivation of both cellular and viral genes (Waltzer, *et al*, 1994, Zimber-Strobl, *et al*, 1994).

A search of primate databases for the core sequence has identified more than 100 matches. It thus seems likely that CBF-1 may be involved in the regulation of the expression of a relatively large number of cellular genes, including other B cell activation antigens. The expression of these same genes could therefore be subject to modulation by EBNA-2, implying that EBNA-2 may have a much greater impact on B cell gene expression than was appreciated from the two known examples of EBNA-2 responsive cellular genes, *c-fgr* and CD23. Recent evidence has shown that other cellular factors are involved in mediating EBNA-2 transactivation - the EBNA-2 mediated transactivation of LMP-1 is dependent on the binding of at least two distinct DNA-binding proteins (RBP-Jκ and PU.1) (Johanssen, *et al*, 1995).

The regulation of CD23 gene expression is likely to be an important factor in EBV-induced B cell growth transformation. The purpose of this study was to explore the regulation of CD23 gene expression by EBV latent proteins. One approach in the definition of DNA sequences important in the regulation of gene expression is the placing of transcriptional control

elements (such as promoter/enhancer sequences) from the CD23 gene upstream of a reporter gene. This facilitates measurement of the activity of the element. Ideally, such a reporter should encode a product which is stable, absent from the cell or organism in which it is being expressed, and be readily detectable in small quantities. In the course of this study, two reporter-based assays were investigated.

(a) Use of E. coli CAT (chloramphenicol acetyltransferase) as a reporter gene.

The E. coli CAT gene, which encodes the enzyme chloramphenicol acetyl transferase has been used as a reporter gene in a variety of cultured animal cell systems. This is a bacterial drug resistance gene which inactivates chloramphenicol by acetylating the drug at one or both of it's two hydroxyl groups. This gene is not found in eukaryotes and thus there is no background activity in a normal mammalian cell. This characteristic, along with the ease and sensitivity of assay, has made CAT the most widely used reporter gene in mammalian gene expression studies. There are a number of chromogenic and radioassays that allow rapid and sensitive detection of CAT, including a simple phase extraction assay. This involves incubating the cell extracts with ^{3}H-chloramphenicol and butyryl CoA. The butyrylated chloramphenicol is separated from the unmodified form by a simple phase extraction.

(b) Use of a human placental alkaline phosphatase gene in transfected cells as a reporter.

Human placental alkaline phosphatase (PAP) can also be used as a reporter gene in transfection assays (Henthorn, *et al*, 1988), its expression being dependent on the presence of exogenous transcription control elements. The enzymatic assay measures the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol (which is a yellow compound that can be measured spectrophotometrically) plus inorganic phosphate. The assay is quantitative, rapid and inexpensive, using nonradioactive substrates and no specialised equipment. The enzyme's resistance to heat allows it's activity to be easily distinguished from other endogenous alkaline phosphatase activities in mammalian cells. Activity can also be detected in individual fixed cells by using a histochemical stain for alkaline phosphatase. Sensitivity has been shown to be comparable to that achieved using CAT as the reporter enzyme (Henthorn, *et al*, 1988).

2. Materials and Methods (Maniatis, *et al*, 1989).

2.1 Bacterial strains and plasmids:

E. coli DH5α Genotype : F^-, *end* A1, hsdR17 (r_k^-,m_k^+), *sup* E44, *thi* -1, λ-, *rec* A1, *gyr* A96, *rel* A1, ϕ80*lac* ZδM15.

Plasmid	Description	Source
pSV_2APap	Human placental alkaline phosphatase gene under the control of the SV40 early promoter.	Dr. Susan McDonnell, D.C.U.
pBCD23	Hind II - Bam HI fragment of the CD23 promoter cloned into pBlueskript SK (Stratagene).	Dr. Aude le Roux, l'Institut Gustave Roussy, Paris.
pGem7ZF	Commercial cloning vector from Promega.	Promega.
pGem3Zf	Commercial cloning vector from Promega.	Promega.
pG7CAT	CAT gene (Cla I - Bam HI) cloned into pGem7Zf	Dr. Aude le Roux.
pCMVCAT	CAT gene under the control of the viral CMV promoter.	Dr. Aude le Roux.
pCD23CAT	CD23 promoter and CAT genes cloned into pBlueskript SK.	Dr. Aude le Roux.
pCMVEBNA2	EBNA2 gene under the control of the CMV promoter.	Dr. Aude le Roux.

Plasmid	Description	Source
pCMVEBNA3A	EBNA3A gene under the control of the CMV promoter.	Dr. Aude le Roux.
pG7CD23	Bam HI-Sal I CD23 fragment from pBCD23 cloned into pGem7Zf (digested with Bam HI and Sal I).	This study.
pG3CD23	BamHI-Sal I CD23 fragment from pBCD23 cloned into pGem3Zf (digested with Bam HI and Sal I).	This study.
pCD23Pap(a)	PAP gene isolated as an Eco RI fragment from pSV$_2$APap cloned in the forward orientation into the Eco RI site of pG7CD23.	This study.
pCD23Pap(b)	PAP gene isolated as an Eco RI fragment from pSV$_2$APap cloned in the reverse orientation into the Eco RI site of pG7CD23.	This study.
pG7CAT	CAT gene cloned as a Hind III fragment into the Hind III site of pGem7Zf.	This study.

2.2 Bacterial media

All media was autoclaved at 15 lbs/in^2 for 20 minutes.

LB

10g tryptone
10g NaCl
5g yeast extract
to 1L with distilled water (final pH 7.5)
for solid media 13g/L of oxoid agar no. 3 was added.

SEB
LB +10 mM $MgCl_2$ + 10 mM $MgSO_4$.

SEC
SEB + 20 mM glucose.
pH 6.9-7.0.

2.3 Selective agents for bacterial culture

Antibiotics

For the selective growth of transformed E. coli, ampicillin was added to the media after autoclaving and cooling to 65°C. Stock solutions (25 mg/ml) were made in sterile water, stored at -20°C, and used at a working concentration of 60µg/ml.

X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside).

For the blue/white selection of recombinant plasmids containing inserts that have disrupted the LacZ gene, x-gal, a chromogenic substrate for β-galactosidase was incorporated into the media after autoclaving and cooling. Stock solutions (20 mg/ml) were made in DMF (dimethyl formamide) and stored at -20°C. A working concentration of 20µg/ml was used.

2.4 Bacterial growth and storage.

DH5α was grown at 37°C and stored short term on LB plates, or long term as a glycerol stock. Glycerol stocks were prepared as follows :
To 1 ml of an overnight 5 ml culture of bacteria containing the appropriate selective agent, 1 ml of 50% glycerol was added. After vortexing, the stocks were transferred to -80°C.

2.5 Preparation of competent cells.

This method is a modification of the method of Hanahan (J. Mol. Biol. 1983 vol 166, p557-580).

(1) From an overnight 5ml culture of DH5α, 1 ml was seeded into 100 ml of SEB medium.

(2) The culture was grown with shaking at 37°C until the O.D. at 550 nm reached 0.45-0.6.

(3) The culture was then spun for 12 min, 2,500 rpm at 4°C.

(4) The pellet was resuspended in 33 ml of FSB (Frozen Storage Buffer)

Frozen storage buffer

10 mM potassium acetate pH 7

100mM potassium chloride

45mM $MnCl_2.4H_2O$

10 mM $CaCl_2.H_2O$

10% glycerol

0.1 M HCl added to final pH 6.4

(5) The cells were then incubated on ice for 10 min, spun at 2,500 rpm for 10 min at 4°C, and resuspended in 8 ml FSB (4°C).

(6) DMSO (280 µl) was added, and the cells were incubated on ice for 10 min.

(7) Aliquots were prepared in prechilled tubes (200 µl), flash frozen in liquid N_2 and stored at -80°C.

2.6 Transformation of competent cells.

(1) Cells (from 2.5) were thawed on ice.

(2) DNA was added and mixed gently.

(3) After incubation on ice for 30 min, the cells were heat shocked for 90 secs at 42 °C.

(4) SEB was added (1 ml), mixed, and incubated at 37°C for 1 hour with agitation.

(5) The cells were then pelleted (4,000 rpm, 5 min), resuspended in 200 µl SEB and plated out on media containing the appropriate antibiotic (+/- x-gal).

2.7 Small scale preparations of plasmid DNA (miniprep)

Harvesting

(1) A single bacterial colony was transferred into 5ml of LB medium containing the appropriate antibiotic in a loosely capped 20 ml tube and incubated overnight at 37°C with vigorous shaking.

(2) The culture was poured into a microfuge tube (1.5 ml) and spun at 10,000rpm for 5 min.

(3) As much of the medium as possible was removed by aspiration.

Lysis

This is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

(1) The bacterial pellet was resuspended in 100µl of ice cold Solution I by vigorous vortexing.

Solution 1

50 mM glucose
25mM Tris.Cl pH 8
10 mM EDTA pH 8

(2) After resuspension was complete, 200 µl of freshly prepared solution 2 was added and mixed thoroughly by inversion.

Solution 2

0.2 N NaOH (freshly diluted from a 10 N stock)
1% SDS

(3) After mixing, 150 µl of ice cold solution 3 was then added and mixed.

Solution 3

5m potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

(4) The lysate was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to a fresh tube, avoiding the white precipitate.

(5) An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added. The mixture was vortexed and centrifuged for 4 min at 10,000 rpm and the upper phase was transferred to a fresh tube.

(6) The DNA was precipitated by adding 2 volumes of ethanol, vortexing, incubating for 5 min at room temperature, and centrifuging for 10 min at 10,000 rpm.
(7) The supernatant was removed by aspiration. The pellet was then washed with 500µl of 70% ethanol, dried, and resuspended in 50 µl of TE (pH 8) containing DNAse-free RNAse (20 mg/ml).

2.8 Large scale preparations of plasmid DNA (maxiprep).

Lysis by alkali.

(1) The bacterial pellet from a 500 ml culture was resuspended in 18 ml of Solution 1.
(2) A freshly prepared solution of lysosyme (10 mg/ml in 10 mM Tris.Cl pH 8) was added (2 ml).
(3) Then, 40 ml of freshly prepared Solution 2 was added, the solution mixed by inversion.
(4) After incubation at room temp for 5-10 min, 20 ml of ice cold solution 3 was added, mixed by inversion and stored on ice for 10 min.
(5) The lysate was centrifuged at 4,000 rpm for 20 min at 4^{o}C in a Sorvall GS3 rotor.
(6) The supernatant was filtered through Whatman no. 1 filter paper into a 250 ml centrifuge bottle, 0.6 volume of isopropanol was added, mixed well and stored at room temp for 10 min.
(7) DNA was precipitated by centrifugation for 20 min at 5,000 rpm.
(8) The supernatant was removed, the pellet washed with 70 % ethanol, and allowed to dry.
(9) The pellet was resuspended in TE pH 8.

Purification by CsCl gradients.

(1) For every ml of DNA solution 1g of solid CsCl was added, the solution warmed to 30^{o}C to facilitate dissolution, and mixed gently.
(2) Ethidium bromide solution (10 mg/ml in water) was added (0.8 ml for every 10 ml of DNA/Cs solution) and immediately mixed. (The final density should be 1.57 g/ml).
(3) The solution was transferred to a Beckmann quickseal tube, the remainder of the tube filled with mineral oil and sealed.

(4) Centrifugation was done in a Ti70.1 rotor at 50,000 rpm for 24 hr at 20 ^{o}C. Two bands should be visible. The upper band contains chromosomal and nicked plasmid DNA, the lower band contains the closed circular plasmid DNA.
(5) To collect the bands :
A 20-gauge needle was inserted into the top of the tube to allow air to enter. An 18-gauge needle was then inserted below the band of interest and the DNA was collected in a 2 ml sterile syringe.
(6) The ethidium bromide was removed from the DNA solution by repeated extractions with TE-saturated isobutanol.
(7) The DNA was dialysed against TE for 24 hr with several changes of buffer to remove the CsCl.

Purification by column isolation.
The plasmid DNA was alternately isolated using the Promega Wizard Maxiprep Column System.

2.9 Solutions for DNA work

0.5 M EDTA :	186.1 g added to 800 ml water. Add 6g NaOH pellets. pH to 8.0 with 5M NaOH. Adjust volume to 1L with water.
50X TAE :	242g Tris 57.1 ml acetic acid. 100 ml 0.5 m EDTA pH 8.0 to 1 L with water.
5X TBE :	54 g Tris 27.5 g Boric acid 20 ml 0.5 M EDTA pH 8.0 to 1L with water.
Ethidium bromide :	0.1 g/ 10 ml water (10 mg/ml). Protect from light.
DNAse-free RNAse :	RNAse A (1 mg/ml) in sterile water. 100^{o}C 30 min. Cool slowly, store -20^{o}C.
Agarose gel loading dye :	40% sucrose 0.25% bromophenol blue.

2.10 Storage of DNA samples

DNA samples should ideally be stored in TE buffer pH 8.0 at 4^{o}C. The use of EDTA chelates heavy metal ions that are commonly needed for DNase activity while storage at pH 8 minimises deamidation

TE buffer

10 mM Tris, 1mM EDTA pH 8.0

2.11 Equilibration of phenol

Before use, phenol was equilibrated to pH 8.0 as DNA partitions into the organic phase <pH 7.8

(1) Solid phenol was melted at 68^{o}C, and hydroxyquinoline was added to a final concentration of 0.1% (acts as an antioxidant, a chelator of metal ions, and an RNase inhibitor).

(2) An equal volume of buffer (0.5 M Tris.Cl pH 8) was added to the liquefied phenol and stirred for 15 minutes. The two phases were then allowed to equilibrate and as much as possible of the upper aqueous phase was removed.

(3) The extraction was repeated using equal volumes of 0.1 M Tris.Cl pH 8 until the pH of the phenol was > 7.8.

(4) Tris.Cl pH 8 (0.1 volume) and 0.2% β-mercaptoethanol were added to the phenol, which was the stored at -20^{o}C in the dark.

2.12 Ethanol precipitation of DNA

Concentration of nucleic acid samples or changing the buffers in which a sample is dissolved was achieved by ethanol precipitation.

(1) In general one tenth volume of 3 M sodium acetate pH 5.5 and 2 volumes of ethanol were added to a DNA sample.

(2) The sample was mixed by inversion and incubated at either -80^{o}C for 30 min or -20^{o}C overnight.

(3) The sample was then centrifuged at 10,000 rpm for 30 min, washed with 70% ethanol to remove excess salts, dried, and resuspended in suitable buffer.

Notes on precipitation :

(a) To prevent the co-precipitation of dNTPs 0.5 volumes of 7.5 M ammonium acetate can be substituted for sodium acetate.

(b) To remove SDS from a sample, the sample can be made 0.2 M in sodium chloride before the addition of ethanol.

(c) If the DNA to be precipitated is small i.e. <200bp, the sample should also be made 10mM in magnesium chloride before ethanol precipitation.

2.13 Spectrophotometric quantification of DNA

In general, for quantification of maxiprep DNA, 5µl of DNA was added to 495µl of distilled water. Absorbance readings were taken at 260 nm and 280 nm. An absorbance reading at 260 nm of 1 corresponds to approx. 50µg/ml of double stranded DNA. The ratio between the readings at 260 and 280 nm provides a measure of the purity of the DNA. A pure preparation of DNA will have a 260/280 ratio of between 1.8 and 2.0.

2.14 Agarose Gel Electrophoresis of DNA

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform, and can be used for the isolation of DNA fragments.

Agarose gels were prepared and run as follows :

(1) An appropriate quantity of agarose was boiled in 0.5X TBE buffer until dissolved and allowed to cool to 60°C before pouring.

(2) DNA samples were mixed with loading buffer and run at a constant voltage of 5V/cm (measured as the distance between the electrodes) for 1-2 hours.

(3) After electrophoresis, the gel was stained in a bath of ethidium bromide solution (0.5µg/ml) for 20 minutes, followed by destaining in water for 15 minutes. The gel was then viewed under UV illumination.

2.15 Isolation of DNA from agarose gels.

A number of methods were used to isolate DNA from agarose gels :

(A) Low melting point agarose

(1) A low melting point agarose was prepared in 1X TAE buffer (gel isolation is not carried out in TBE buffer as borate ions are difficult to remove from the resultant DNA solution).

(2) After electrophoresis, the gel was stained in an ethidium bromide bath and viewed under 70% UV illumination. The time of exposure to UV light was kept to a minimum, as overexposure to UV causes damage to the DNA.
(3) The DNA band to be isolated was excised from the gel using a scalpel and placed in a sterile microfuge tube. About 5 volumes of 20 mM Tris.Cl, 1 mM EDTA (pH 8) was added to the gel slice and incubated at 65°C for 5-10 minutes to melt the gel.
(4) After cooling to room temperature, an equal volume of phenol (pH 8) was added, the sample vortexed and centrifuged for 10 min at 4000 rpm.
(5) The aqueous phase was re-extracted once with phenol:chloroform and once with chloroform.
(6) 10 M ammonium acetate (0.2 volumes) and 2 volumes of ethanol at 4°C were added to the sample. After incubation at -20°C overnight, the DNA was recovered by centrifugation at 10,000 rpm for 20 minutes.

(B) Electroelution
(1) An agarose gel was prepared in TAE buffer. For DNA isolation a high grade agarose was used (Seakem GTG agarose). This is to minimise the chance of introduction of contaminants such as DNAses into the DNA sample.
(2) The gel slice containing the DNA band of interest was placed into a dialysis bag containing a small amount of running buffer. This was then immersed in a shallow layer of 1X TAE in an electrophoresis tank and subjected to an electric current of 4-5 V/cm for 2 hours. During this time the DNA is eluted on to the surface of the dialysis bag.
(3) The current was reversed for 1 minute to release the DNA from the wall of the bag, and the buffer was removed from the bag.
(4) The DNA was purified by phenol:chloroform extraction and ethanol precipitation. (The process was checked by staining the gel slice to ensure all the DNA had been eluted).

(C) Spin columns
(1) The DNA was isolated from a seakem agarose gel as before and transferred to a spin column. These columns are commercially available (from Costar) and contain a 0.2µm cellulose acetate membrane.

(2) The agarose slice is first frozen at -80°C for 30 min, then centrifuged for 20 min at 10,000 rpm.
(3) The resulting solution is then cleaned by phenol:chloroform extraction, and the DNA isolated by ethanol precipitation.

2.16 Preparation of dialysis tubing.

Dialysis tubing was boiled for 10 min in 1 mM EDTA, rinsed thoroughly with distilled water, and stored at 4°C.

2.17 Decontamination of ethidium bromide solutions.

This method was developed by Lunn and Sansone (1987).
(1) Sufficient water was added to reduce the concentration of ethidium bromide to <0.5 mg/ml.
(2) 0.2 volume of fresh 5% hypophosphorus acid and 0.12 volume of fresh 0.5M sodium nitrate was then added. The solution was mixed carefully and the pH was checked (should be <3).
(3) After incubation for 24 hr, a large excess of sodium bicarbonate was added and the solution was discarded.

2.18 Restriction digestion of DNA

Restriction enzymes bind specifically to and cleave double stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site.

The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). DNA digests were done by adding 1 unit of enzyme/μg of DNA solution, and incubating for 2 hours at the optimum enzyme temperature (usually between 30 and 37°C). The final DNA concentration in the digest should be between 100 and 300 ng/μl for maximum digestion.

2.19 Ligation of DNA molecules

Several strategies for ligation of DNA molecules are available depending on the nature of the termini.

(1) Cohesive termini.

Ligations of equimolar amounts of vector and insert DNA were carried out overnight at 16°C in a commercial ligation buffer (5mM ATP) with 10 Weiss units of ligase/ml. After ligation, the samples were heat treated for 10 min at 70°C to inactivated the ligase (this appears to improve transformation efficiencies).

(2) Blunt termini.

Compared to cohesive termini ligations, the ligation of blunt termini is considerably less efficient. The reaction conditions used were the same, except for the use of low concentrations of ATP (0.5 mM), the addition of high concentrations of ligase (50 Weiss units/ml) and the use of condensing agents. These are substances that increase macromolecular crowding and hence increase the concentration of blunt ended termini. Hexamine cobalt chloride was used at a concentration of 1μM.

During ligation, T_4 DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one contains a 5' phosphate group and the other contains a 3' hydroxyl group. Recircularisation of plasmid DNA can therefore be minimised by removing the 5' phosphate groups by treatment with calf intestinal phosphatase enzyme (CIP).

Dephosphorylation of linearised plasmid DNA.

(1) Vector DNA (5μg) was digested with the appropriate restriction enzyme in a volume of 20μl.

(2) The sample volume was brought to 150μl with water (including dephosphorylation buffer to a concentration of 1X). CIP was added (1 unit/100 pmoles for cohesive termini and 1 unit/2 pmole for blunt termini) and the sample incubated at 37°C for 1 hour.

(3) The DNA was then purified by phenol extraction and ethanol precipitation.

2.20 In situ colony screening by hybridisation.

Principle

DNA is labelled by random primed incorporation of digoxygenin-labelled deoxyuridine-triphosphate. The dUTP is linked via a spacer arm to the steroid hapten digoxygenin (DIG-dUTP). After hybridisation to the target DNA, the hybrids are detected immunologically using an anti-DIG-alkaline phosphatase antibody conjugate and colorimetric assay.

Preparation of colony lifts

(1) The transformants to be analysed were transferred to duplicate LB plates containing the appropriate antibiotic using sterile cocktail sticks.

(2) Plates were incubated at 37 ^{o}C overnight, then chilled for one hour at 4^{o}C.

(3) A nitrocellulose filter was placed on the plate for one minute, then transferred colony side up to blotting paper for 5-10 min to allow the cells to bind.

(4) The filter was placed colony side up on blotting paper soaked with denaturation solution for 15 min.

Denaturation solution

0.5 N NaOH
1.5 M NaCl
0.1% SDS

(5) The filter was then placed onto blotting paper soaked with neutralisation solution for 5 min, followed by transfer to paper soaked with 2 x SSC for 15 min.

Neutralisation solution

1 M Tris.Cl pH 7.5
1.5 M NaCl

20xSSC

3 M NaCl
0.3 M sodium citrate
pH 7.0, autoclaved.

(6) The DNA was fixed by baking for 2 hours at 80^{o}C and cellular debris was then removed by incubation in 3xSSC/0.1% SDS with shaking for 1-3 hours at 68^{o}C.

DNA probe labelling by random priming with digoxygenin-labelled nucleotides

(1) The DNA was purified by phenol:chloroform extraction and ethanol precipitation.

(2) The DNA was denatured by boiling for 10 min followed by quick chilling on ice.

(3) The labelling reaction was carried out as outlined in the Boehringer Mannheim DIG kit protocol.

Hybridisation

(1) The filters were prehybridised as outlined in the kit instructions for 2 hours.

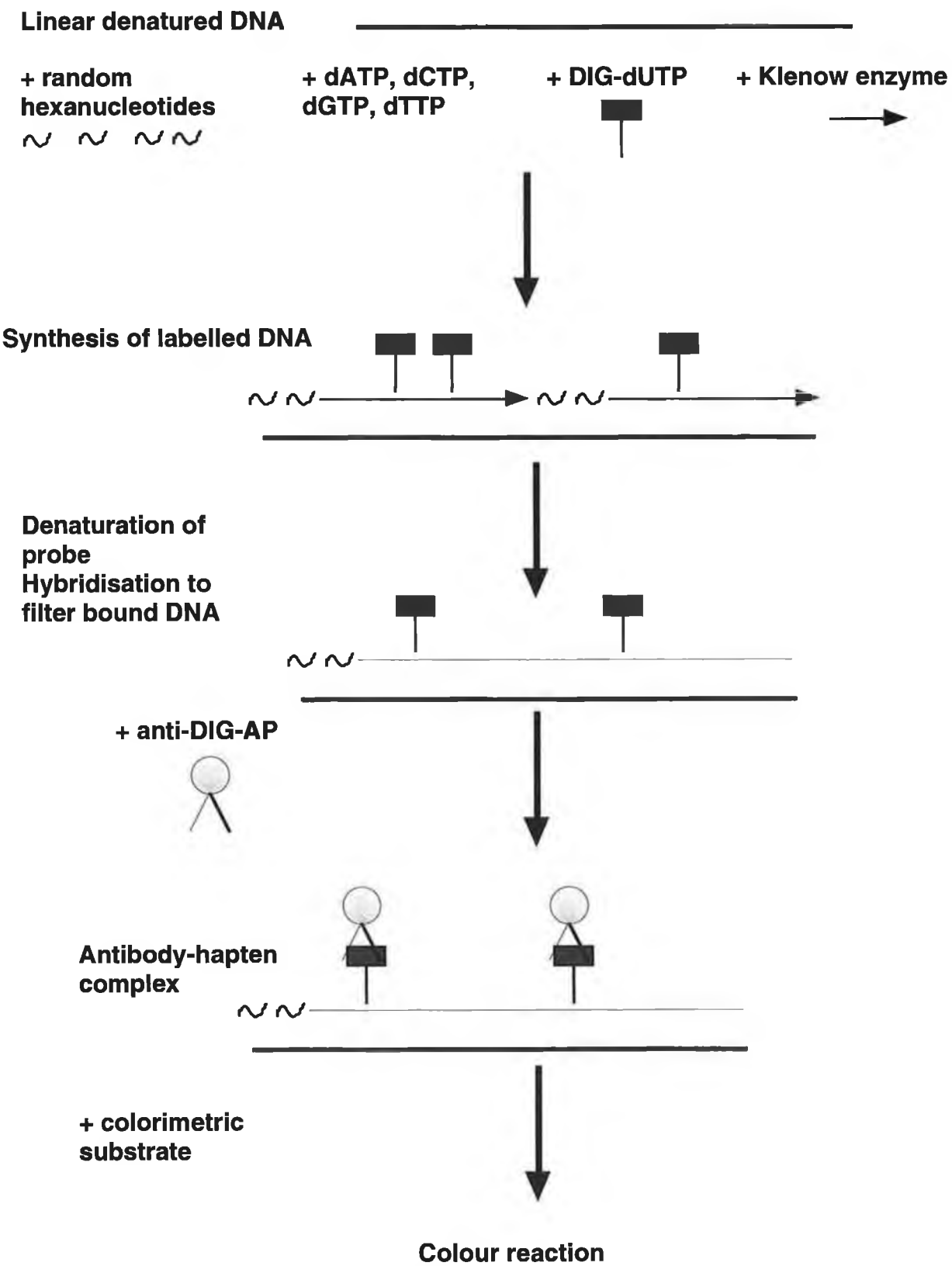
(2) Hybridisation was carried out overnight in the presence of 50% formamide at 42^{o}C.

(3) 50 ng of probe was used.

Detection

(1) Detection was carried out as outlined in the kit instructions and the filters were air dried for storage.

Principle of DIG DNA labelling and detection system



2.21 Amplification of the CD23 5' promoter region by the polymerase chain reaction (PCR)

DNA was amplified by PCR in a 100µl reaction volume containing the following :

10X PCR amplification buffer:		10µl
dNTP mix	:	200µM final conc of each dNTP
primer A	:	250 ng
primer B	:	250 ng
Magnesium chloride	:	1.5 mM
Template DNA	:	10 ng
Taq polymerase	:	0.5 units

Sterile water added to final volume of 100µl.

Samples were overlaid with 60µl of mineral oil and the amplification was carried out as follows :

Cycles	Denaturation	Annealing	Polymerisation
1	94°C, 2 min		
30	94°C, 1.5 min	51°C, 1 min	72°C, 1.5 min
1			72°C, 5 min

Primers used for CD23 amplification :

(1) Forward primer 1 : 5' gggag**ctctgtgattcggccatagt** 3'
Sac I

52% GC, Tm=58°C, Ta=53°C.

(2) Forward primer 2 : 5' gggag**ctcacatcttgacgctacc** 3'
Sac I

50% GC, Tm=54°C, Ta=49°C.

(3) Forward primer 3 : 5' gggag**ctctagttctcacccaat** 3'
Sac I

45% GC,Tm=56°C, Ta=51°C.

(4) Reverse primer 4 : 5' ggccc**gggtgagaactagagatgt** 3'
Sma I

47% GC, Tm=56°C, Ta=51°C.

(5) Reverse primer 5 : 5' ggcccgggtttaagcaggaagagag 3'
Sma I
48% GC, Tm=56°C, Ta=51°C.

Annealing sequences are given in bold type.
Tm : melting temperature given by 2(A+T) + 4(g+C)
Ta : annealing temperature.

2.22 Generation of nested sets of deletion mutants by BAL 31 nuclease digestion.

BAL 31 Reaction Buffer (5X)

40 mM Tris.HCl pH 7.2
25 mM $MgCl_2$
25 mM $CaCl_2$
2 mM EDTA
1.2 M NaCl

A time course reaction for the generation of deletion mutants was carried out :

(1) The plasmid DNA was linearised, phenol extracted, precipitated, and resuspended in TE at a concentration of 1µg/µl

(2) The reaction mix was set up as follows :

60 µl of DNA
60 µl of BAL 31 reaction buffer
2 µl of BAL 31 enzyme (1/10 dilution)
178 µl of sterile distilled water

(3) Tubes were set up on ice containing 5 µl of 0.2 M EGTA.

(4) The reaction mixture was incubated at 30°C and 45 µl samples were removed to the tubes containing EGTA at selected time points (e.g. T = 0, 10, 20, 30, 40, 50 min).

(5) The samples were heated to 65°C for 10 min to inactivate the BAL 31. To each sample, 5 µl of 3M sodium acetate pH 5.5 and 100 µl of ethanol were then added, mixed, the samples incubated for 30 min at - 80°C, and spun for 20 min at high speed.

(6) The pellets were dried and resuspended in 22 µl of TE buffer.

2.23 Repair of DNA molecules to generate blunt ends

The DNA from BAL 31 digestion was repaired using a combination of T_4 DNA polymerase and Klenow. The reaction was set up as follows :

BAL 31 digested DNA	22µl
dNTP mix (2mM each)	4µl
10X polymerase buffer	3µl
T_4 DNA polymerase (5 units)	1µl

The samples were incubated for 30 min at room temp, followed by the addition of 2µl (4 units) of Klenow enzyme and incubation for 15 min. The samples were then phenol cleaned and precipitated.

2.24 Construction of a T vector for the cloning of PCR products.

Attempts to clone PCR products as blunt-ended fragments are generally very inefficient, due to the template-independent terminal transferase activity of Taq polymerase, which results in the addition of a single nucleotide at the 3' end of the fragment. This nucleotide is almost exclusively an adenosine, due to the strong preference of the polymerase for dATP (Hu, 1993) This characteristic of Taq polymerase can be exploited for cloning if a T vector is used. The following reaction can be used to create a vector with a single thymidine addition at each 3' end (Marchuk, *et al*, 1990).

Procedure :

(1) The plasmid (1.5 µg) was digested with a blunt-cutting enzyme in a volume of 20 µl for 2 hours.

(2) After digestion was complete, 6µl of 3 M sodium acetate pH 5.5, 14 µl of water and 120 µl of ethanol were added. After mixing, the sample was stored at -70°C for 20 min, spun at 10,000 rpm for 20 min, dried and resuspended in 20 µl.

(3) The T reaction mix was prepared as follows :

10 µl 10X PCR buffer (including $MgCl_2$)
10 µl 20 mM dTTP
20 µl plasmid
0.5 µl Taq DNA polymerase
59.5 µl of water

(4) The reaction was overlaid with mineral oil and incubated at 70°C for 2 hours.

(5) The sample was then extracted with phenol:chloroform and precipitated with sodium acetate, resuspended at 50 ng/µl.

2.25 Cell lines.

(1) Suspension cultures :

DG75 - an EBV negative Burkitt's lymphoma cell line (a gift from Dr. Aude le Roux, L'institut Gustave Roussy, Paris).
(Ref. : Ben-Basat, *et al*, (1977) Int. J. Cancer, 19, 27-33).

HuT 78 - ATCC TIB 160 (Human cutaneous T cell lymphoma)
(a gift from Stephen Ahern, Dept. of Clinical Medicine, Trinity medical School, St. James' Hospital, Dublin 8).

HuT 78 is a human T cell line which was derived from peripheral blood of a patient with Sezary syndrome. This line has properties of a mature T cell line with inducer/helper phenotype and releases T cell growth factor (IL-2). Biologically active IL-2 could also be eluted from the surface of these cells and subcellular fractionation showed that almost all the IL-2 activity was associated with the plasma membrane. IL-2 activity is adsorbed by this line and HuT 102 (ATCC TIB 162) but not by other human tumour cell lines. Growth rate can be increased by the addition of IL-2. Ref : J. Exp. Med. 154 : 1403-1418, 1981.

(2) Adherent cultures :

SW480 ATCC CCL 228 (Colon, adenocarcinoma, human)
(a gift from Helen O' Shea, Biology Dept. DCU).

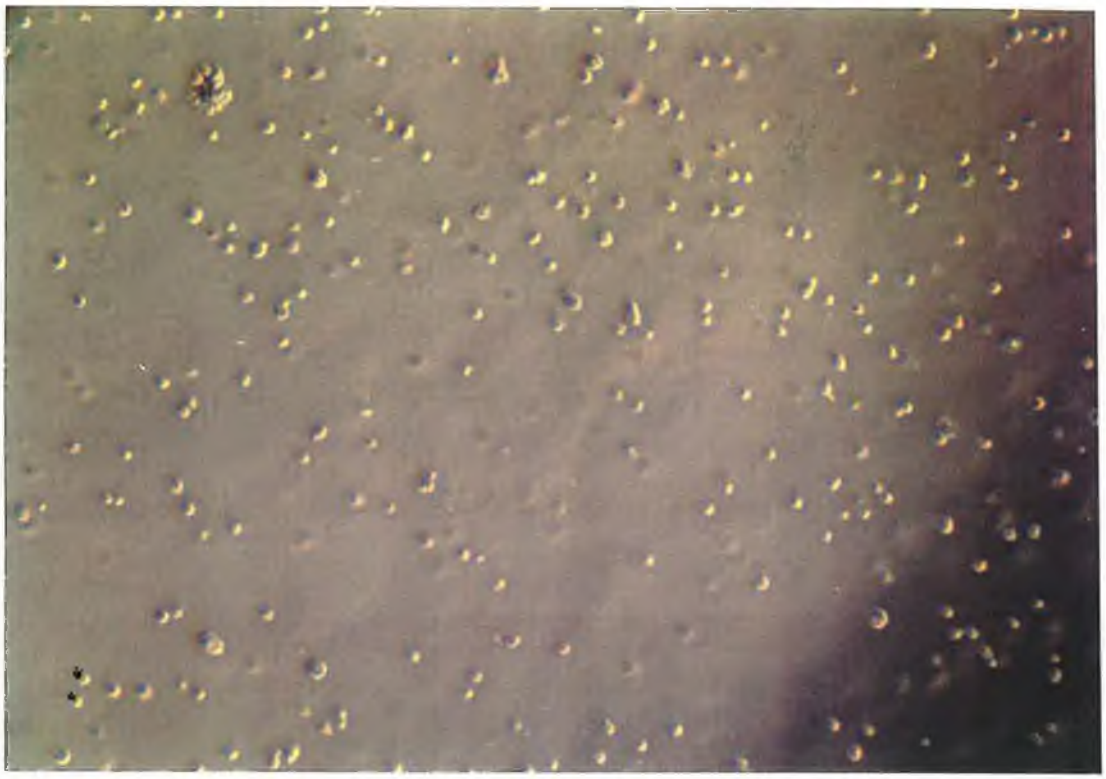
The tumour of origin (Grade III-IV adenocarcinoma of the colon) was minced and various fractions of the resultant fragments were seeded to culture flasks in derivatives of L-15 medium supplemented with 10 % FCS, insulin (0.1 u/ml) and cortisol (10 u/ml). The population grew initially as small islands of epithelial cells and individual bipolar cells becoming predominantly epithelial-like once established. Microvilli were apparent, carcinoembryonic antigen (CEA) was produced and the cells were tumorigenic in nude mice.

Hep G2 ATCC HB 8065 (Human hepatocellular carcinoma).
(a gift from Barbara Fingleton, Biology Dept, DCU).

This line was derived from tissue of a 15 year old male Caucasian. The cells are epithelial in morphology, have a modal chromosome number of 55 and are not tumorigenic in nude mice. The cells produce α-fetoprotein, albumin, α2-macroglobulin, α1-antitrypsin, transferrin, α1-antichymotrypsin, haptoglobin, ceruloplasmin, plasminogen, complement (C3, C4), C3 activator, fibrinogen, α1-acid glycoprotein, α2-HS glycoprotein, β-lipoprotein and retinol binding protein. There is no indication that this cell line harbours an hepatitis B virus genome.

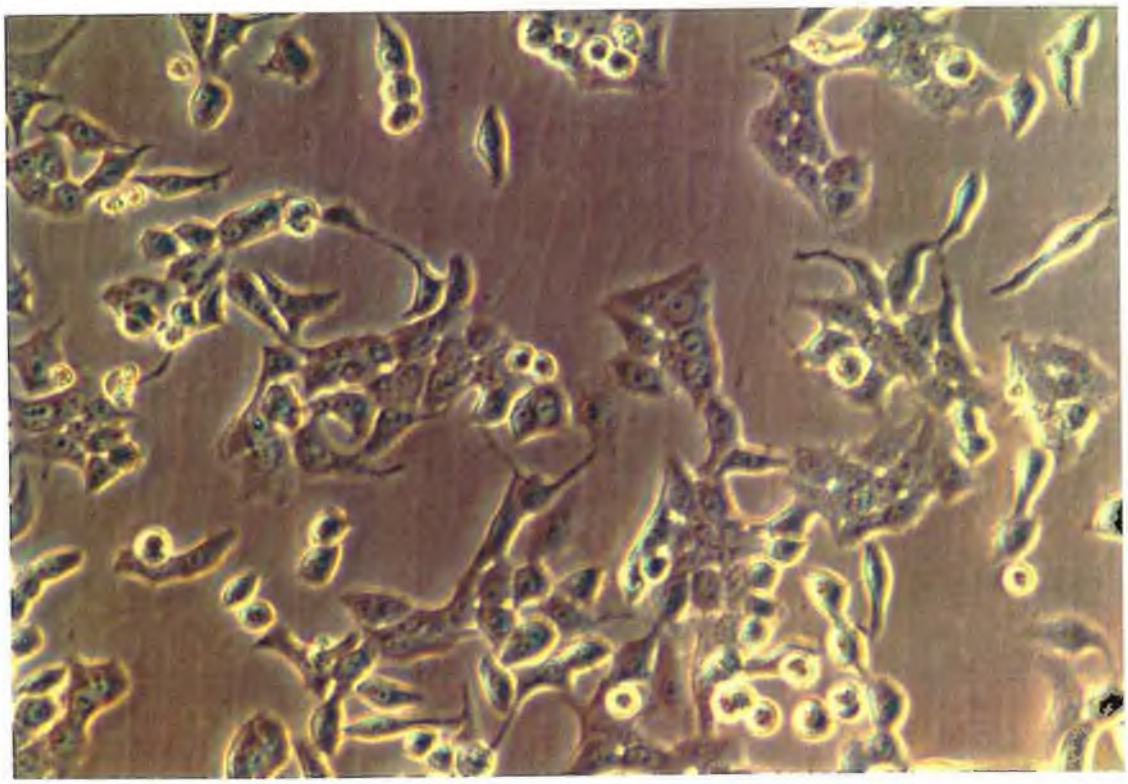
Suspension cell cultures

DG75 (100X)



Adherent cell cultures (200X)

SW480



2.26 Growth media for cell culture

The culture of suspension cells DG75 and HuT 78 was carried out in supplemented RPMI media (10% foetal calf serum - FCS):

176 ml RPMI-1640
20 ml FCS
2 ml L-glutamine
2 ml Pen/strep

DG75 and HuT 78 cells were seeded at 5 X 10^5 cells/ml and split when they had reached a density of 1.5 x 10^6 cells/ml. This was done by counting the cells, centrifuging them at 800 rpm for 5 min, and resuspending them in fresh growth media.

The culture of adherent cell lines SW480 and Hep G2 was carried out in supplemented DMEM media (5% FCS) :

186 ml DMEM
10 ml FCS
2 ml L-glutamine
2 ml pen/strep

SW480 and HepG2 cells were seeded at 1 x 10^6 cells/75cm^2 flask and split when they had reached 75% confluency. This was done by trypsinisation, counting, centrifugation at 2000 rpm for 5 min and resuspension in fresh growth media.

2.27 Viable cell counting.

This is done by the addition of trypan blue to the cells - dead cells stain blue while viable cells remain clear.

(1) Cell suspension (90 µl) was added to 10 µl of trypan blue dye and mixed well.

(2) Cells were counted using a haemocytometer.

2.28 Preparation of cell stocks.

(1) Stocks were prepared in 1 ml aliquots using 10^7 cells per stock.

(2) Cells were harvested in exponential phase by centrifugation.

(3) The pellet was resuspended in 900 µl of supplemented RPMI (20% serum) per 10^7 cells and incubated on ice for 10 min.

(4) DMSO was added (100 µl per 10^7 cells) as a cryoprotective agent and

the mixture was incubated on ice for 5 min.
(5) The mixture was transferred in 1 ml aliquots to cryotubes.
(6) Freezing was carried out at -20°C for 30 min, followed by -80°C for 12 hours. The cells were then transferred to liquid nitrogen.

2.29 Reconstitution of frozen stocks

(1) Vials were thawed at 37°C.
(2) The cells were immediately transferred to 10 ml of growth medium to dilute the DMSO and centrifuged for 5 min at 800 rpm (suspension cells) or 2000 rpm for 5 min (adherent cells).
(3) The pellet was then transferred to 10 ml of fresh growth medium in a culture flask.

2.30 Trypsinisation of adherent cells.

(1) The medium was removed from the cells and they were rinsed once in serum free medium and once in PBS (phosphate buffered saline). This removes the serum from the cells which would inhibit the action of the trypsin.
(2) 2 ml of 0.25% trypsin/EDTA were added to the cells and they were incubated at 37 ^{0}C for 10 min.

0.25% trypsin/EDTA

88 ml sterile PBS
2 ml sterile EDTA (1% w/v)
10 ml 2.5% commercial trypsin solution.

PBS (phosphate buffered saline

8g NaCl
0.2g KCl
1.44g $NaHPO_4$
0.24g KH_2PO_4
800ml of distilled water
pH was adjusted to 7.4 with HCl and the volume was made up to 1L.

(3) When the cells have detached, 5 ml of complete growth medium was added to stop the action of the trypsin. At this stage a sample can be taken for cell counting. The remainder was centrifuged at 2000 rpm for 5 min.

(4) Flasks were seeded at 1 x 10^6 cells/75cm^2 flask.

2.31 Transfection of adherent cell lines : $CaPO_4$ technique.

Principle :

Plasmid DNA is mixed with calcium chloride and added to a solution containing phosphate ions. A calcium phosphate-DNA co-precipitate is formed which is taken up by mammalian cells in culture.

Solutions :

(1) 2x HBS (Hepes Buffered Saline)

280 mM NaCl 8.18g
50 mM Hepes 5.96g
1.5 mM Na_2HPO_4 0.13g
500 ml ultra pure water
Filter sterilise pH 7.12, store 4°C.

The pH is critical for efficient transfection (this can vary widely between batches of 2x HBS) - the optimal pH is between 7.05 and 7.12. Efficiency can be checked by mixing 0.5 ml 2x HBS and 0.5 ml 2.5 M $CaCl_2$ and vortexing. A fine precipitate should develop that is readily visible in the microscope.

(2) 2.5 M $CaCl_2$

183.7 g $CaCl_2$
500 ml ultra pure water
Filter sterilise, store 4°C.

(3) Tris-EDTA (TE)

10 mM Tris
1 mM EDTA
pH 8.0

(4) 10% Glycerol/HBS

10 ml glycerol
50 ml 2x HBS
40 ml ultra pure water.

Procedure :

Day prior to transfection :

(a) Cells were trypsinised, counted and set up at 5 x 10^5 cells into 10 cm tissue culture plates. On the day of transfection it is important that the cells are thoroughly separated on the dish as the ability to take up DNA is related to the surface area of the cell exposed to the medium.

(b) Plasmid DNA was diluted to 1 μg/ul in TE. Sterile water was added to the DNA (final volume 420 μl) and this was placed in a sterile 3 ml tube at 4 °C overnight.

Day of transfection:

(c) The tube containing the DNA was placed at 37 °C for 1 hour. At the same time, 480 μl 2x HBS was placed into a 3 ml sterile tube at room temperature.

(d) The 2.5 M $CaCl_2$ (60 μl) was added dropwise into the tube containing the DNA with continual mixing using a vortex. The DNA-$CaCl_2$ mixture was immediately added into the 2x HBS. The DNA must be added **dropwise** into the HBS with **continuous vortexing.** This is the most important step - the finer the precipitate the better the transfection efficiency.

The samples were left in the laminar flow for exactly 30 min - this time is critical so if there are a number of samples then they must be staggered so that each gets exactly 30 min.

(e) The cells were removed from the incubator and the DNA was added dropwise to the cells with gentle swirling of the plate to ensure even mixing. The plates were then incubated at 37 °C for 4 hours.

(f) The cells were then glycerol shocked to aid the entry of the DNA into the cells :

1. The medium was removed.
2. Immediately, 3 ml of 10% glycerol/HBS was added.
3. The cells were left for 3 min exactly - overexposure to glycerol will kill the cells.
4. The glycerol was removed, the cells were rinsed once in sterile PBS and once in serum free medium. They were then refed with 10ml of fresh growth medium.

2.32 Transfection of B lymphocytes by electroporation.

The following procedure works well for DG75 and T cells (gives about 90% kill).

(1) The cells were passaged 2 days before transfection. Cells should be less than 10^6/ml(5-7 X 10^5/ml).

(2) On the day of transfection 60 mm dishes were prepared with 5 ml RPMI-1640/glutamine/10% FCS (complete) and incubated at 37°C.

(3) Cells were spun at 800 rpm for 5 min ($1X10^7$ cells / transfection), and washed in one fifth volume of cold complete RPMI.
(4) During this cuvettes were prepared - labelled, on ice, and containing DNA.

DNA preparation
Transfected DNA was in 30 µl TE pH 7.4. There must be the same total quantity of DNA in each tube. The appropriate quantities of DNA were co-precipitated, washed in 70% ethanol, dried and then resuspended in TE pH7.4.
(5) Cells were resuspended in cold complete RPMI at 250µl per 10^7 cells.
(6) Cells/DNA mix was pulsed at 220V/960µF (with capacitance extender). The time was recorded in millisecs.
(7) Cells must not stay longer than 10 min in the cuvettes after pulsing.
(8) The contents of the cuvettes were gently transferred to culture dishes using micropipette and yellow tip. The cuvettes were washed with medium from the dish.
(9) Transfected cells were incubated from 24 to 48 hours (37°C, 5% CO_2).
(10) The cells were harvested for reporter enzyme assays (see 2.33)

2.33 Harvesting of cells post-transfection.

(a) Adherent cells
1. The medium was removed from the plates and the cells were washed twice with 5 ml of sterile TBS.
2. After washing, 1 ml of TBS was added and the cells were scraped off the plates and transferred to a sterile microfuge tube on ice.
3. Cells were spun at high speed for 1 minute.
4. The cell pellet was resuspended in 100 µl ice cold TBS.
5. Cells were frozen for 5 min in dry ice/ethanol, followed by incubation for 5 min at 37 °C. This is repeated twice more. (Successive freezing and thawing lyses the cells).
6. The lysate was cooled on ice and then spun for 5 min at 4 °C. The supernatant was removed and stored at -80 °C. (This is the cell extract that contains the transfected protein).

(b) Suspension cells

1. Cells were pelleted at 1000 rpm for 5 min.
2. The pellet was washed with 1 ml of TBS and transferred to a sterile microfuge tube.
3. Preparation of the lysate was as per adherent cells.

2.34 PAP assay

PAP (placental alkaline phosphatase) is resistant to heat and is also restricted in expression to the placenta. The assay measures the hydrolysis of PNPP (p-nitrophenyl phosphate) which gives rise to a yellow colour (Henthorn, *et al*, 1988).

Reagents :

DEA buffer :	20 ml diethanolamine 10 mg $MgCl_2$ 20 mg sodium azide Made to 100 ml with water, pH to 9.85 with conc. HCl, stored in the dark at 30 °C.
Substrate stock solution :	PNPP sigma 104 phosphate substrate. (a) A 0.1 M solution was made in DEA buffer. (b) This was stored in glass bottles in 3-5 ml aliquots in the dark. (Discarded if the solution turns yellow).
TBS :	154 mM NaCl 50 mM Tris pH 7.5 1 mM $MgCl_2$
Reaction mixture :	Dilute substrate stock 1:20 just before use (5 mM PNPP).

Method :

(1) Cells were transfected and centrifuged, 800 rpm, 5 min.
(2) The pellet was washed with 1 ml TBS, and transferred to an eppendorf.
(3) After centrifugation at 10,000 rpm for 5 min, the pellet was resuspended in 100 µl TBS pH 7.8.

(4) The lysate was prepared by the freeze-thaw method as for adherent cells.
(5) Heat inactivation was carried out at 65 °C for 30 min.
(6) After cooling to room temperature, 20 µl of lysate was added to 400 µl of reaction mixture, and incubated at 37 °C for 30-60 min (or longer to get measurable activity).
(7) The O.D. was read at 405 nm.
Endogenous PAP activity is absent from a large number of cell lines so far tested.

2.35 Phase extraction CAT Assay

Reagents and materials :

0.01 µCi/µl ^{3}H-chloramphenicol
5 mg/ml butyryl CoA
100 mg/ml unlabelled chloramphenicol
2 M Tris.Cl pH 8.0
2:1 (vol/vol) tetramethylpentadecane (TMPD)/xylenes
Scintillation fluid

Procedure:

(a) Preparation of 0.01 µCi/µl ^{3}H-chloramphenicol solution for phase extraction assay.
(1) A 0.2 µCi/µl ^{3}H-chloramphenicol stock was prepared by adding 960µl of 100% ethanol and 40µl of 100mg/ml unlabelled chloramphenicol to 250µl of ^{3}H-chloramphenicol (250 µCi/250µl in ethanol : 42.0 to 58.2 Ci/mmol).
(2) The 0.2 µCi/µl ^{3}H-chloramphenicol stock was preextracted by first diluting it 20-fold in water, and then extracting this mixture with an equal volume of xylenes by vigorous shaking. The sample was centrifuged for 2 min at room temp and the top xylenes phase removed and discarded. This extraction was repeated once more, creating a working solution of 0.01 µCi/µl ^{3}H-chloramphenicol.

(b) Phase extraction CAT assay.
(1) Transfected cells were harvested in PBS and resuspended in 100µl ice cold 0.25 M Tris.Cl pH7.5.
(2) Cells were lysed by four successive cycles of freezing and thawing (dry ice/ethanol 5 min, 37°C 5 min.)

(3) The lysate was then spun for 5 min and the supernatant was removed and saved (this is the extract that contains the CAT enzyme).
(4) The lysate was heat treated at 65°C for 10 min to inactivate potential inhibitors.
(5) To assay 30µl of cell extract, the following reaction mix was made per sample :
20µl 0.01 µCi/µl ^{3}H-chloramphenicol
5µl 5mg/ml butyryl CoA
5µl 2M Tris.Cl pH 8.0
40µl H_2O
(6) For each assay 30µl of cell extract was added to 70µl CAT assay mix. The samples were incubated for 30 to 90 min at 37°C.
(7) The acetylated chloramphenicol was extracted with 200µl of 2:1 TMPD/xylenes by vigorous shaking, followed by centrifugation. The top organic phase was removed to a scintillation vial.
(8) 3 to 5 ml of scintillation fluid was added to each sample and the samples were counted to determine CAT activity.

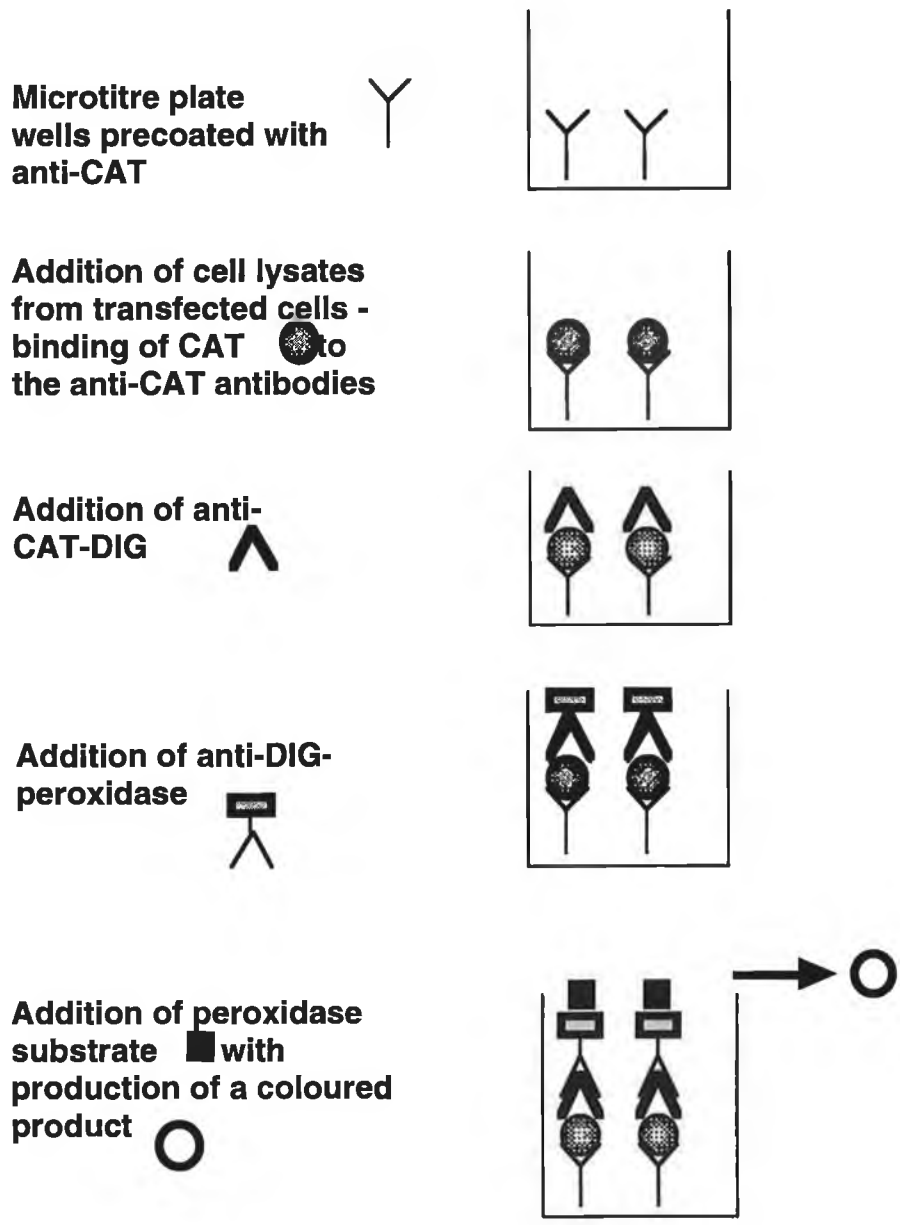
2.36 CAT ELISA

The procedure was followed exactly as outlined in the Boehringer Mannheim CAT ELISA kit instructions. The substrate enhancer was used in this case.

Principle

The ELISA is based on the sandwich-enzyme immunoassay principle. Antibodies to CAT (anti-CAT) are prebound to the surface of a microtitre plate. Following lysis of the transfected cells, the cell lysates containing the CAT protein are added to the microtitre plates. After washing, a digoxygenin-labelled anti-CAT is added, followed by an anti-digoxygenin-peroxidase conjugate. The activity of the peroxidase enzyme is then assayed by the addition of a colorimetric substrate, and subsequent absorbance measurement.

Principle of the CAT ELISA.



2.37 Biorad Protein Assay

Principle of assay :

This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The method used was an adaptation of the Biorad Microassay procedure.

(1) The samples and standards were diluted to 160 µl with water and added to 40 µl of undiluted dye reagent.

(2) The samples were mixed by gentle vortexing, avoiding excess foaming.

(3) After a period of 5 min to one hour the OD_{595} was read versus a reagent blank, using an ELISA plate reader.

(4) The OD_{595} was plotted versus concentration of standards, and the sample concentrations were read from the standard curve.

3. Results and Discussion.

3.1 Construction of a CD23 reporter plasmid.

The most common reporter gene used in transfection studies is CAT (chloramphenicol acetyl transferase). This enzyme is usually assayed using a radioisotopic method. To obviate the need to use a radioassay, we constructed and tested a reporter plasmid involving the CD23 promoter cloned upstream of PAP (placental alkaline phosphatase). This is a heat stable enzyme - the assay involves a colorimetric reaction, and is a simpler and cheaper alternative to the CAT assay.

Two cloning strategies were investigated for pCD23Pap:

Cloning strategy (1)

(a) pSV_2APap was used as the source of the PAP gene.

(b) pBCD23 was used as the source of the CD23 promoter.

(c) pGem3Zf was used as the cloning vector.

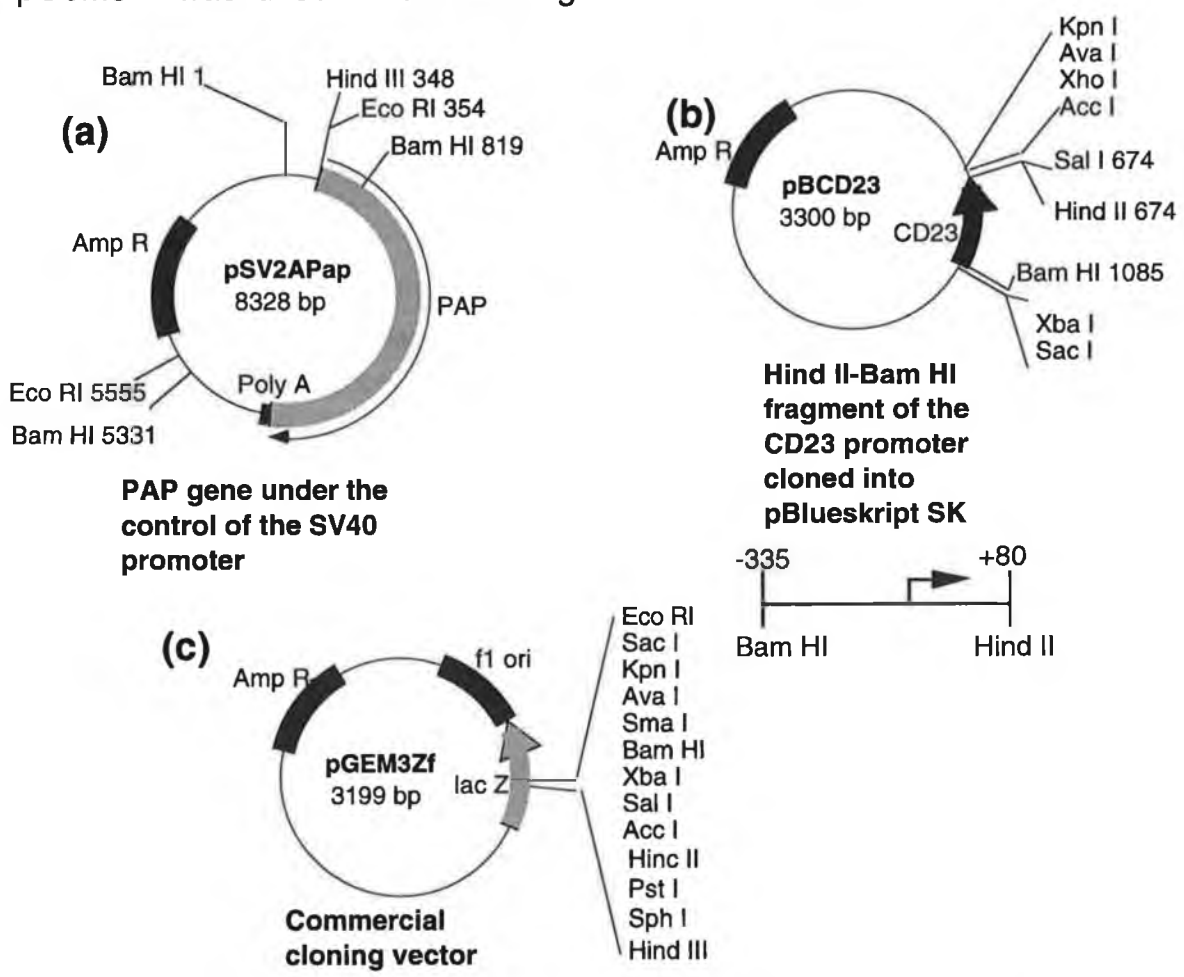
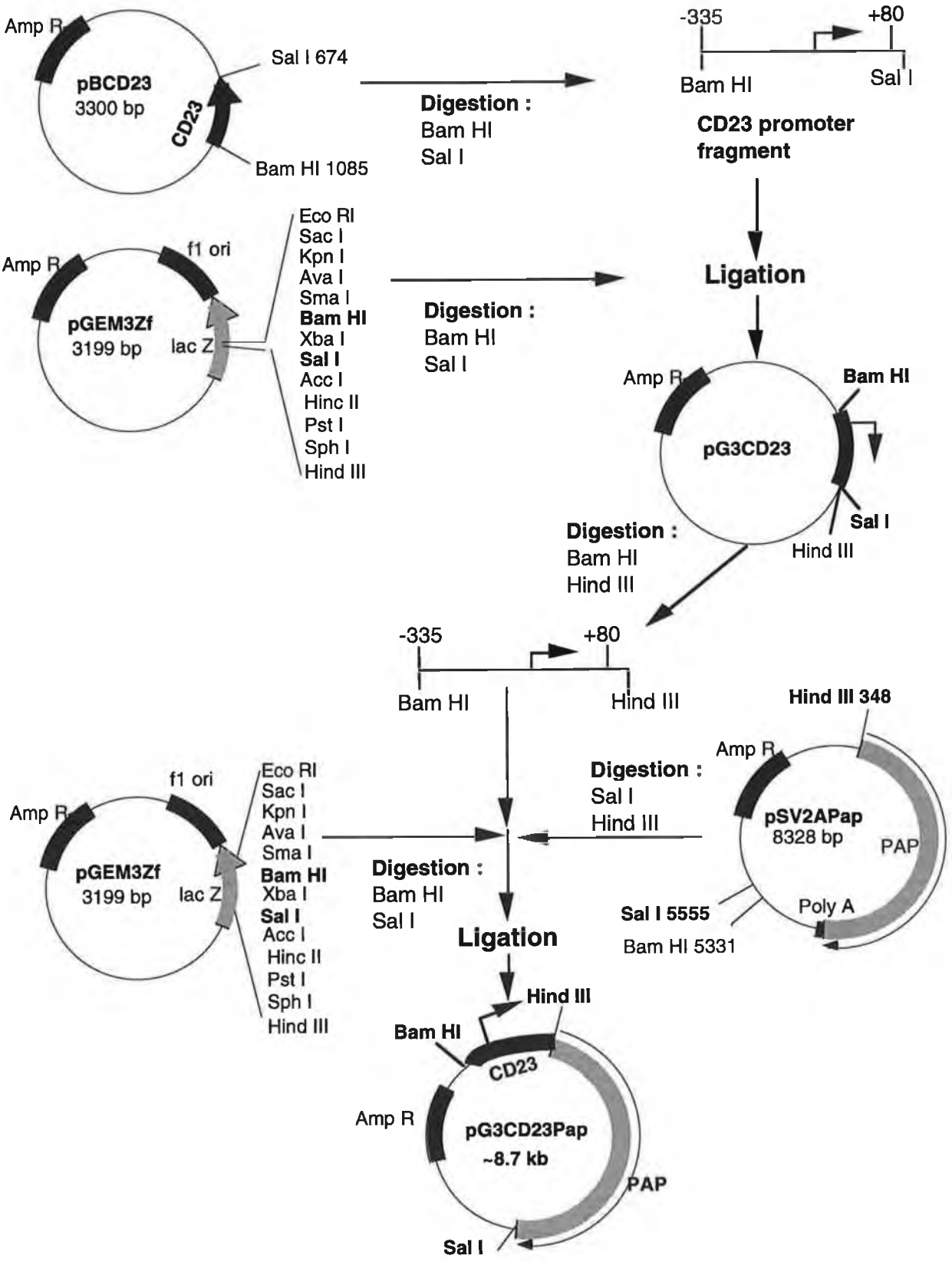


Figure 4. Plasmids used in cloning strategy 1.

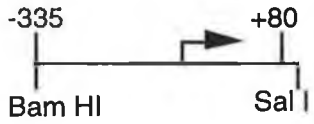
Figure 5 : STRATEGY 1 :



Strategy (1) (fig. 5) : summary

(a) Sub-cloning the CD23 promoter fragment

pBCD23 was digested with Bam HI and Sal I to excise the CD23 promoter



CD23 promoter fragment

Figure 6.

This fragment was then subcloned into pGem3Zf digested with Bam HI and Sal I. This step created a construct containing the CD23 promoter fragment flanked with a number of useful restriction sites :

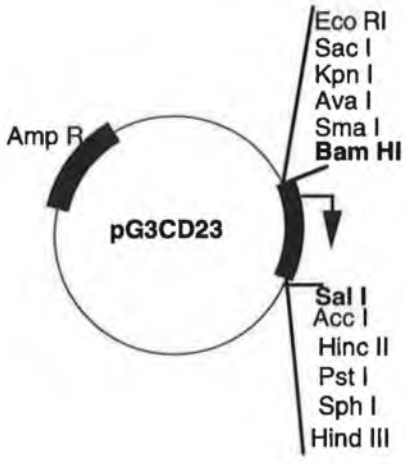


Figure 7. pG3CD23

(b) Cloning PAP downstream of CD23

pBCD23 was then used as the source of the CD23 promoter. A Bam HI-Hind III digestion excised the CD23 fragment. pSV_2APap was the digested with Hind III and Sal I to isolate the PAP gene. A three fragment ligation was carried out with pGem3Zf (digested with Bam HI and Sal I) :

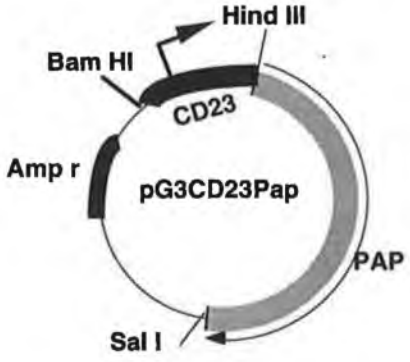


Figure 8. pG3CD23Pap

Procedural comments :

(1) Double digests of vector were prepared as follows :

After digestion with the first enzyme, the vector was phenol/chloroform extracted and ethanol precipitated. This allows buffer conditions to be changed for the second digest. A sample was run on a 1% agarose gel to ensure digestion was complete before the subsequent digest was carried out.

(2) The insert was prepared by double-digestion, followed by isolation from a seakem agarose gel (1.5%) by spin-x column centrifugation (Materials and methods section 2.15).

(3) The following controls were included in the ligation :

(a) Single-digested pGem3Zf (50 ng) +/- ligase.

(b) Double-digested pGem3Zf (50 ng) +/- ligase.

A comparison of these samples indicates if the double-digestion has been efficient. The restriction enzymes used have incompatible ends and so should not support ligation in a vector that has been successfully digested with each.

The single-digested vector gave a >100 fold increase in the number of colonies produced after ligation. The double-digested vector gave no significant difference in colony numbers before and after ligation indicating successful double-digestion.

The competency of the cells was tested when they were made and was found to be $>10^7$ cfu/μg DNA.

Analysis of pG3CD23:

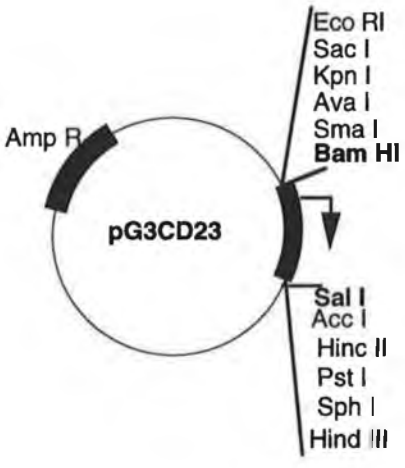


Figure 9.

The use of pGem3Zf allows blue/white selection of recombinants. Cloned inserts interrupt the lac Z gene which codes for β-galactosidase. Production of this enzyme gives rise to blue colonies on plates containing the chromogenic substrate x-gal. Recombinants can be selected as white colonies.

This cloning creates a construct with a Hind III site at the 3' end of the CD23 promoter fragment of interest (fig. 9). This can then be isolated with a Bam HI-Hind III digest. Clones were analysed by using this digest (a Bam HI/Hind III digest of the pBCD23 parent would linearise the plasmid as there are no Hind III sites present). Hinf I digests were also carried out for confirmation.

Predicted sizes of fragments from a Hinf I digest (from sequence information) :

pGem3Zf : 1382, 517, 452, 396, **244**, 75, 65, **46**, 22.

pG3CD23 : 1382, **552**, 517, 452, 396, **137**, 75, 65, 22.

(differences in the banding patterns are highlighted in bold type).

Approximate sizes of fragments observed on a 1.5% agarose gel :

pGem3Zf : 1382, 517, 452, 396, **244**.

pG3CD23 : 1382, **552**, 517, 452, 396, **137**.

(gel not shown).

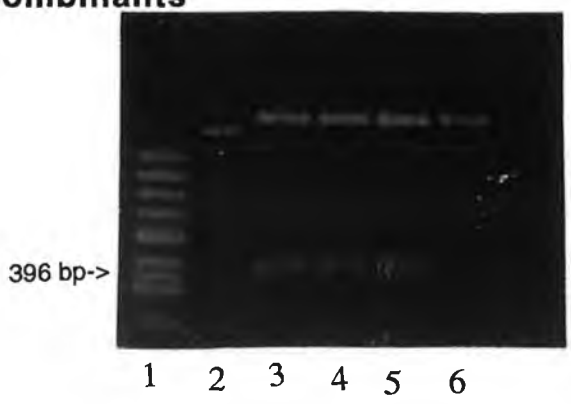
The above results show that the fragments expected from a digest of pG3CD23 were observed indicating the cloning had been successful. The bands smaller than 137 bp were not seen due to the limited resolution of the gel.

Predicted sizes of fragments from a Bam HI/Hind III digest (from sequence information) :

pGem3Zf : 3199.

pG3CD23 : 2919, 411.

Figure 10 : Restriction analysis of recombinants



1.5% agarose

Lane :	Sample :
1	Molecular weight markers
2	3000 bp marker
3-7	Isolated colonies A,B,C,D,E (Bam HI-Hind III digest).

The gel (fig. 10) shows the expected banding pattern for pG3CD23 digested with Bam HI and Hind III (2919, 411 bp).

Analysis of pG3CD23Pap

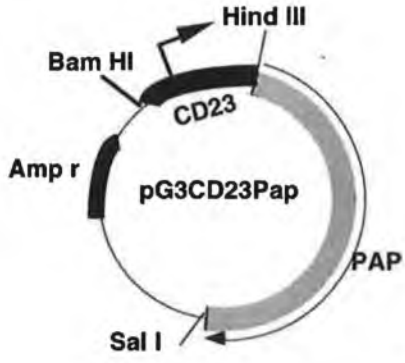


Figure 11.

Several attempts were made to construct this plasmid, but in all cases the three fragment ligation resulted in very few colonies (<30) and of these only 10% were white. None of those tested were found to be the required plasmid.

The ligase activity was tested by digesting plasmid DNA with a frequently cutting enzyme and religating. The result was (a) viewed on a gel before and after ligation, and (b) transformed (10 ng).

Transformation gave greater than a 100 fold increase in colonies after ligation.

From the 1% gel (fig. 12) it appears the ligase is working.

Figure 12 : Analysis of ligase activity

Lane :	Sample :
1	Molecular weight markers
2	Plasmid DNA digested with Hae III.
3	Ligation of Hae III digest.

1 2 3

As can be seen from the gel, the DNA bands have all moved to high molecular weight bands in the ligated sample indicating ligation was successful.

The cells were tested for competency and found to be $>10^8$ cfu/μg.

Ligations were run on a 1% gel and the DNA before ligation appeared as 2 bands (PAP ~5 kb and CD23 ~400 bp) as expected. After ligation the DNA appeared as high molecular weight bands (CD23 band disappearing) as expected.

However, transformation produced few colonies, none with the required plasmid. An alternative strategy for the construction of this plasmid was then employed.

Cloning strategy (2)

(a) pSV_2APap was used as the source of the PAP gene.

(b) pBCD23 was used as the source of the CD23 promoter.

(c) pGem7Zf was used as the cloning vector (fig. 13).

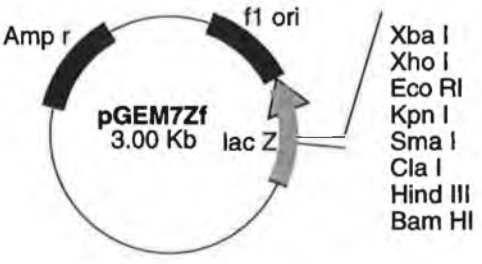


Figure 13.

Strategy (2) : summary (fig. 16)

(a) Subcloning the CD23 promoter into pGem7Zf.

pBCD23 was digested with Sal I, repaired to create a blunt end, and then digested with Bam HI. The CD23 fragment was gel isolated and cloned into pGem7Zf (digested with Bam HI and Sma I) : pG7CD23

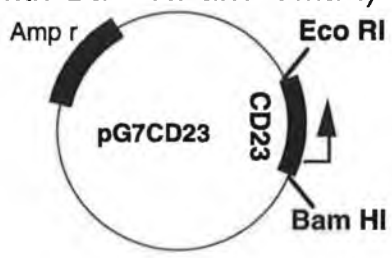


Figure 14.

(b) Cloning PAP downstream of CD23.

pG7CD23 was then used as the vector in the next step. The PAP gene was gel isolated as an Eco RI fragment from pSV_2APap, and cloned into pG7CD23 digested with Eco RI. The PAP gene orientation was then determined by restriction analysis : pG7CD23Pap.

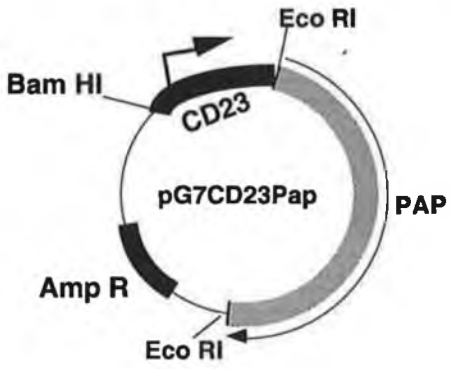


Figure 15.

Procedural comments :

(a) The CD23 insert was prepared by digestion with Sal I, repair to generate a blunt end (Materials and methods section 2.23) and digestion with Bam HI. DNA was purified from seakem agarose as previously described.

(b) Any background of single-digested religated vector (pGem7Zf) was reduced by digesting the ligation with Hind III before transformation. (Any double-digested vector has lost the Hind III site between Sal I and Bam HI in the polylinker).

(c) To reduce the background of religated pG7CD23 in the pCD23Pap ligation, the vector was treated with calf intestinal phosphatase (CIP) before ligation (materials and methods section 2.19).

(d) Controls were as in strategy (1).

STRATEGY 2 :

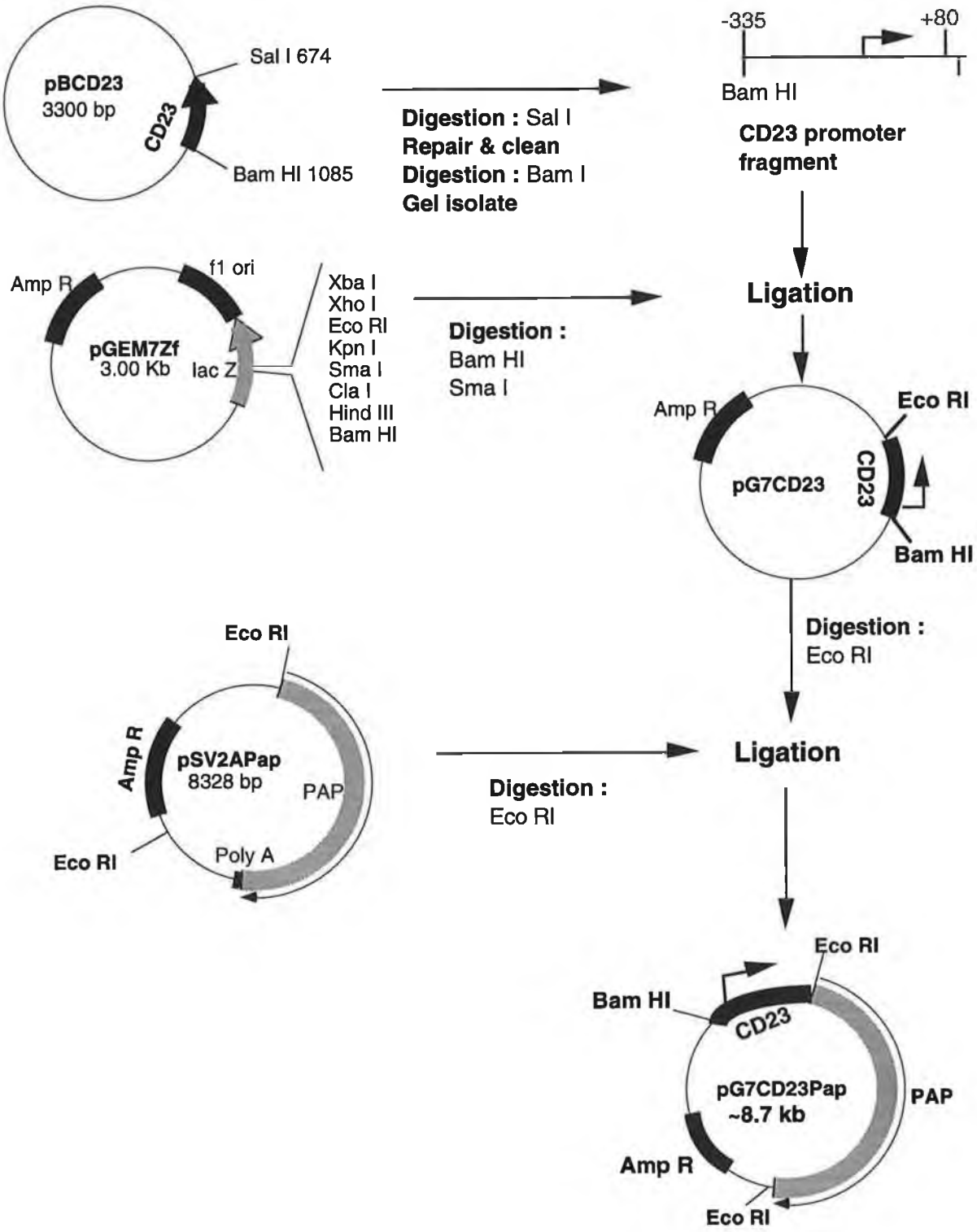


Figure 16.

Analysis of pG7CD23 :

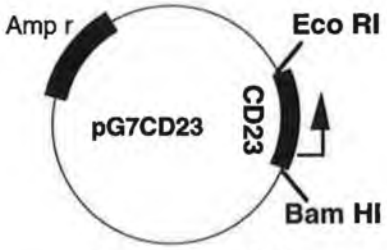


Figure 17.

Blue/white selection was used to isolate a clone containing an insert. The construct has an Eco RI site at the 3' end of the CD23 fragment (fig. 17). As the parent pBCD23 plasmid does not contain this site, the construct can be analysed for an insert using a Bam HI/Eco RI digest. Hinf I digests were used for confirmation.

Predicted sizes of fragments from a Hinf I digest (from sequence information) :

pG7CD23 : 1129, 517, 452, 396, **356**, **332**, 75, 65, **45**, 22.
pGem7Zf : 1129, 517, 452, 396, **299**, 75, 65,**34**, 22, **11**.
pGem3Zf : **1382**, 517, 452, 396, **244**, 75, 65, **46**, 22.

Approx. sizes of fragments observed on a 1.5% agarose gel :

pG7CD23 : **1129**, 517, 452, 396, **356**.
pGem3Zf : **1382**, 517, 452, 396.

Figure 18 : Analysis of pG7CD23 recombinants

Lane :	**Sample :**
1	Molecular weight markers
2	pG7CD23-Hinf I digest
3	pGem3Zf-Hinf I digest
4	pG7CD23-Bam HI/Eco RI
5	pGem7Zf-Bam HI/Eco RI

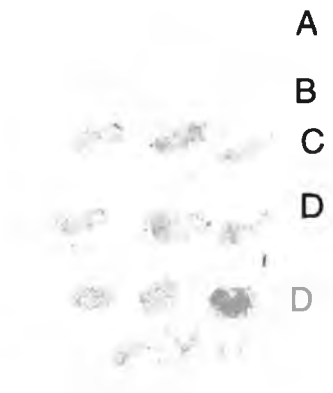
As can be seen from the gel, the clone contains the expected Bam HI/ Eco RI fragment (CD23 insert ~424 bp).
This was confirmed by the Hinf I digest - fragments were as expected.

In order to clone PAP downstream of the CD23 promoter, the next step involved using the pG7CD23 clone as a vector. The pG7CD23 clone (maxiprep) was digested with Eco RI and treated with CIP to prevent recircularisation. The phosphatase treatment was shown to be effective by carrying out a ligation of plasmid before and after CIP treatment, and analysing the result on an agarose gel. Transformation of these samples showed greater than 100 fold decrease in the number of colonies after CIP treatment.
The PAP insert was isolated from pSV$_2$APap digested with Eco RI and purified by column chromatography as previously described.

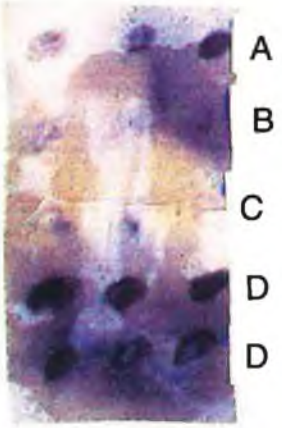
Detection of pG7CD23Pap clones
As the vector already contained the CD23 insert, a blue/white selection was no longer possible. Ligation produced >500 colonies. In spite of an apparently effective phosphatase treatment, no clones were isolated from 60 minipreps, so an *in situ* colony hybridisation technique was used (see section 2.20).
The PAP gene was isolated as before and prepared for use as a probe by labelling with digoxygenin. A Bam HI/Eco RI digest of pG7CD23, followed by gel isolation produced CD23 DNA for use as a second probe.
400 colonies were regrown in an ordered array and nitrocellulose colony lifts were prepared in duplicate.

A combination of 2 probes was used to enable elimination of anything other than the plasmid of interest. A PAP probe would detect the clone of interest pG7CD23Pap, but it would also detect the PAP parent pSV$_2$APap. A CD23 probe would detect the clone of interest, but also the CD23 parent pG7CD23. **Only** the clone of interest would give a positive result with **both** probes. Bacteria transformed with pSV$_2$APap or pG7CD23 were used as controls. An example of a duplicate filter detection is shown below :

Filter 1 : probed with PAP
a : pG7CD23
b : pGem7Zf
c : pSV$_2$APap
d : clones 1-6



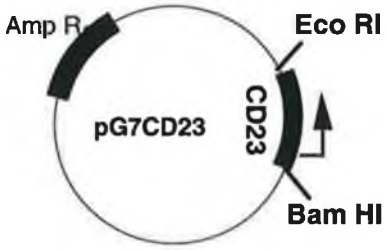
Filter 2 : probed with CD23
a : pG7CD23
b : pGem7Zf
c : pSV$_2$APap
d : clones 1-6



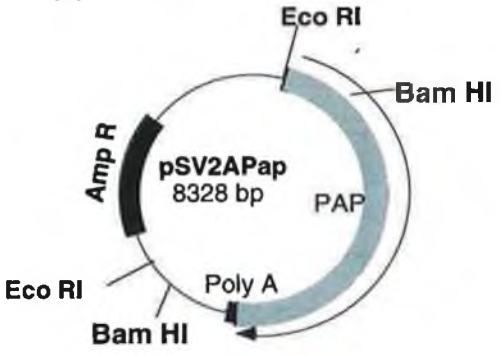
The higher background staining in filter 2 is likely to be due to overdevelopment and insufficient washing during the antibody detection step. As can be seen from the filters, all clones 1-6 are examples of positive clones as they hybridise to the PAP and CD23 probes. As expected the PAP probe does not hybridise to pBCD23 and the CD23 probe does not hybridise to PAP DNA. Neither probe hybridises to pGem7Zf DNA. These results indicate the probe is specific and cross-hybridisation is weak.

Figure 19. Analysis of clones :
The following possibilities existed after cloning strategy 2 :

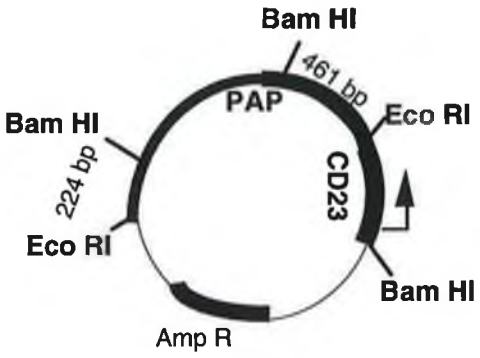
(a) CD23 parent



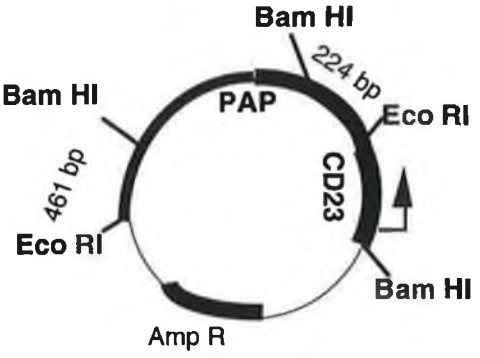
(b) PAP parent



(c) Clone pG7CD23Pap (forward orientation)



(d) Clone pG7CD23Pap (reverse orientation)



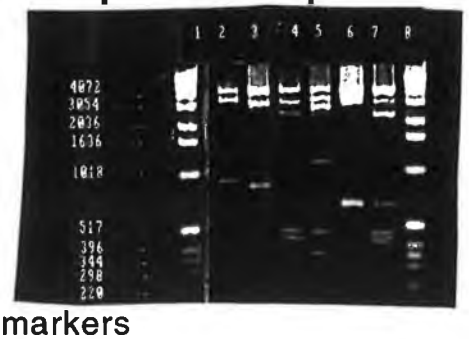
Predicted digests (from sequence information) :

Plasmid	Enzyme(s)	Sizes (bp)
pG7CD23	Bam HI/Eco RI	2965, 424
pSV$_2$APap	Bam HI/Eco RI	4512, 2425, 461, 348
pG7CD23Pap(a)	Bam HI/Eco RI	4512,2970,461,415, 224
pG7CD23Pap(b)	Bam HI/Eco RI	4512,2970,461,415, 224
pG7CD23	Bam HI	3389
pSV$_2$APap	Bam HI	4512, 2650, 800
pG7CD23Pap(a)	Bam HI	4512, 3200, 876
pG7CD23Pap(b)	Bam HI	4512, 3410, 636

Fragments observed on 1.5% agarose gel (Bam HI digests):

pSV$_2$APap : 4512, 2650, 800
pG7CD23Pap : 4512, 3200, 876
pG7CD23Pap : 4512, 3410, 636

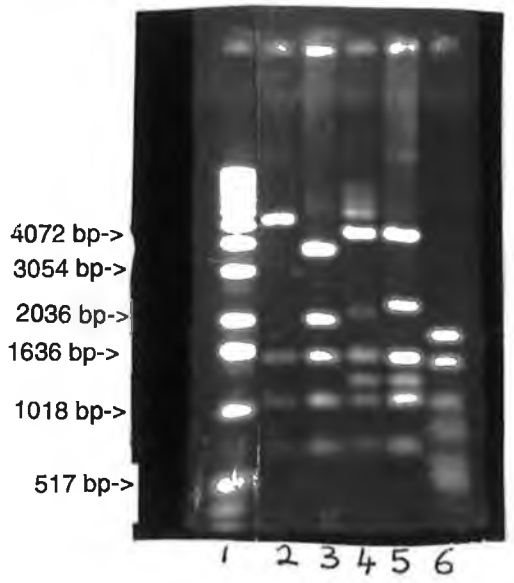
Figure 20 : Restriction analysis of pG7CD23Pap recombinants



Lane :	Sample :
1	Molecular weight markers
2	pG7CD23Pap(a)(forward orientation clone)-Bam HI digest
3	pSV$_2$APap - Bam HI digest
4	Clone 9 Bam HI digest
5	Clone 10 - Bam HI digest
6	pG7CD23Pap(b)(reverse orientation clone)-Bam HI digest
7	Clone 12 Bam HI digest
8	Molecular weight markers

As can be seen from the gel (fig. 20), clones were isolated with the PAP gene cloned in both orientations. This was confirmed by a digest with Sac I- the three plasmids (pSV_2APap, pG7CD23Pap(a), pG7CD23Pap(b)) gave different banding patterns when cut with this enzyme.

Figure 21 : Restriction analysis of pG7CD23Pap



Lane :	Sample :
1	Molecular weight markers
2	pSV_2APap - Bam HI digest
3	pG7CD23Pap(a)(forward orientation clone)-Sac I digest
4	pG7CD23Pap(b)(reverse orientation clone)-Sac I digest
5	Clone 8 - Sac I digest
6	Molecular weight markers

The forward clones were now suitable for CD23 promoter assay by transfection. Subsequent analysis of these clones by transfection however, gave no detectable activity (section 3.5). Further studies on the CD23 promoter were therefore conducted using CAT as the reporter enzyme.

3.2 CD23 promoter studies part 1: Deletion mutagenesis of CD23CAT

To investigate the active regions of the CD23 promoter, a set of nested deletion mutants of the plasmid pCD23CAT (fig. 22) was constructed. This plasmid contains CD23 (Bam HI-Xho I fragment from pBCD23) and CAT (Xho I-Nsi I fragment) cloned into pBlueskript SK digested with Bam HI and Pst I (Stratagene). The nuclease BAL 31 was used for progressive deletion of the promoter in a time course reaction.

BAL 31 Nucleases

BAL 31 nucleases are extracellular nucleases purified from the culture medium of Alteromonas espejiana BAL 31. They are multifunctional enzymes exhibiting a highly specific stranded endodeoxyribonuclease activity which simultaneously degrades both 3' and 5' termini of duplex DNA without internal strand scission. Most of the DNA fragments generated have fully base paired ends but a fraction has 5' generated protruding single strands, indicating that BAL 31 acts sequentially as a 3' to 5' exonuclease followed by endonucleolytic removal of the protruding strand. Activity is absolutely dependent on the presence of calcium and therefore the reaction can be stopped by the addition of the chelating agent EGTA.

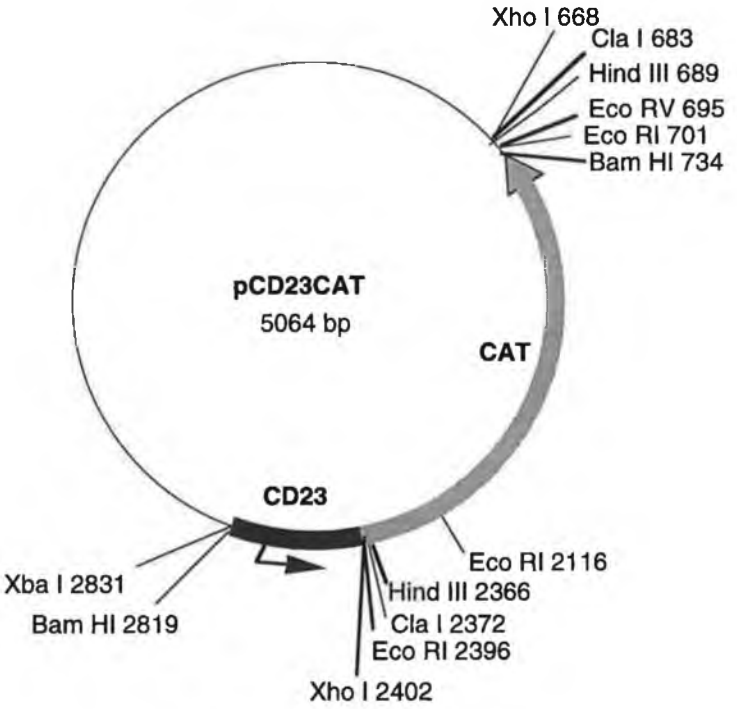


Figure 22 : pCD23CAT

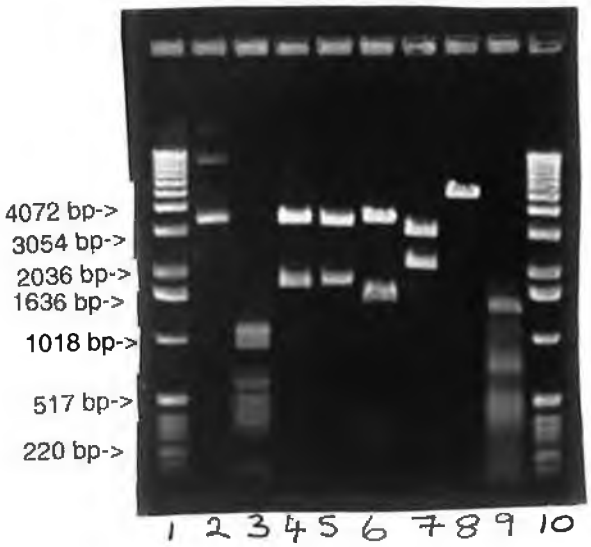
The structure of the pCD23CAT plasmid was first confirmed by restriction analysis.

Enzyme	**Predicted fragments (bp)**
Hinf I	1077,952,616,517,456, 396,369,303,109
Cla I	3375,1689
Xho I	3330,1734
Eco RI	3369,1415,280
Bam HI	2979,2085
Xba I	5064

The fragments observed on a 1% agarose gel were as expected (fig. 23).

Figure 23 : Restriction analysis of pCD23CAT

Lane :	**Sample :**
1	Molecular weight markers
2	Uncut pCD23CAT 200 ng
3	pCD23CAT - Hinf I
4	pCD23CAT - Cla I
5	pCD23CAT - Xho I
6	pCD23CAT - Eco RI
7	pCD23CAT - Bam HI
8	pCD23CAT - Eco RV
9	Partial digest - markers
10	Molecular weight markers



Construction of deletion mutants : summary (fig. 24).

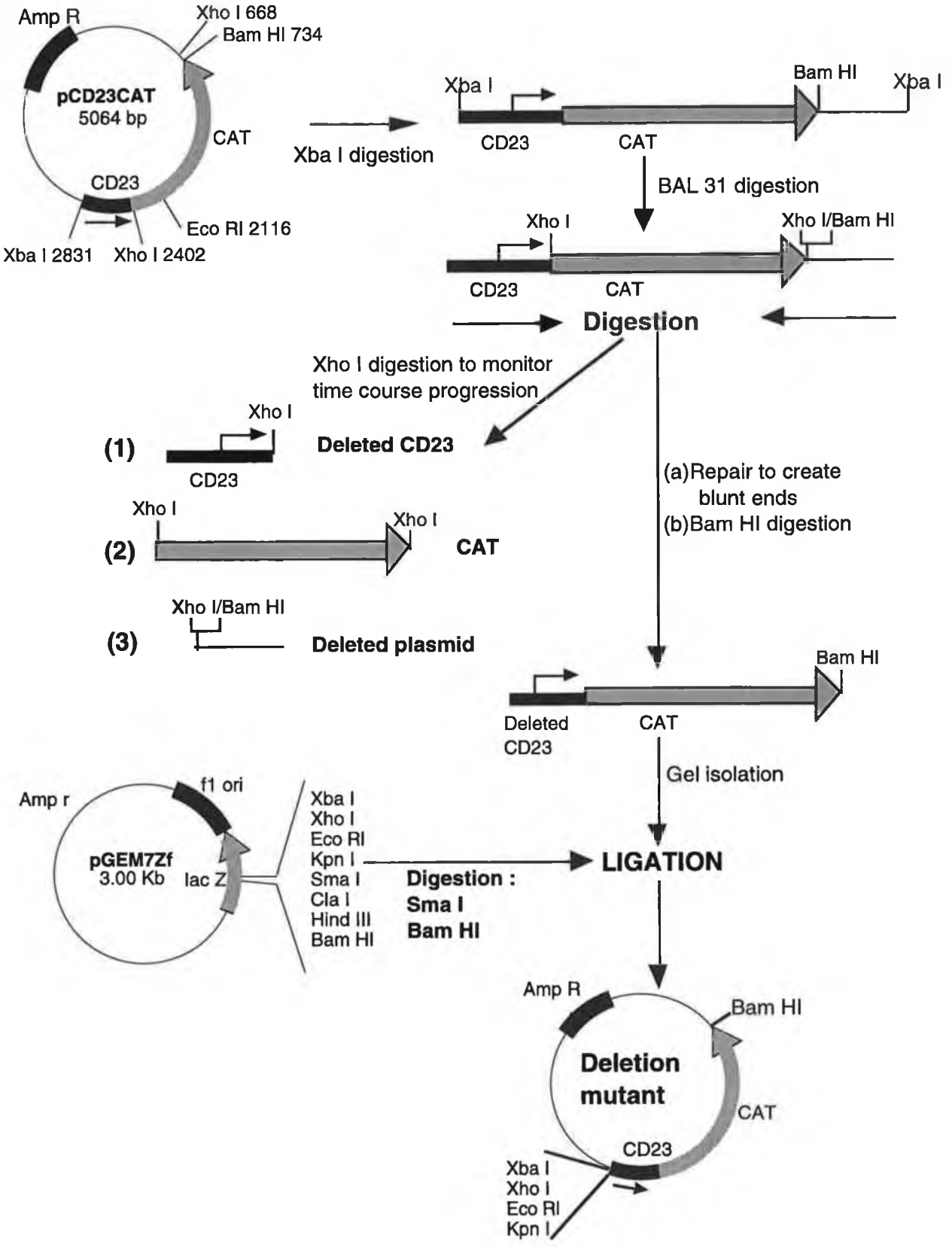
(a) pCD23CAT was digested with Xba I to linearise the plasmid at the 5' end of the CD23 promoter.

(b) The linear DNA was digested with BAL 31 nuclease in a time course reaction, with samples being removed at appropriate intervals and quenched with 20 mM EGTA .

(c) Samples of each time point were analysed by digestion with Xho I and viewed on an agarose gel. This digest cuts at the 5' and 3' ends of the CAT gene giving 3 fragments : the CD23 fragment, the CAT gene and the vector fragment (the CD23 and vector bands should decrease in size as digestion proceeds from both ends of the linear DNA, while the CAT band remains constant).

(d) The time points of interest were repaired to create blunt ends, followed by digestion with Bam HI. This cuts out the fragments containing the CD23CAT fragment for subcloning. These can then be cloned into either pGem7Zf or pBlueskript SK (digested with Bam HI and Sma I).

Figure 24 : BAL 31 Deletion Mutagenesis



Results :

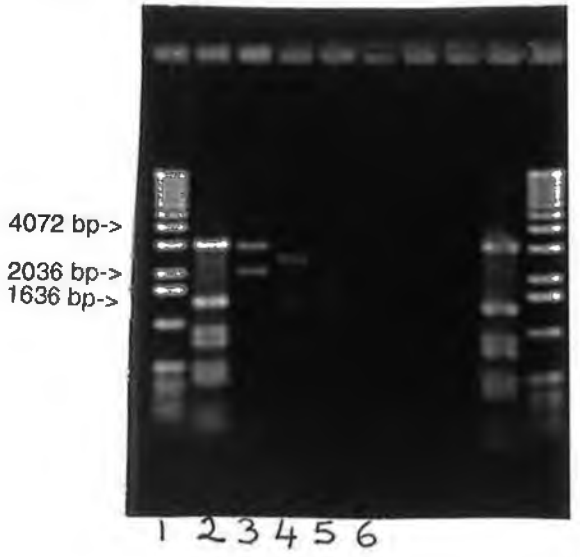
1. Analysis of enzyme activity.

Preparations of BAL 31 show considerable variability in activity, depending on batch, length of time stored, supplier etc. The CD23 promoter fragment of interest is 415 bp in length (-335+80) so the time course reaction should allow digestion to the extent that would give less than 415 bp digested (otherwise the deletion would have proceeded into the start of the CAT gene, rendering the construct useless for promoter analysis).

The activity of the BAL 31 preparation was analysed by using a series of enzyme dilutions and time points. The neat preparation of enzyme (1 U/µl) gave an activity that was too high - as can be seen from the gel (fig. 25) after only 5 min approx. 800 bp had been digested.

Figure 25 : Analysis of BAL 31 enzyme activity.

Lane :	Sample :
1	Molecular weight markers
2	Molecular weight markers
3	Time point 0 min - Bam HI digest
4	Time point 5 min - Bam HI digest
5	Time point 15 min - Bam HI digest
6	Time point 30 min - Bam HI digest



The Bam HI digest gave 2 fragments : the CD23CAT fragment and the plasmid fragment (undigested at T = 0 min).

The dilution of enzyme selected was 1/10 (0.1 U/µl) with time points T = 0, 10, 20, 30, 40, 50 min. 2µl of diluted enzyme was used in each time course reaction.

Figure 26 : BAL 31 time course reaction

Lane :	Sample :
1	Molecular weight markers
2	Time point 0 min - Bam HI digest
3	Time point 10 min - Bam HI digest
4	Time point 20 min - Bam HI digest
5	Time point 30 min - Bam HI digest
6	Time point 40 min - Bam HI digest
7	Time point 50 min - Bam HI digest
8	Molecular weight markers

The CD23CAT fragments from each time point were gel purified for cloning into pGem7Zf (Bam HI-Sma I digested). Several methods were used for gel purification of the insert : spin-x column chromatography from Seakem agarose, electroelution and low melting point agarose.

Ligation results :

1. Transformation of 100 ng pGem7Zf (Bam HI/Sma I digest) showed greater than 100-fold increase in the number of blue colonies recovered after ligation.
2. pGem7Zf (Bam HI/Sma I digest) was ligated to gel isolated time course DNA. Of the resulting transformants, 50% were white.
3. Distilled water was used as the negative control.

Controls :

(1) Transformation control : uncut pGem7Zf (50 ng) gave blue colonies as expected when plated on media containing x-gal.

(2) Repair control : pGem7Zf (Bam HI digested, repaired and ligated) - if the repair was successful this plasmid when transformed and plated on media containing x-gal, should show increased white colonies after repair. A 4-5 fold increase in colony number was observed after repair.

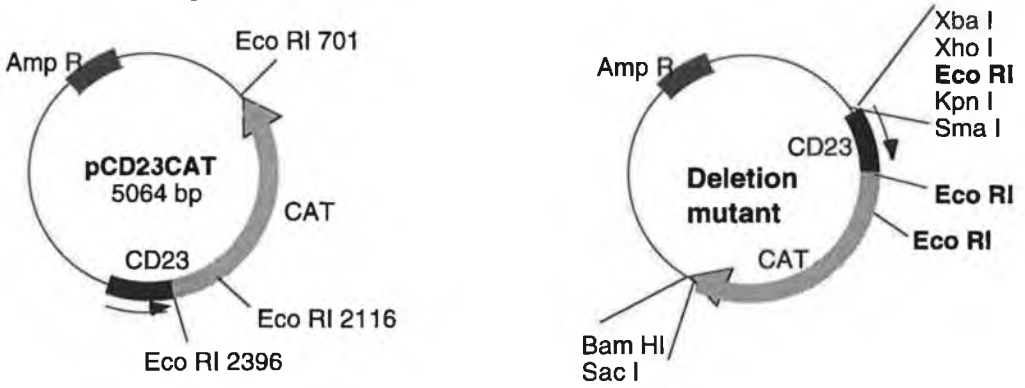
(3) Ligation controls: (a) Cohesive - pGem7Zf (Bam HI digested) +/- ligase.
(b) Blunt - pGem7Zf (Sma I digested) +/- ligase.

Both samples showed greater than 100 fold increase in the number of colonies after ligation.

(4) Cloning control : Hinf I digested pGem7Zf repaired and ligated into pGem7Zf digested with Sma I should result in clones with inserts of varying sizes.

After ligation and analysis of the white transformants it was found that 20% contained inserts.

Figure 27 : Analysis of clones :



Digestion with Eco RI shows if an insert is present and distinguishes between clones and the parent plasmid pCD23CAT (fig. 27).

Plasmid	**Expected fragments from an Eco RI digest (bp)**
pGem7Zf	3000
pCD23CAT	3369, 1415, 280
Clones	4316, 280, <411 (depending on the extent of digestion)

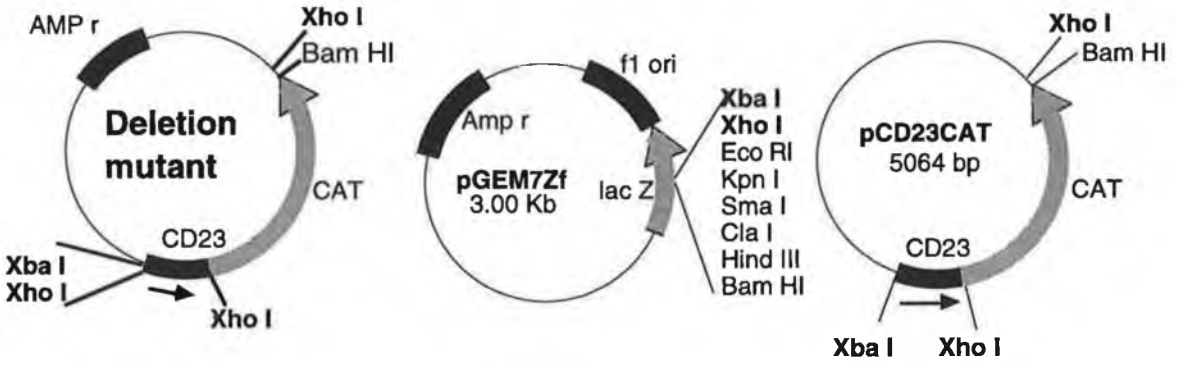


Figure 28.

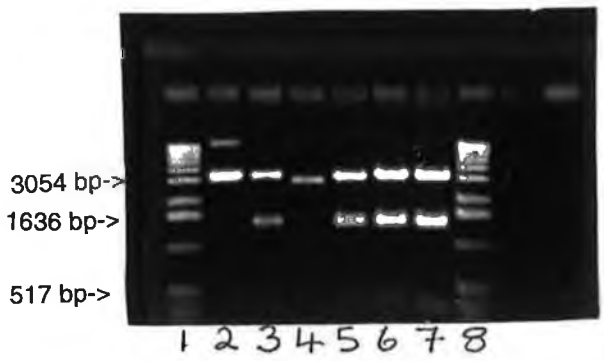
Digestion with Xba I/Xho I allows direct comparison of the insert sizes to the CD23 fragment from pCD23CAT (fig. 28) and thus shows the extent of deletion.

Plasmid	**Expected fragments from an Xho I/Xba I digest**
pGem7Zf	3 kb
pCD23CAT	3 kb, 1.7 kb, 429 bp
Clones	3 kb, 1.7 kb, < 429 bp (depends on the extent of deletion)

Transformants were analysed by miniprep of DNA, followed by restriction analysis.

Figure 29 : restriction analysis of putative deletion mutants

Lane :	**Sample:**
1	Molecular weight markers
2	uncut pCD23CAT
3	miniprep a - Eco RI digest
4	miniprep b - Eco RI digest
5	pCD23CAT - Eco RI digest
6	miniprep c - Eco RI digest
7	miniprep d - Eco RI digest
8	molecular weight markers

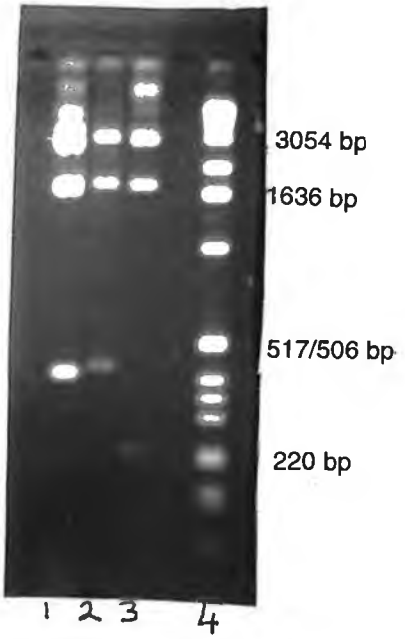


As can be seen from the gel (fig. 29), most of the white transformants analysed were due to the parent plasmid pCD23CAT. This appears to have been carried over in the gel isolation step in trace amounts, and transformed very efficiently.

Several attempts were made to clone the time points but despite screening over 200 colonies by miniprep, only one clone was isolated (miniprep 48).

Figure 30 : Restriction analysis of deletion mutant CD23CATδ1

Lane :	Sample :
1	pCD23CAT - Xho I/Xba I
2	miniprep 27 - Xho I/Xba I
3	miniprep 48 - Xho I/Xba I
4	Molecular weight markers



The expected size of the CD23 fragment from the parent plasmid in this digest is 429 bp. In clone 48 the insert size appears approx. 220 bp. This indicates a deletion of approx. 200 bp (CD23CATδ1). A deletion of this size would remove both of the EBNA-2 responsive elements upstream of the promoter.

This would be expected to render the construct unresponsive to transactivation by EBNA-2 (see transfection results 3.5).

Given the unsatisfactory yield of deletion constructs using this method, an alternative was employed.

3.3 CD23 promoter studies part 2: Cloning by PCR

Objective :

The objective was to PCR amplify specific CD23 promoter sequences for insertion upstream of the CAT gene for use in transfection experiments. The activity of the promoter fragments in these constructs could then be measured by co-transfection with plasmids encoding EBNA-2/EBNA-3, followed by assaying for CAT. Promoter sequence elements e.g. the EBNA-2 responsive elements could also be amplified and cloned upstream of heterologous promoters in CAT vectors and the level of activity measured to examine the effects of these sequence elements.

Overall Strategy :

The first step was the construction of a suitable CAT vector i.e. a plasmid containing the CAT gene flanked with restriction sites useful for cloning. This was achieved by subcloning the CAT gene into pGem7Zf as a Hind III fragment - pG7CAT. This results in a construct containing the CAT gene flanked by Bam HI and Sma I sites. The primer pairs designed to amplify the CD23 promoter sequences carry restriction enzyme sites at their 5' ends to facilitate cloning (Sma I and Sac I). Using the double-digested vector pGem7Zf (Bam HI-Sac I), a three-fragment ligation was carried out to clone CD23 (Sma I-Sac I) upstream of the CAT gene (Sma I-Bam HI) (fig. 36).

Strategy step 1: cloning CAT as a Hind III fragment

(1) pGem7Zf was used as the cloning vector (digested with Hind III). The vector was treated with CIP to prevent recircularisation.

(2) A plasmid which contains CAT cloned downstream of EBV DNA was used as the source of the CAT gene. The insert was prepared as a Hind III fragment and isolated from low melting point agarose.

Figure 31 : Construction of pG7CAT

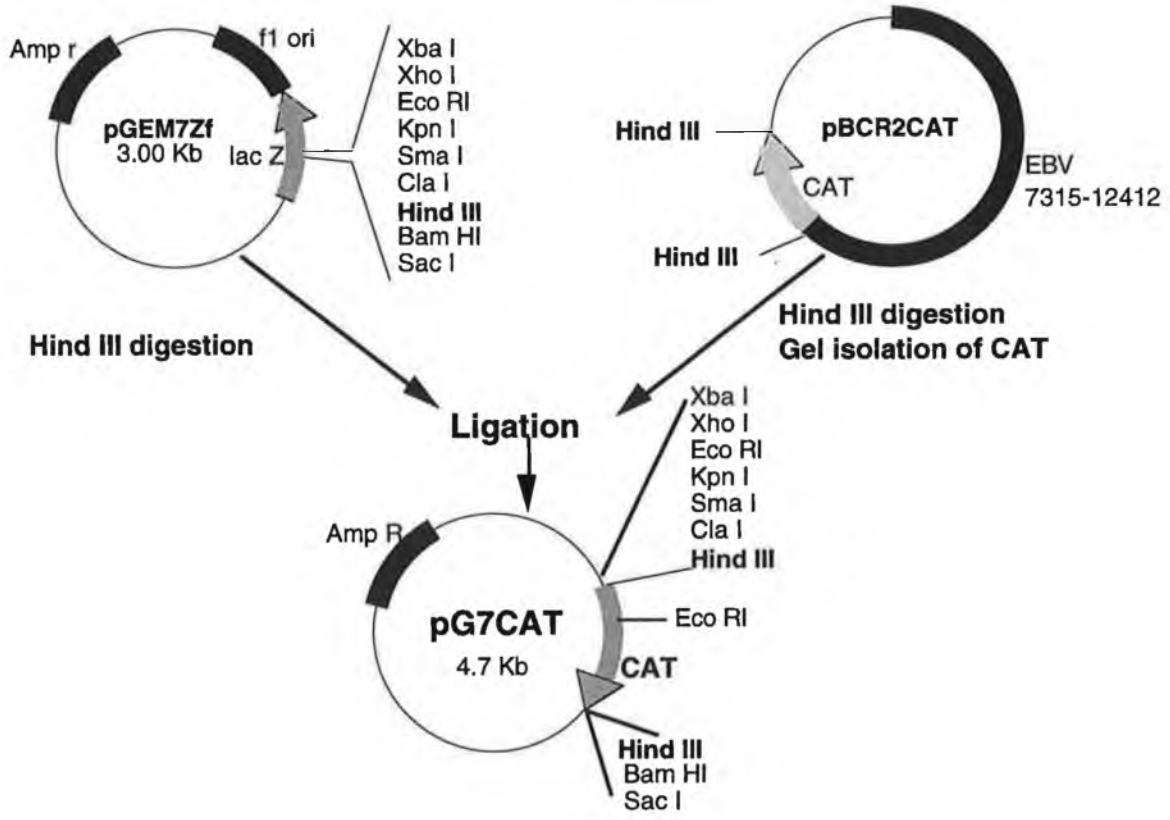
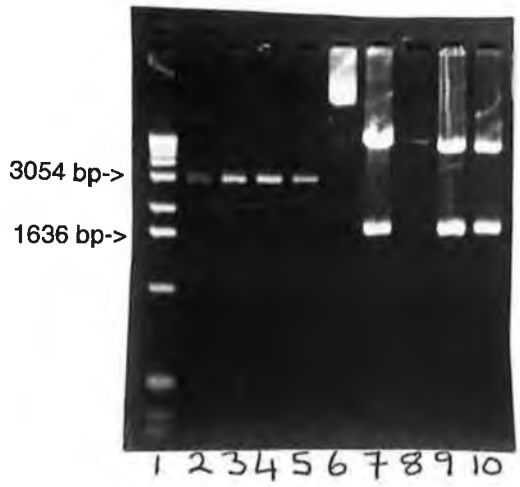


Figure 32 : Gel isolation of CAT gene



Lane :	Sample :
1	Molecular weight markers
2	uncut pGem7Zf
3-5	pGem7Zf - Hind III digest
6	uncut CAT plasmid
7, 9, 10	CAT plasmid - Hind III digest

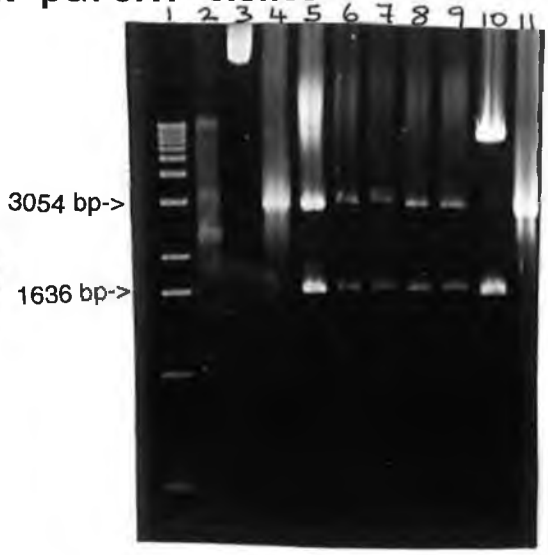
Ligation results :

After ligation of the vector and CAT insert, 20% of the transformants were white and of these, 12 were selected for restriction analysis.

Predicted fragments from restriction digestion (bp) :

Plasmid	Hind III	Eco RI	Rsa I
pGem7Zf	3000	3000	1821, 1179
CAT plasmid	8279, 1708	5100, 4606, 281	2654, 2095, 1225, 708, 676, 567, 538, 425, 400, 260, 224, 215.
pG7CAT clone	3000, 1708	4429, 279.	2133, 1179, 708, 538, 150.

Figure 33 : Analysis of recombinant pG7CAT clones

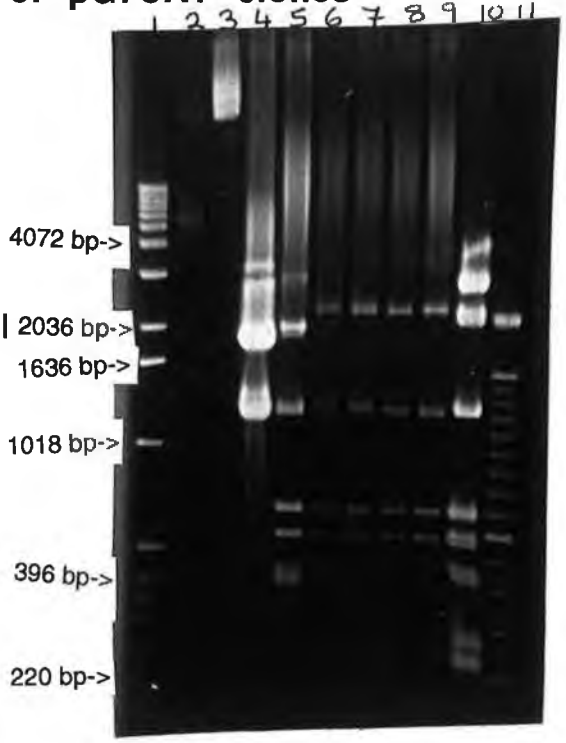


Lane :	Sample :
1	Markers
2	uncut pGem7Zf
3	uncut CAT plasmid
4	pGem7Zf - Hind III
5-9	pG7CAT minipreps - Hind III
10	CAT plasmid - Hind III
11	pGem7Zf - Hind III

As can be seen from the gel (fig. 33), the pG7CAT minipreps showed the expected insert at 1.7 kb.

Figure 34 : Restriction analysis of pG7CAT clones

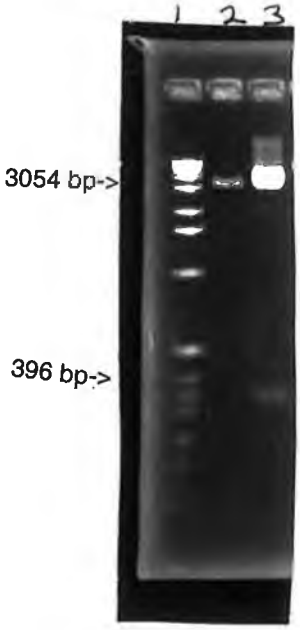
Lane:	Sample :
1	Markers
2	pGem7Zf uncut
3	CAT plasmid uncut
4	pGem7Zf - Rsa I
5 - 9	pG7CAT minipreps - Rsa I
10	CAT plasmid - Rsa I
11	Markers



Five clones were isolated (fig. 34) - four in the desired orientation (lanes 6-9) and one in the reverse orientation (lane 5).
The Rsa I digest for the reverse clone gave a different banding pattern to the forward clones which were all the same.

Figure 35 : Restriction analysis of pG7CAT

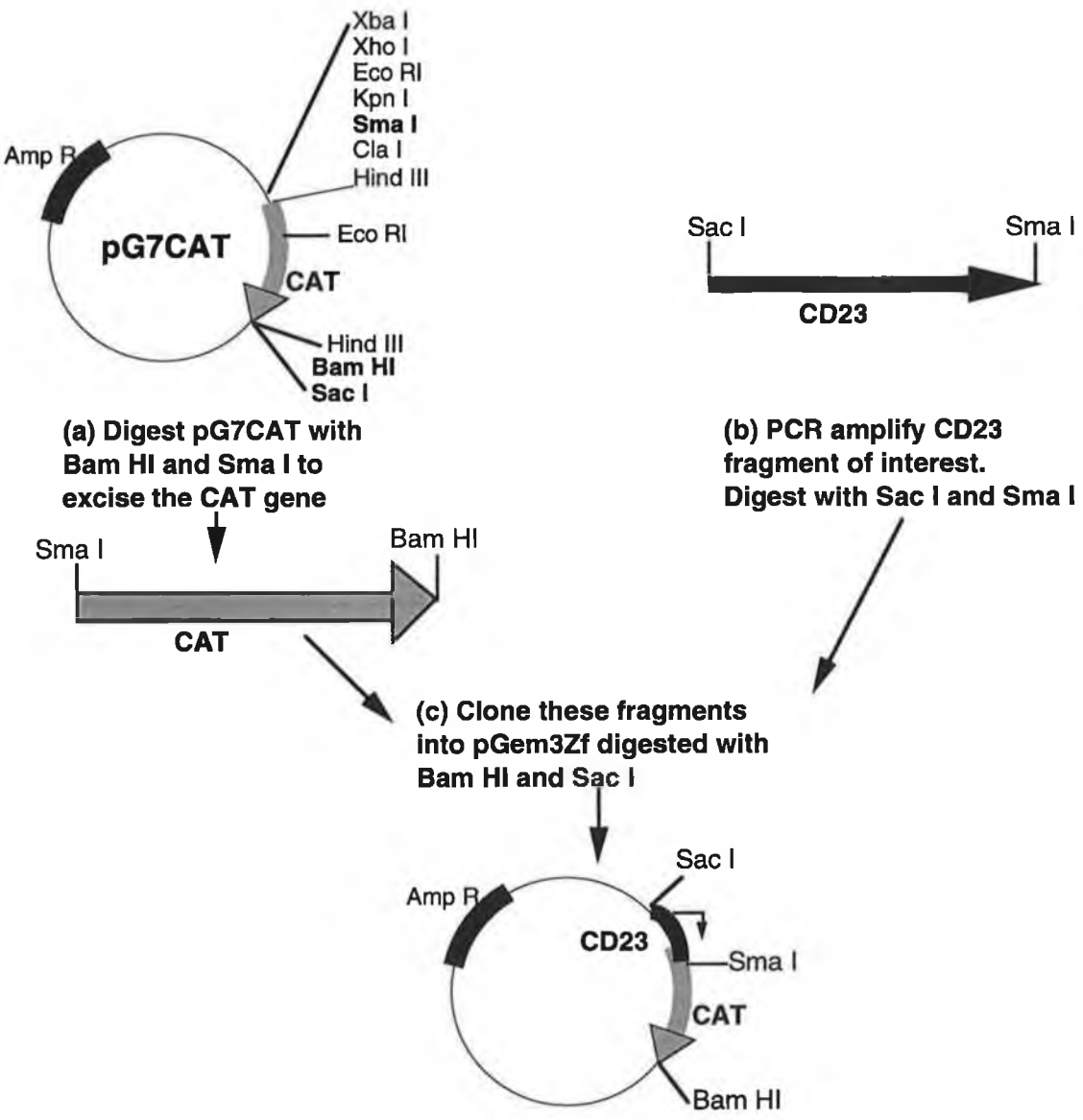
Lane :	Sample :
1	Markers
2	pGem7Zf - Eco RI
3	pG7CAT - Eco RI



Cloning Strategy step 2: cloning CD23

CD23 promoter sequences were PCR amplified using primer pairs with Sma I and Sac I sites at their ends and cloned upstream of CAT (fig. 36).

Figure 36 : Construction of CD23CAT plasmids



CD23 sequence (Suter, *et al*, 1987):

-332

GATCCCAACTCCAGGCCGTCCTTCTAACCCAAGAGGCCCTGCCTCTGCCT
AGAGCCTTCCGTGGCTCCCCAGGGCC**CTCTGTGATCGGCCATAGT**GGTATGATT
CAGTGTGCAGTAACAGTGGT**TCACATCTTGACGCTACC**ACTCACCTCCTTCAG
CCCTGTGGGAACTTGCTGCTTA**ACATCTCTAGTTCTCACCCAAT**TCTCTT
ACCTGAGAAATGGAGATAAAATAACACGGACTTCACCGGGTGTGGGGAGCACCAGG
AGAGGCCATGCGTGTAATGTTATCCGGGTGGCAAGCCCATATTTAGGTCTATGAAA
ATAGAAGCTGTCAGTGGCTCTACTTTCAGAAGAAAGTG**TCTCTCTTCCTGCTTA**
AACCTCTGTCTCTGACGGTCCCTGCCAATCGCTCTGGTC

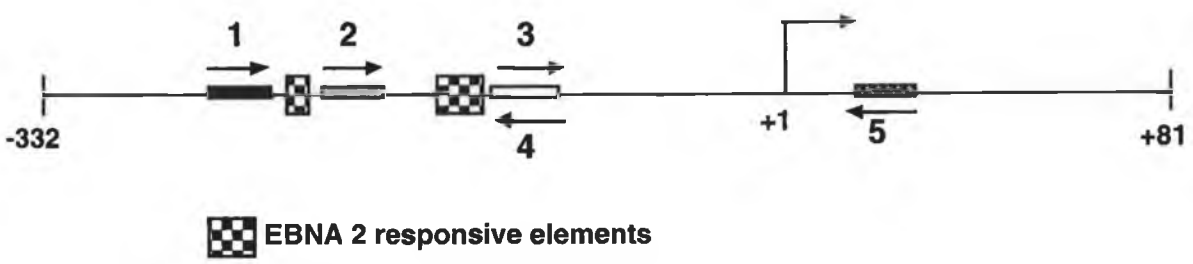
+81

EBNA 2 Responsive Elements : CAGTGTG

CCTGTGGGAACTTGC

Primer sequences are in bold type and underlined.

Figure 37 : Location of primers on the CD23 promoter sequence



For primer sequences see materials and methods section 2.21.

Figure 38 : PCR amplification of CD23

Lane :	Sample :
1	Molecular weight markers
2	CD23 PCR - primers 1+4
3	CD23 PCR - primers 1+5
4	CD23 PCR - primers 3+5
5	CD23 PCR - primers 2+5
6	Molecular weight markers

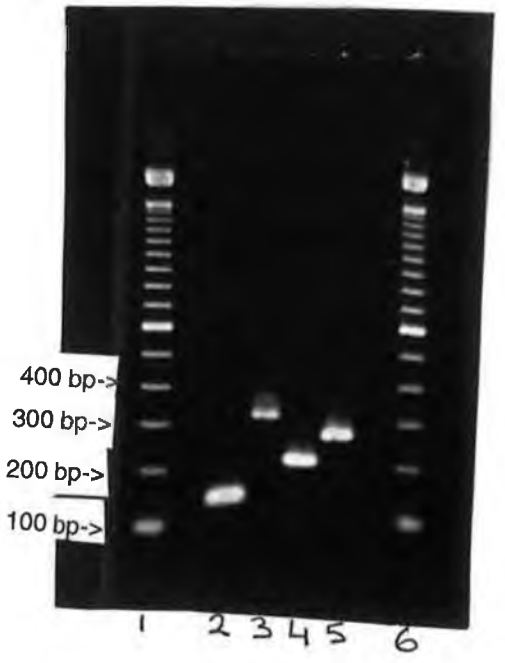


Figure 39 : PCR amplification of CD23

Lane :	Sample :
1	Molecular weight markers
2	CD23 PCR - primers 2+4

Primer combination	Expected product size (bp)
1 and 5	312
1 and 4	132
2 and 5	266
2 and 4	85
3 and 5	206

The gels show (fig. 38 and 39) that the sizes of the fragments obtained by PCR amplification of CD23 were as expected.

The vector pGem3Zf was digested with Bam HI and Sac I to clone the PCR products and the CAT gene.
The CAT insert was prepared by digesting pG7CAT with Bam HI and Sma I followed by a gel isolation.
The PCR products were gel isolated and digested with Sac I and Bam HI.

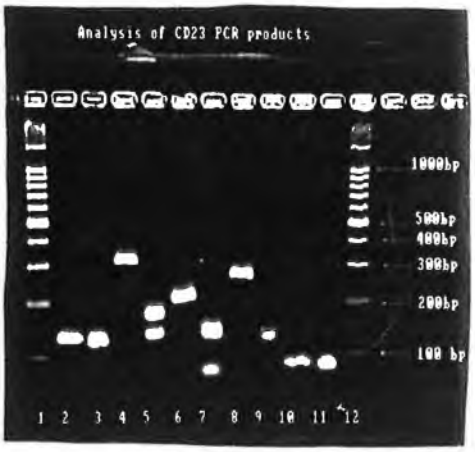
Ligations were set up for each of the PCR products with controls as before:
No white colonies were observed in any case.

Although this experiment was repeated several times, no white colonies resulted. A possibility for the failure of this experiment was that the digests of the PCR products were not successful (some restriction enzymes such as Sal I fail to cut when they are close to the ends of a DNA molecule). To investigate this possibility, the PCR products were digested with both enzymes - Sma I and Sac I - individually, and run on a high percentage agarose gel (3.5%) to compare the sizes with the products before restriction.

Analysis of the published sequence for the CD23 promoter (Suter, *et al*, 1987) showed there should be no internal Sma I site in this sequence. However, digestion of the PCR products with Sma I contradicted this observation (fig. 40).

Figure 40 : Restriction analysis of PCR products
3.5 % agarose.

Lane :	**Sample:**
1	Molecular weight markers
2	PCR product (primers 1+4)
3	PCR product (primers 1+4) - Sma I digest
4	PCR product (primers 1+5)
5	PCR product (primers 1+5)- Sma I digest
6	PCR product (primers 3+5)
7	PCR product (primers 3+5)- Sma I digest
8	PCR product (primers 2+5)
9	PCR product (primers 2+5)- Sma I digest
10	PCR product (primers 2+4)
11	PCR product (primers 2+4)- Sma I digest
12	Molecular weight markers



As can be seen from the gel, three of the PCR products appeared to contain an internal Sma I site. The other two PCR products were analysed on a higher percentage gel and it was noted that Sma I cuts these products as expected - with 5 bases cleaved from the ends as expected (not shown).

The PCR products were also digested with Xma I - this has the same recognition site as Sma I. The same result was observed, eliminating the possibility that the Sma I enzyme preparation was contaminated with another enzyme or that samples had been mixed up.

To investigate if this apparent Sma I site was present in the plasmid from which the CD23 promoter sequences were amplified i.e. pCD23CAT, this plasmid was digested with Sac I and Sma I.

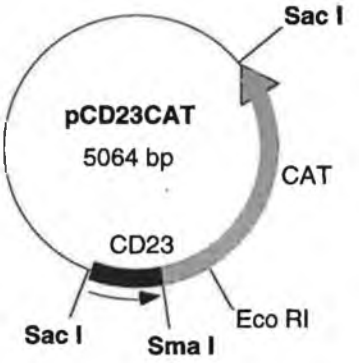


Figure 41. Sma I and Sac I sites in pCD23CAT.

From sequence information the predicted sizes of fragments obtained from digestion of pCD23CAT (fig. 41) are :

Sma I	5064
Sac I + Sma I	2934, 1660, 470

If an additional Sma I site is present in the CD23 sequence, the 470 bp band would be expected to be replaced by 2 smaller bands.

Figure 42 : Restriction analysis of CD23CAT

Lane :	Sample :
1	Markers
2	pCD23CAT uncut
3	pCD23CAT - Xba I
4	pCD23CAT - Sma I
5	pCD23CAT - Sma I/Sac I
6	Markers

As can be seen from the gel (fig. 42), there appears to be an additional internal Sma I site present in the CD23 sequence of this plasmid. Instead of linearising the plasmid as predicted, the Sma I digest gave 2 bands, one of which was approx. 210 bp. The predicted sizes of the fragments from a Sma I/Sac I double digest are 2934 (plasmid), 1660 (CAT).and 470 bp (CD23). The fragments observed were : approx. 2.9 kb, 1.65 kb, 280 bp and 200 bp. This indicates that an additional Sma I site is present in the CD23 promoter sequence.

As the pCD23CAT plasmid was constructed originally by using a sequence amplified by PCR from a B lymphoma cell line (DG75) it is possible that a clone was isolated which contained a mutation resulting from a misincorporation in the PCR reaction. To investigate this further, genomic DNA was amplified from DG75 cells and analysed using restriction digests.

A rapid method for the isolation of genomic DNA was used : 10^4 cells were pelleted by centrifugation, washed in PBS and boiled in 50µl of distilled water for 15 min. This lysate was then used in the PCR reaction. The biggest of the PCR products was amplified from genomic DNA (312 bp product amplified using primers 1 and 5).

Figure 43 : genomic CD23 amplification from DG75

2% agarose

Lane :	**Sample:**
3	CD23 amplified from pCD23CAT
4	Negative control - no template
5	Negative control - no primers
6-8	CD23 amplified from genomic DNA
9	Molecular weight markers

The PCR product from the genomic amplification was then analysed by digestion with Sma I, and compared to the results obtained from the PCR product from pCD23CAT.

Figure 44 : Analysis of genomic CD23 PCR products

Lane :	Sample:
1	Molecular weight markers
2	312 bp product from pCD23CAT
3	312 bp product from genomic DG75 DNA
4	312 bp product from pCD23CAT (Sma I digested)
5	312 bp product from genomic DG75 DNA (Sma I digested)
6	Molecular weight markers

From this gel (fig. 44) it appears that the sequence difference is not an artefact of PCR but is also present in the genome of DG75 cells. This result was reproducible.

Analysis of results : mapping the location of the potential sequence mutation.

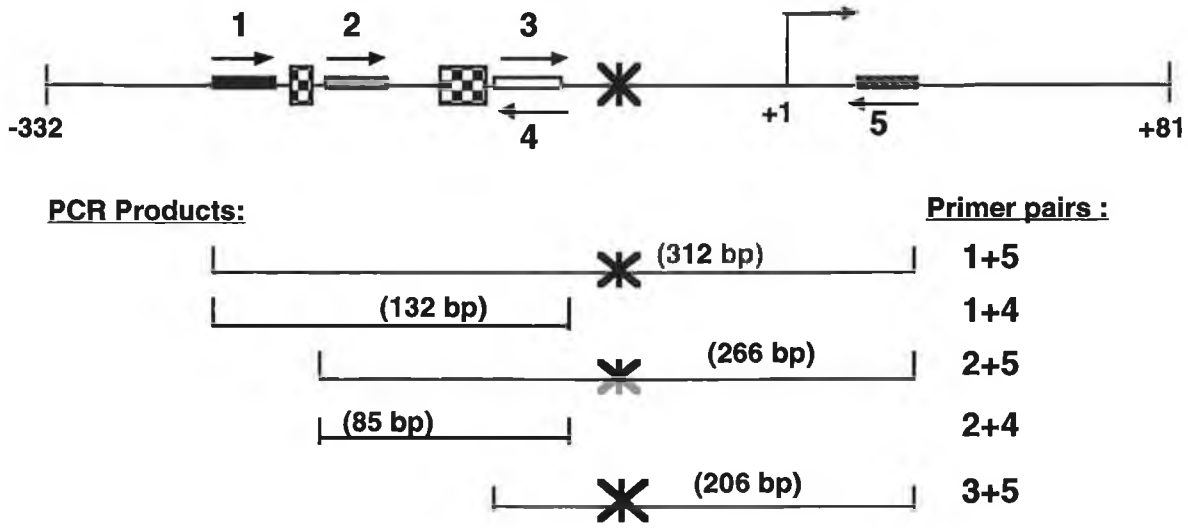


Figure 45.

PCR products	Approx. size of fragments from a Sma I digest
312 bp	180, 130 bp
132 bp	132 bp
266 bp	130, 130 bp
85 bp	85 bp
206 bp	130, 70 bp

From this restriction analysis it appears that the potential Sma I site is located approx. 130 bp from the 5' end of the reverse primer (primer 5). Examination of the CD23 sequence at this location reveals this sequence : (-92) T**C**A**CCGGG**TGT (-82). A single base change from A to C, or deletion of the A could have generated the observed Sma I site (**CCCGGG**).

Digestion of the pCD23CAT plasmid confirmed this : the Sma I digest yielded fragments approx. 4800 and 210 bp in length. If the theoretical Sma I site is present then the digestion of pCD23CAT with Sma I would give the following :

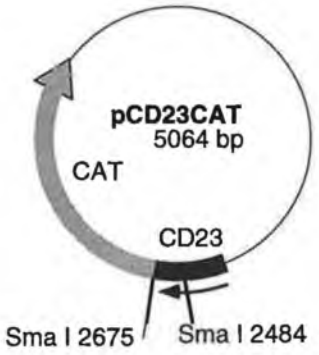


Figure 46.

An alternative strategy must be found for cloning the PCR products : either by using a T vector or by repairing and cloning into a blunt restriction site e.g. the Sma I site of pG7CAT.

In order to assay the activity of the CD23 promoter in the constructs, co-transfections with EBNA-2/EBNA-3 were carried out in a range of cell backgrounds, including the EBV-negative Burkitt's lymphoma cell line, DG75. The successful growth of this cell line depends very much on the batch of fetal calf serum used to supplement the growth medium. To establish optimum growth conditions for DG75, a set of serum tests were set up.

3.4 Serum tests.

Serum samples from three different sources were used (Gibco, Advanced protein products and Hyclone), as well as a semi-synthetic substitute (Fetal Clone I from Stratagene). RPMI-1640 was supplemented with 10% serum in each case and the growth of cells was measured over a week. The results are as follows :

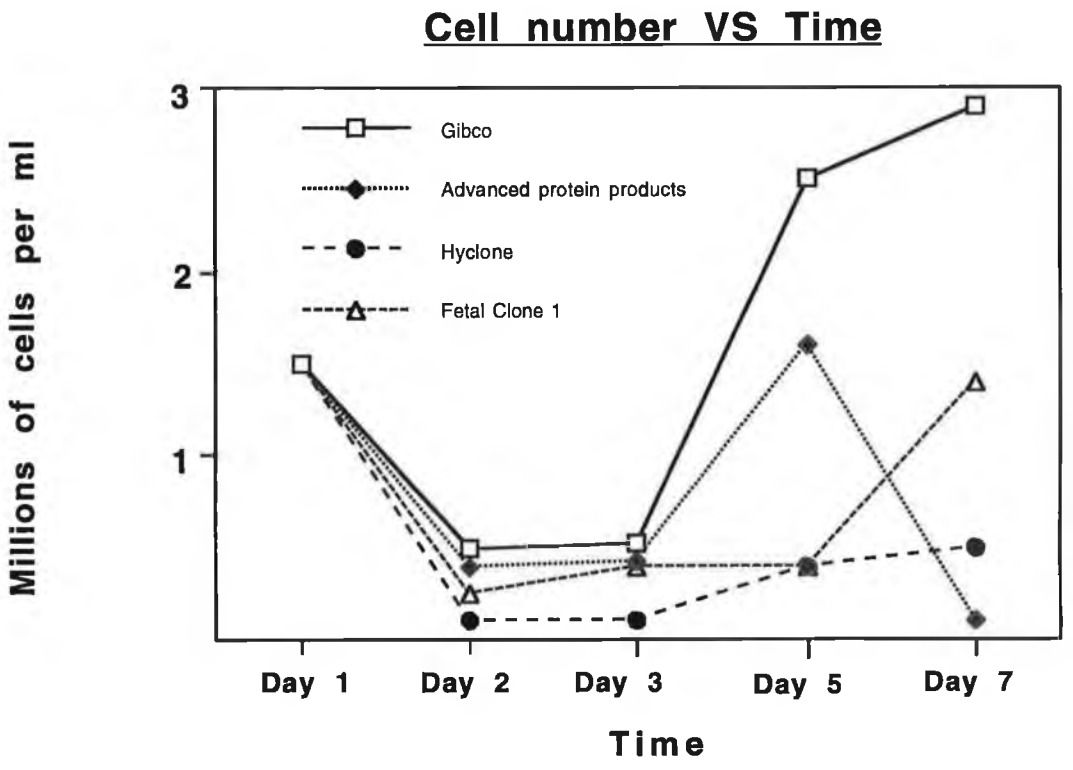


Figure 47.

All subsequent culture of DG75 was carried out in media supplemented with 10% serum from Gibco Batch number 40G9635F.

3.5 Transfections.

To examine the regulation of the CD23 promoter by EBV latent proteins EBNA-2 and EBNA-3A, co-transfection assays were carried out in a range of cell backgrounds using the CD23 promoter-reporter constructs and the appropriate EBNA expression vectors. Each cell line was transfected with a plasmid containing the CAT gene under the control of the CMV promoter (pCMVCAT). This is the positive control for the CAT assay and should be detected in all cell lines transfected (CAT activity indicating successful transfection).

Placental alkaline phosphatase (pSV2APap) was used as the internal transfection control - an internal control functions as a positive indication that transfection has occurred. In samples where CAT activity is expected to vary (e.g. in the case of EBNA-2 upregulation) it is necessary to show that any variation is not due to differences in transfection efficiencies between samples. Hence the inclusion of equal quantities of PAP DNA in each transfection should indicate the efficiency of transfection (if transfection efficiencies between samples are comparable, then the amount of PAP detected should also be comparable).

The plasmids carrying CAT under the control of the entire and deleted CD23 promoter fragments (pCD23CAT and pCD23CATδ1) were also transfected alone, and in combinations with EBNA 2 and EBNA 3A.

The construct containing the PAP gene under the control of the CD23 promoter was tested alone, and in combinations with EBNA 2 and EBNA 3A. In this case, the internal transfection control used was β-galactosidase.

All harvesting and reporter assays were carried out 48 hours post-transfection (as outlined in section 2.33). The resulting cell extracts do not contain identical numbers of cells and so must be normalised for protein content before analysis. This ensures that any variations in reporter enzyme expression seen are not due to variations in protein concentrations between samples, allowing samples to be compared directly. This is done using the Biorad protein microassay.

In summary, the results are as follows :

(a) Hep G2 cells were found to have an endogenous heat stable PAP activity which means pSV2APap cannot be used as a transfection control. Attempts to transfect these cells appear to have failed (no CAT activity detected from the pCMVCAT control). For this reason, no further research was carried out using this cell line.

(b) SW480 cells were successfully transfected (PAP and CAT activities detected). No transactivation of the CD23 promoter by EBNA-2 was seen in this cell line.

(c) In the T cell lymphoma line, HuT 78, transfection efficiencies varied widely between experiments. Successful transfection was achieved in one case (PAP activity was detected in transfected cell extracts). CAT activity was seen in the extract from the cells transfected with pCMVCAT but no transactivation of the CD23 promoter was apparent in this cell line.

(d) In DG75, EBNA-2 was seen to upregulate CD23 gene expression by a factor of 4-5. This effect was repressed by EBNA-3A, with CAT reporter enzyme activity returning to the basal levels. The deletion mutant of the CD23 promoter, pCD23CATδ1, was not sensitive to transactivation by EBNA-2 (this is as expected as this construct is deleted for the DNA elements known to be EBNA-2-responsive).

(e) The construct pCD23Pap showed no activity in any of the cell lines tested, either alone, or in combination with plasmids expressing EBNA-2.

Results :

A. Biorad protein assay.

Standard curve :

BSA (µg)	O.D. @ 595 nm.
0.00	0.000
1.40	0.102
2.70	0.185
4.10	0.290
8.20	0.495
16.40	0.837

Biorad Protein Assay Standard Curve

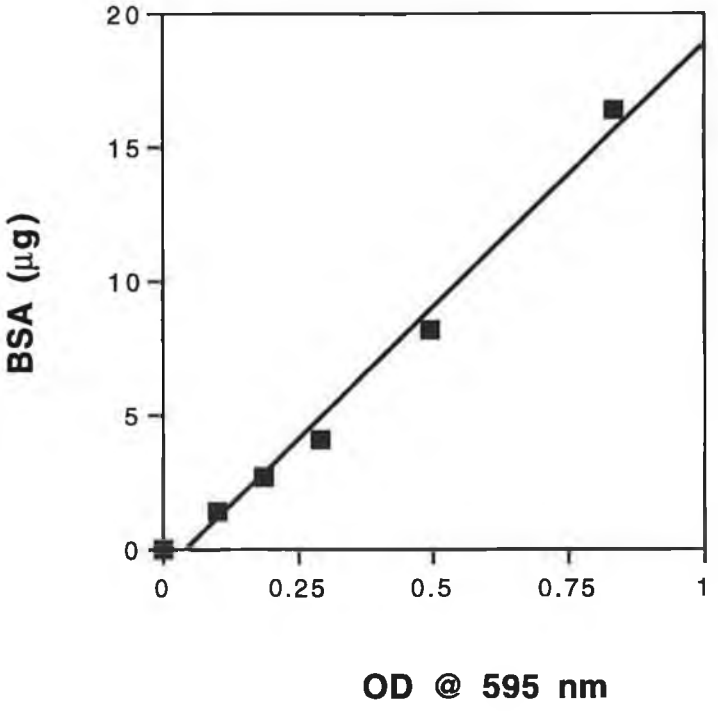


Figure 48.

Transfections set 1 : Results

The conditions established for the transfection of DG75 were 250µl of cells (10^7 cells), 30 µl (up to 90µg) of DNA, 220 V, 960 µF.

Protein Assay : 10µl cell extract + 150µl water + 40µl dye reagent.

Samples (plasmids used in each transfection):

A : CD23CAT (40µg) + PSV2APap (20 µg)

B : CD23CAT (40µg) + PSV2APap (20 µg) + 10µg pCMVEBNA2

C : CD23CAT (40µg) + PSV2APap (20 µg) + 10µg pCMVEBNA2 + 20µg pCMVEBNA3A

D : CD23CAT (40µg) + PSV2APap (20 µg) + 20µg pCMVEBNA3A

E : PSV2APap (20 µg)

(pG7CAT was used as carrier DNA in all cases - the total DNA quantity in each transfection should be the same)

Sample	O.D.@ 595 nm	Protein (µg/µl)
DG75 A	0.414	0.80
DG75 B	0.211	0.40
DG75 C	0.218	0.41
DG75 D	0.171	0.34
DG75 E	0.338	0.66
HuT 78 A	0.312	0.59
HuT 78 B	0.369	0.72
HuT 78 C	0.347	0.68
HuT 78 D	0.358	0.69
HuT 78 E	0.382	0.73
Hep G2 A	0.242	0.47
Hep G2 B	0.226	0.44
Hep G2 C	0.220	0.44
Hep G2 D	0.190	0.37
Hep G2 E	0.231	0.45

Table 1. Protein concentrations in transfected cell extracts.

Transfections set 2 : results.

Protein assay : 10µl of extract + 150µl of water + 40µl of dye reagent.

Samples (plasmids used in each transfection) :

1 : pCMVCAT (40µg) + pSV2APap (20µg)
2 : pCD23CAT (40µg) + pSV2APap (20µg)
3 : pCD23CAT (40µg) + pSV2APap (20µg) + pCMVEBNA2 (10µg)
4 : pCD23CAT (40µg) + pSV2APap (20µg) + pCMVEBNA2 (10µg) + pCMVEBNA3A (20µg)
5 : pCD23CAT (40µg) + pSV2APap (20µg) + pCMVEBNA3A (20µg)
6 : pCD23Pap (40µg)
7 : pCD23Pap (40µg) + pCMVEBNA2 (10µg)
8 : pCD23Pap (40µg) + pCMVEBNA2 (10µg) + pCMVEBNA3A (20µg)
9 : pCD23Pap (40µg) + pSV2APap (20µg)
10: pG7CAT (90µg)

(a) DG75

Sample	O.D. @ 595 nm	Protein (µg/µl)
1	0.202	0.39
2	0.282	0.54
3	0.532	1.00
4	0.244	0.47
5	0.317	0.59
6	0.218	0.41
7	0.195	0.37
8	0.258	0.50
9	0.170	0.34
10	0.331	0.66

Table 2. Protein concentrations in cell extracts from transfected DG75.

(b) HuT 78

Sample	O.D. @ 595 nm	Protein (μg/μl)
1	0.207	0.39
2	0.196	0.37
3	0.251	0.50
4	0.309	0.59
5	0.471	0.90
6	0.063	0.14
7	0.219	0.44
8	0.310	0.59
9	0.305	0.59
10	0.376	0.73

Table 3. Protein concentrations in cell extracts from transfected HuT 78.

(c) SW480

Sample	O.D. @ 595 nm	Protein (μg/μl)
1	0.408	0.79
2	0.411	0.79
3	0.345	1.68
4	0.402	0.79
5	0.430	0.82
6	0.259	0.50
7	0.368	0.70
8	0.455	0.87
9	0.388	0.73
10	0.334	0.66

Table 4. Protein concentrations in cell extracts from transfected SW480.

Transfections set 3 : Results

Protein Assay : 10µl cell extract + 150µl water + 40µl dye reagent.

Samples (plasmids used in each transfection):
A : CMVCAT (40µg) + PSV2APap (20 µg)
B : CD23CAT (40µg) + PSV2APap (20 µg)
C : CD23CAT (40µg) + PSV2APap (20 µg) + 10µg pCMVEBNA2
D : CD23CAT (40µg) + PSV2APap (20 µg) + 10µg pCMVEBNA2 + 20µg pCMVEBNA3A
E : CD23CAT (40µg) + PSV2APap (20 µg) + 20µg pCMVEBNA3A
F : pG7CAT (90 µg)
G : CD23CATδ1(40µg) + PSV2APap (20 µg)
H : CD23CATδ1 (40µg) + PSV2APap (20 µg) + 10µg pCMVEBNA2
(pG7CAT was used as carrier DNA in all cases).

Sample	O.D.@ 595 nm	Protein (µg/µl)
DG75 A	0.487	0.91
DG75 B	0.511	0.93
DG75 C	0.403	0.79
DG75 D	0.440	0.82
DG75 E	0.387	0.73
DG75 F	0.261	0.50
DG75 G	0.414	0.80
DG75 H	0.461	0.87
HuT 78 A	0.415	0.80
HuT 78 B	0.521	0.99
HuT 78 C	0.444	0.86
HuT 78 D	0.278	0.53
HuT 78 E	0.403	0.79
HuT 78 F	0.388	0.73

Table 5. Protein concentrations in extracts from transfected cells.

B. PAP Assay

Standard curve (blanked against reaction mixture without enzyme)

Phosphatase (ng)	O.D. @ 405 nm
0.00	0.000
0.05	0.126
0.10	0.189
0.15	0.288
0.20	0.398
0.25	0.491

Table 6. PAP assay standard curve.

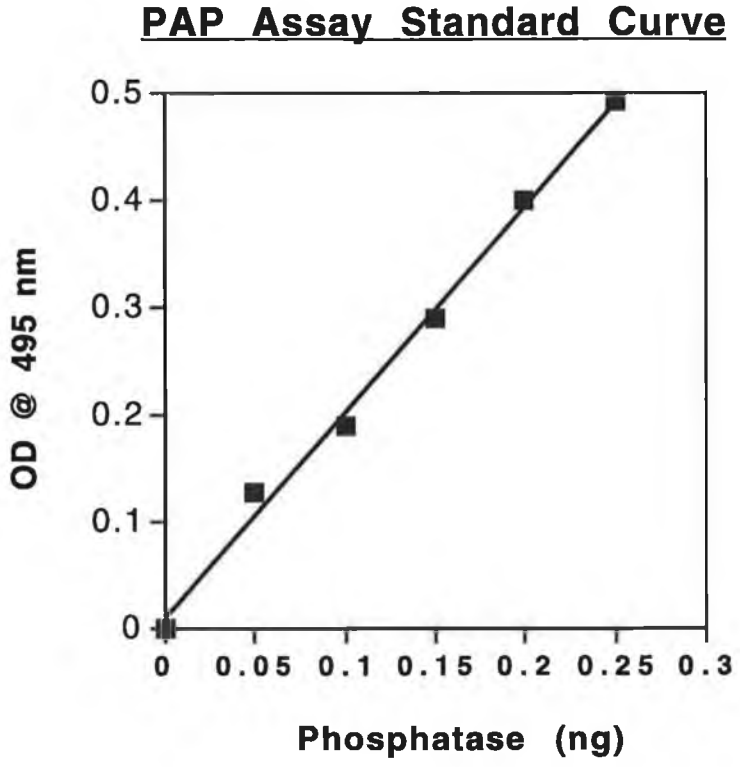


Figure 49.

Transfections set 1 : results.
PAP assay : 10µl of extract assayed in each case.

(a) DG75 - no PAP activity detected in any of the samples.

(b) HuT 78

Sample	O.D. @ 405 nm	ng PAP/µg protein
A	0.144	0.0047
B	0.160	0.0102
C	0.300	0.0189
D	0.100	0.0074
E	0.000	0.0000

Table 7. PAP concentrations in cell extracts from transfected HuT 78.

(c) Hep G2 : endogenous heat stable activity seen in all samples.

Transfections set 2 : results.
PAP assay : 20µl of extract (diluted 1/10) for DG75 samples.
10µl of extract (diluted 1/10) for SW480 samples.

(a) DG75

Sample	O.D. @ 405 nm	ng PAP/µg protein
1	0.139	0.089
2	0.168	0.083
3	0.192	0.150
4	0.127	0.069
5	0.210	0.089
6	0.019	-
7	0.018	-
8	0.015	-
9	0.012	-
10	0.020	-

Table 8. PAP concentrations in cell extracts from transfected DG75.

(b) HuT 78 : No PAP detectable in any of the HuT 78 samples.

(c) SW480

Sample	O.D. @ 405 nm	ng PAP/μg protein
1	0.283	0.186
2	0.273	0.112
3	0.261	0.228
4	0.253	0.103
5	0.277	0.118
6	0.080	-
7	0.060	-
8	0.030	-
9	0.030	-
10	0.080	-

Table 9. PAP concentrations in cell extracts from transfected SW480.

Transfections set 3 : results.

PAP assay : DG75 : 20μl of extract, HuT 78 : 20μl of extract.

Sample	O.D.@ 595 nm	ng PAP/μg protein
DG75 A	1.317	0.038
DG75 B	2.344	0.065
DG75 C	1.424	0.047
DG75 D	0.823	0.026
DG75 E	1.833	0.058
DG75 F	1.863	-
DG75 G	1.907	0.039
DG75 H	1.680	0.050
HuT 78 A	0.390	-
HuT 78 B	0.365	-
HuT 78 C	0.429	-
HuT 78 D	0.285	-
HuT 78 E	0.312	-
HuT 78 F	0.337	-

Table 10. PAP concentrations in extracts from transfected cells.

C. Phase extraction radioassay for CAT.

Samples were counted for 10 min after addition of scintillation fluid.
The background was 16.5 cpm.
The standard (101700 dpm) was read as 24383.0 cpm.

Standard curve :

Units CAT enzyme	pg CAT enzyme	cpm
0.000	0	2763.4
0.005	60	4397.7
0.010	120	6674.9
0.020	240	8024.0
0.040	480	11582.5
0.060	720	15280.9

Table 11. CAT assay standard curve

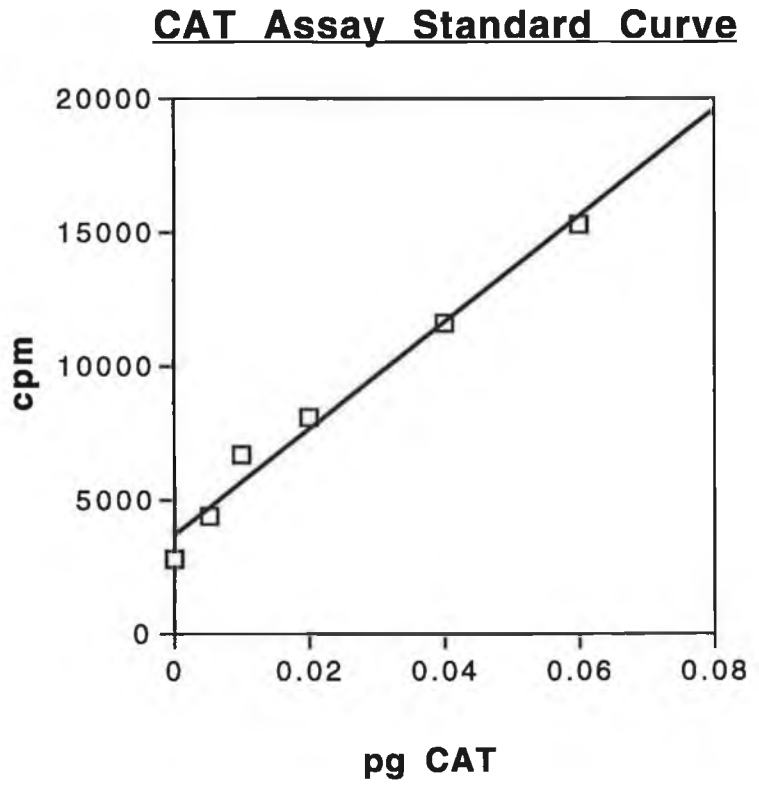


Figure 50.

Transfections set 1 : results

Samples that had shown positive transfection i.e. PAP activity, were assayed for CAT.

CAT assay :

30 µl samples assayed in each case (each sample normalised to 15 µg of total protein).

Sample	cpm
HuT 78 A	15423.3
HuT 78 B	2027.1
HuT 78 C	1466.3
HuT 78 E	2477.7

Table 12. CAT activity in cell extracts from transfected HuT 78.

Transfection with pCMVCAT gave CAT activity (table 12), but transfection with pCD23CAT alone or in combination with EBNA2 gave no detectable signal.

Transfections set 2 : results

CAT assay :

30 µl samples assayed in each case (each sample normalised to 15 µg of total protein).

(a) DG75

Sample	cpm
1	15754.7
2	6101.1
3	20574.7
4	3840.1
5	5749.8
10	3758.2

Table 13. CAT activity in cell extracts from transfected DG75.

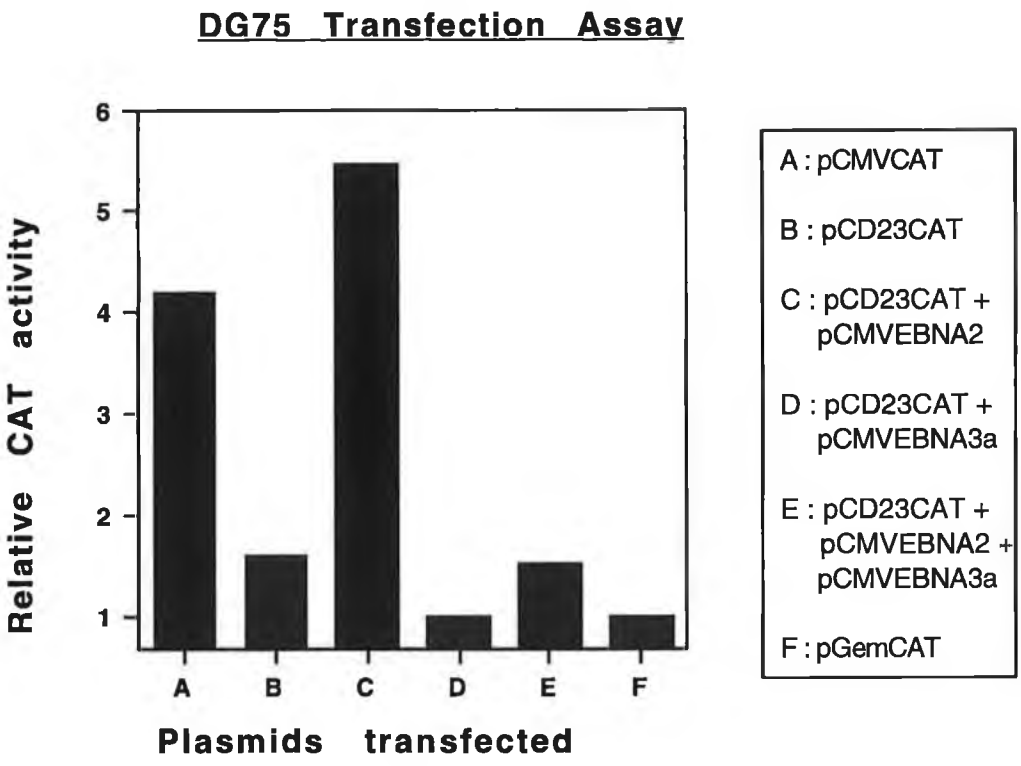


Figure 51. DG75 transfection assay 1.

(b) SW480

Sample	cpm
1	14984.9
2	6182.3
3	7510.1
4	2821.6
5	2592.8
10	4182.8

Table 14. CAT activity in cell extracts from transfected SW480.

The CMV promoter appears to be functioning in these cells, giving a detectable level of CAT.

Comparison with ELISA :

The SW480 sample was also assayed using the Boehringer CAT ELISA. The radioassay gave a result of 350 pg of CAT when corrected for background. The ELISA result for the same sample was 270 pg.

Transfections set 3 : results.

CAT assay :

5µl of sample for DG75 samples (normalised to 5µg total protein).
5µl of sample for HuT 78 samples (normalised to 5µg total protein).

Background was 15.4 cpm.
Standard was read as 24406.4 cpm (101700 dpm).

Sample	cpm
DG75 A	19232.4
DG75 B	234.1
DG75 C	870.1
DG75 D	246.0
DG75 E	257.1
DG75 F	232.6
DG75 G	244.2
DG75 H	238.1
HuT 78 A	275.7
HuT 78 B	271.2
HuT 78 C	204.7
HuT 78 D	227.6
HuT 78 E	301.4
HuT 78 F	266.2

Table 14. CAT activity in extracts from transfected cells.

DG75 Transfection Assay

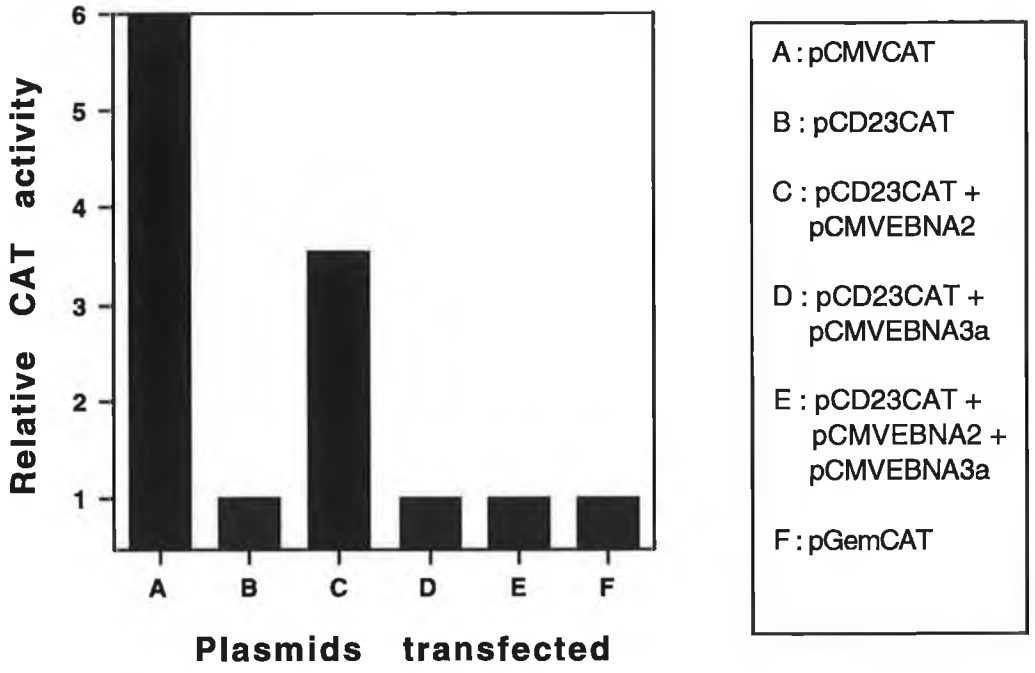


Figure 52 : DG75 transfection assay 2.

4. General Discussion :

CD23 is an early, B cell surface antigen marker induced by antigen, mitogen or IL-4 stimulation. All EBV-transformed B lymphocytes express high CD23 levels, and EBV-immortalised B cells arise from the subpopulation of cells expressing CD23 soon after infection (Azim, *et al,* 1988). EBNA-2 is essential for B lymphocyte growth transformation and has been shown to specifically upregulate mRNAs encoding CD23 (Wang, *et al,* 1991). Activation of CD23 expression could be implicated in the establishment of an autocrine loop of growth factors, the surface molecule acting as a receptor, and the processed molecule acting as a growth factor (Sarfati, *et al,* 1984). Hence EBNA-2 upregulation of CD23 expression is likely to be an important pathway for EBV-induced B cell growth transformation. The purpose of this study was to explore the regulation of CD23 gene expression by EBV latent proteins.

The basis of one of the most common approaches to promoter study is the placing of controlling elements such as promoter/enhancer sequences from the 5' end of the gene upstream of a reporter gene. Reporter enzyme activity is easily assayed, and is usually a direct indication of the activity of the upstream element. Deletions or mutations can thus be introduced into the promoter region of interest and the effects on activity studied by measuring activity of the reporter enzyme.

To facilitate measurement of the activity of the CD23 promoter, it was first cloned upstream of a suitable reporter gene. The most popular reporter used in studies of gene expression is E. coli CAT (chloramphenicol acetyl transferase). CAT is usually assayed using a radioisotopic method. To avoid the use of radioactive substrates, an alternative reporter was investigated. Henthorn *et al* have reported the use of human placental alkaline phosphatase (PAP) as a suitable reporter gene for transfection studies. This enzyme can be rapidly assayed using a simple and cheap colorimetric assay with a sensitivity comparable to CAT (Henthorn, *et al,* 1988).

Two strategies were employed for construction of a CD23PAP reporter plasmid (as outlined in section 3.1). The CD23 promoter element (-335/+80) was prepared for cloning as this is the promoter element containing the DNA sequence element shown to be transactivated by EBNA-2 (Wang, *et al,* 1991). The resultant clone (prepared by strategy 2) was used in transfection studies with plasmids encoding EBNA-2 and EBNA-3 (fig. 53).

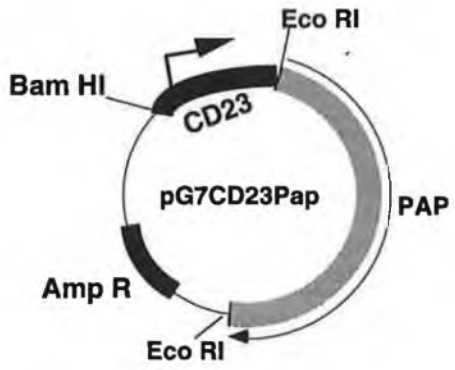


Figure 53 : pG7CD23Pap

Investigation of the CD23 promoter active regions was carried out by constructing a set of deletion mutants of the promoter for use in transfection studies. Two strategies were employed : the use of the nuclease BAL 31, and the amplification of selected regions of the promoter by PCR.

The nuclease BAL 31 was used for progressive deletion of the CD23 promoter in a time course reaction (see section 3.2). The enzyme activity was first analysed to enable selection of time points that would give an appropriate digestion (as the promoter is 415 bp in length deletions larger than this would not be useful). A suitable time course was obtained, however subsequent attempts to clone the resultant deletion mutants yielded only one clone. The resulting transformants analysed were found to contain mostly the parental pCD23CAT. This plasmid appears to have been carried over in the gel isolation steps, and as it is a supercoiled plasmid it transforms with a very high efficiency in trace amounts that would not necessarily be visible on a gel. The experimental controls also showed that the repair step should be modified as it appears to have a low efficiency. While the transformation and ligation efficiencies appear high, a blunt-ended cloning control showed a low efficiency of transformants with inserts (only 20% of those selected by blue/white screening).

The deletion mutant isolated (pCD23Papδ1) contained a deletion of approx. 200 bp (from restriction analysis) - this would remove both of the EBNA-2 responsive elements upstream of the promoter and hence should render the construct unresponsive to transactivation by EBNA-2. This was subsequently analysed in transfection studies.

A second strategy was employed due to the low yield of deletion mutants produced by this method. PCR was used to amplify CD23 promoter sequence elements of different sizes for cloning upstream of a reporter gene for use in transfection studies. Firstly, the CAT gene was cloned into a commercially available cloning vector pGem7Zf to generate a cloning vector containing the reporter gene flanked by multiple restriction sites. This was done to facilitate cloning of the CAT gene and CD23 PCR fragments in a three-fragment ligation scheme (Fig. 36). The CD23 promoter sequence elements were amplified using primer pairs with restriction sites Sma I and Sac I at their ends. The sites were incorporated into the primers to facilitate cloning of the PCR products without the need for a repair step. The enzymes were chosen as they were not present internally in the published CD23 sequence (Suter, *et al*, 1987) and were situated upstream of the CAT gene in pG7CAT. Failure to clone the PCR products and subsequent analysis led to the discovery of an internal Sma I site in the CD23 sequence (see section 3.3). Amplification of genomic CD23 from DG75 cells and subsequent restriction analysis showed that this site is present in genomic DNA and is not an artefact of PCR (the CD23 promoter used was originally cloned from PCR-amplified DNA). Examination of the published CD23 sequence and restriction analysis of the PCR products indicated that this Sma I site may be the result of a single base change from A to C or deletion of an A (position -90) (see section 3.3). The CD23 DNA originally sequenced and published was isolated from human placental cells. This apparent sequence mutation found may be unique to DG75 cells. To investigate this, genomic CD23 from other cell lines should be analysed. However, as genomic DNA isolation from other cell lines (SW480, HuT 78) was unsuccessful, PCR could not be carried out. The DG75 mutation does not appear to affect the putative EBNA-2 responsive element or create any new known sequence elements. It was concluded an alternative strategy is needed to clone these PCR products - potentially by using T vectors (Marchuk, *et al*, 1990).

The objective of these studies was to examine the regulation of the CD23 promoter by EBV latent proteins in B cells, and compare the results with those obtained in different cell backgrounds. This was done by analysis of CAT activity from the reporter constructs after transfection into a variety of cell lines. The cell lines used were DG75, HuT 78, SW 480 and Hep G2. Each cell line was transfected with a plasmid containing the CAT gene under the control of the CMV promoter. This is the positive control for the CAT assay and should be detected in all cell lines transfected. The plasmid carrying the CAT gene under the control of the CD23 promoter was also transfected alone, and in combinations with EBNA-2 and EBNA-3A. Placental alkaline phosphatase (pSV2APap) was used as the transfection control. The construct containing the PAP gene under the control of the CD23 promoter was also tested alone, and in combinations with EBNA-2 and EBNA-3A.

The CD23 promoter (-335/+80) has previously been shown to be transactivated by EBNA-2 in studies where it was cloned upstream of CAT and analysed in co-transfection assays with EBNA-2 in a B cell background (Wang, *et al*, 1991). Studies of the mechanism of EBNA-2 transactivation of the EBV TP-1 promoter have mapped an EBNA-2 responsive element (Le Roux, *et al*, 1993). Using reporter plasmids in which DNA fragments from the 5' controlling region of the TP-1 promoter were positioned upstream of CAT and co-transfected with EBNA-2 expression vectors, it has been shown that an 81 bp sequence mediates the EBNA-2 transactivation effect. This sequence which is situated between -256 and -175 bp upstream from the TP-1 mRNA CAP site, contains two copies of an 11 bp sequence motif. Deletion of the more 5' copy results in almost complete loss of EBNA-2 responsiveness from the TP-1 promoter. This 81 bp fragment has also been shown to confer EBNA-2 responsiveness on heterologous promoters (Le Roux, *et al*, 1993).

Studies have also shown that while EBNA-3A itself has no effect on the TP-1 promoter, it can repress the EBNA-2-mediated transactivation in a B cell background (Le Roux, *et al*, 1994). Homologies have been shown between sequences present in the EBV TP-1 EBNA-2 responsive element and other functionally similar sequences which are found in the CD23, the EBV LMP-1 and EBNA BC-R2 promoters (Fig. 54) (Le Roux, *et al*, 1994).

Promoter	Homology I	Homology II
TP-1	-255 C TC GTGGGAA AAT G G -241	-199 C AGTG T G -193
	-230 A CC GTGGGAA AAT A G -216	-301 C AGTG T G -295
LMP-1	-290 G TT GTGGGAA GCG G C -304	-258 C AGTG C G -264
	-215 G CT GTGGGAA TGC G G -229	-279 G AGTG C G -285
CD23	-174 C CT GTGGGAA CTT G C -160	-228 C AGTG T G -222
BC-R2	-378 G CC GTGGGAA AAA A T -364	-355 C AGTG C G -349

Figure 54. Sequence homologies in viral and cellular promoter regions.

This indicates that the mechanism of promoter transactivation by EBNA-2 may be similar for at least some viral and cellular promoters, and EBNA-3A repression of this effect may also occur in the regulation of CD23 gene expression (which this study aimed to investigate and has now shown).

The results obtained in transfection experiments using various cell lines will now be discussed in turn :

(A) Suspension cells.

(1) DG75 (B cell lymphoma).

The initial attempts to transfect these cells used pSV_2APap to establish successful electroporation conditions. In subsequent transfections PAP activity was detected as the transfection control. Positive samples were subsequently assayed for CAT activity. The results indicate that EBNA-2 upregulates the activity of the CD23 promoter, increasing the amount of CAT activity by a factor of 4-5 (see graphs of DG75 transfection assays). The EBNA-3A protein suppresses the EBNA-2-mediated upregulation of the CD23 promoter, with CAT activity returning to basal levels in the presence of both EBV proteins. The construct CD23CATδ1 containing the deleted CD23 promoter gave no basal CAT activity and was not transactivated by EBNA-2. This was as expected, as this construct contains a deletion of approx. 200 bp which should

remove the EBNA-2 responsive elements. In assays for PAP, no PAP activity was seen in any of the samples containing the CD23PAP construct. As the CD23CAT plasmid produced CAT activity, it appears that the CD23 promoter is active in these cells and that the CD23PAP construct is not functioning.

(2) HuT 78 (T cell lymphoma).

Initial attempts to transfect this cell line by electroporation failed. Using the same conditions as DG75 failed to produce any detectable PAP activity. Varying the voltage to 250 V (as suggested by Marianne Ostermann; Internet) initially produced no transfection, but when this was repeated, a low level of PAP was detected. The positive transfection for CAT produced a level of activity readily detectable by the radioassay, but none of the samples transfected with CD23CAT showed any detectable CAT. This could be due to failure of the CD23 promoter to function in these cells (the transfection control PAP confirmed that the transfections had worked in these samples). Subsequent attempts to transfect this cell line have failed to produce any detectable CAT or PAP. Conditions for transfection need to be optimised for this cell line, as the transfections so far have not been reproducible with the same set of conditions. Within a set of transfections, the efficiency appears to be comparable (see HuT 78 transfection assay set 1). However, the variability in efficiency between batches of transfections is considerable (no transfection was seen in transfections set 2 or 3). Recently, it has been suggested that the conditions of the cells before transfection is critical for optimum efficiency. Some groups have been using a protocol that involves feeding the cells every day for a week prior to transfection, keeping the cell density at a level of 0.5-0.6 x 10^6 cells/ml (Dominic Voon, Internet). This appears to improve transfection efficiencies.

(B) Adherent cells.

(1) Hep G2 (liver carcinoma).

These cells were transfected with pSV2APap, CMVCAT, CD23CAT +/- EBNA2/3A, and pG7CAT as a negative control.

During the calcium phosphate procedure, it was noted that the expected precipitate failed to form on the cells. This is thought to be essential for successful transfection to occur. When the cells were harvested and assayed for PAP activity, it was noted that these cells have an endogenous heat stable phosphatase activity. Hence all the samples were positive for PAP, and the results of the assay were inconclusive. The positive transfection control for CAT (sample transfected with pCMVCAT) was assayed for CAT activity and none was found to be present relative to a positive assay control (commercial CAT enzyme). It was concluded that the transfection failed to work in this case (probably due to the lack of precipitate on the cells). It may also be possible that the CMV promoter is not functional in this particular cell line and hence CAT expression was not detected. This experiment was not repeated due to the endogenous level of PAP activity.

(2) SW480 (colon carcinoma).

These cells proved to be very easily transfected by the calcium phosphate procedure, and showed a high transfection efficiency when compared to the suspension cells. The transfections were carried out twice. Transfection with pSV2APap gave high phosphatase activity which was easily detected in the extract after 20 minutes of incubation with the colorimetric substrate. The CD23Pap construct gave no activity when used either alone or in combination with EBNA 2/3A. This construct appears to be non-functional in this case. The cells were assayed for CAT activity using the phase extraction radioassay. In both sets of transfections CAT activity was detected in the positive control (pCMVCAT). The commercially available CAT ELISA (Boehringer Mannheim) was also used to test these samples and the sensitivity of both assays was found to be comparable. No CAT activity was detected in the extracts from the co-transfections with CD23CAT +/- EBNA 2/3A.

It was concluded that the CD23 promoter may not be functional in these cells. Although identified as a B cell promoter, the basal level of expression in B cells is low unless transactivating factors such as EBNA-2 are present also. However, the inclusion of these factors caused no detectable rise in activity of the CD23 promoter in the SW480 cells. It may be possible that the EBNA-2 plasmid used was not producing any protein. However, as this plasmid contains EBNA-2 under the control of the CMV promoter, and this promoter functions well in these cells (easily detected CAT activity from CMVCAT), this was not thought to be the case. A western blot using antibodies to EBNA-2 could be used to confirm the presence of this protein in transfected cell extracts. It is possible that additional cell-specific factors necessary for the activity of this promoter were absent in this case. The transfections themselves were working well (PAP and CAT controls) so this was not thought to be the problem.

The pG7CD23Pap construct appears to be non-functional in the cell lines so far tested. The expected sequence was constructed to ensure that there is no extra sequence in the polylinker that may cause problems with transcription and to ensure that all the PAP sequence from pSV_2APap was taken :

From the paper (Henthorn, *et al*, 1988): pSV_2APap was originally constructed by taking an Eco RI-Sca I fragment for cloning. pG7CD23Pap was constructed by taking an Eco RI fragment so none of the PAP sequence was lost in the construction of pG7CD23Pap.

The theoretical sequence was examined in further detail to see if an ATG was present in the junction between CD23 and PAP:

.....TCTGCTGA(Sal I repaired) (Sma I)GGGGTACC GA(Eco RI) ATTC.....
CD23 **Polylinker pGem7Zf** **PAP**

As can be seen from above, there is no extra ATG in the polylinker or in the junction.

Restriction analysis of the plasmid confirmed it had the expected structure. Four clones were isolated in this experiment. It may be possible that the clone chosen for use in the transfection assays had a mutation -

repeating the experiments using the other clones would eliminate this possibility.

It may be that the pG7CD23Pap construct is functional, but that the PAP activity produced is too low to be detected. As seen from the CAT assays, the basal level of CD23 promoter activity in DG75 is very low and the upregulation is only by a factor of 4-5. As the PAP assay appears to be less sensitive than the CAT assay, it is possible that even an upregulated PAP activity would not give a signal above the background in the enzyme assay. To examine if this is the case the levels of PAP mRNA before and after transfection could be measured to determine if a transactivation effect was occuring. It may also be the case that the novel CD23Pap mRNA is highly unstable.

It should be noted that this approach to promoter analysis in some circumstances can be limited. There may be controlling elements present at other locations that are missed in this analysis. For example, sequences that influence EBV promoter activity have previously been shown to be located outside the immediate 5' region (Walls, *et al*, 1990). In some cases other means of gene regulation e.g. genomic methylation of coding sequences/promoter regions, may also be important.

In conclusion, this study has shown that in a B cell background, EBNA-2 transactivates the CD23 promoter by a factor of 4-5 and this effect is repressed by the EBNA-3A protein, which alone has no effect on promoter activity. This effect is not seen in other cell backgrounds. EBNA-3A-repression of EBNA-2-mediated effects may occur in a number of ways. The mechanism of action of EBNA-3 may be mediated by direct binding of the protein to the DNA, i.e. EBNA-3 may compete with an activation molecule e.g. CBF-1, by attaching to the CBF-1 binding site or a site close by and hence prevent binding of either CBF-1 to it's binding site, or binding of EBNA-2 to CBF-1. The EBNA-3 effect may also be mediated by protein-protein interactions, i.e. binding of EBNA-3 to either CBF-1 or EBNA-2 itself, could prevent EBNA-2 promoter transactivation effects. Gel retardation experiments could be carried out to investigate these theories in detail.

A functional antagonism between EBNA-2 and the EBNA-3 proteins may be important in the overall viral strategy. Depending on the time course of their synthesis and the amounts of proteins produced, the

EBNA-3 proteins may offer a means to regulate EBNA-2 transactivating functions in a qualitative and/or quantitative manner. Previous studies have shown that EBNA-2 is the first protein to be expressed, with EBNA-3 appearing later. EBNA-2 is necessary for immortalisation to occur, but is not required to maintain the immortalised state, and may have effects that are toxic to the cell if prolonged e.g. EBNA-2 upregulates the expression of LMP which when over-expressed can be toxic to the cell (Hammerschmidt, *et al*, 1989). Thus EBNA-3 may repress EBNA-2-mediated effects, ensuring survival of the EBV-infected cell *in vivo* .

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