# Differentiation of Lung Cancer Cell Lines: A Role for Translational Regulation of Gene Expression

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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 Ph.D. 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 own work.

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## *Abstract*

1) Treatment with the differentiating agent, 5-Bromo-2-deoxyuridine (BrdU) was capable of inducing keratin (K8/ K18) expression in the poorly differentiated stem cell-like lung cancer cell line, DLKP and the more differentiated adenocarcinoma line, A549. The absence of keratin induction by BrdU in the Leukaemic cell line, HL60 suggested that this effect may be epithelial-specific. Immunocytochemical analysis of DLKP, treated with BrdU for one week and then continuously cultured in the absence of BrdU for 3 months, revealed that keratin filament formation was maintained in these cells.

2) RT-PCR and Northern blotting analysis revealed equal levels of K8/18 mRNAs before and after BrdU treatment suggesting that induction occurred at a post-transcriptional level. Since treatment of DLKP with a cocktail of protease inhibitors failed to induce K8 and K18, it would appear that proteolytic mechanisms are not in operation and that control is likely to be at the level of translation.

3) BrdU-treated lung cancer cell lines exhibited significant increases in the levels and phosphorylation of the important translation initiation factor, eIF-4E. On the other hand, levels and phosphorylation of this factor were decreased in BrdU-treated HL60s. eIF-4E may represent a key effector molecule in BrdU-mediated induction of K8/18 expression in epithelial lines, but since little increase in eIF-4E mRNA is observed it may not be the primary target gene for BrdU activation. BrdU almost certainly acts at the transcriptional level, ultimately.

4) Protein levels of the general translation initiation factor, eIF-2$\alpha$ also increased following BrdU treatment of both epithelial lung cancer cell lines.

5) Levels of c-Myc1 protein, the growth inhibitory c-Myc isoform, increase following treatment of epithelial lines with BrdU. (Expression of this factor is known to be

translationally regulated and dependent upon eIF-4E availability. In addition, c-*myc* has been reported to increase gene transcription of both eIF-4E and eIF-2α).

6) Since the transcription factor, YY1, has been shown to be induced by BrdU treatment, and it is known to be a regulator of c-myc gene transcription and c-Myc protein activity, we investigated its expression in this system. Levels of YY1 protein increase significantly in BrdU-treated epithelial cell lines, while they decrease in BrdU-treated HL60 cells.

7) Investigations using *in-vitro* translation suggested that DLKP may contain an inhibitor of K8/18 translation, whose activity is abrogated by BrdU treatment.

8) Transfection of DLKP cells with eIF-4E cDNA results in increased expression, not only of eIF-4E protein, but also of K8/18, YY1 and c-Myc1 proteins.

9) Transfection of DLKP with YY1 cDNA resulted in increased expression, not only of YY1 protein, but also of K8/18, eIF-4E and c-Myc1 proteins.

10) From these results we have outlined a possible differentiation-inducing cascade that may be a target for BrdU activation. Emanating from the transcription factor, YY1 it culminates in the activation of a proposed differentiation-inducing feedback loop revolving around eIF-4E and c-Myc1 expression, and the induction of keratin expression.

11) Exposure of DLKP to the physiological differentiating agent, Retinoic Acid (RA) resulted in growth inhibition but did not influence the expression of keratins 8/18. The growth inhibitory affects of RA were attributed to the expression of Retinoic Acid Receptor-α in DLKP. The lack or expression of a truncated form of the Retinoic Acid Receptor-β is offered as an explanation for the inability of RA to affect keratin expression in DLKP. RA was shown to be capable of altering K8/18 expression in the RAR-β-positive cell line, A549. These findings may offer an insight into the poorly differentiated and aggressive nature of DLKP.

*This thesis is dedicated to my parents and family*

*who have been so supportive throughout my life,*

*and to my grandfather, Christopher (George),*

*an inspirational figure.*

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# *ABBREVIATIONS*

| | | |
|---|---|---|
| 4E-BP | - | eIF-4E-Binding Protein |
| Ab | - | Antibody |
| ATCC | - | American Tissue Culture Collection |
| BrdU | - | Bromodeoxyuridine |
| cDNA | - | complementary Deoxyribonucleic Acid |
| c-*myc* | - | Refers to c-myc gene or mRNA (italicised *myc*) |
| c-Myc | - | Refers to c-myc protein (Capital M) |
| Da | - | Daltons |
| DEPC | - | Diethyl Pyrocarbonate |
| DMEM | - | Dulbecco's Minimum Essential Medium |
| DMSO | - | Dimethyl sulfoxide |
| DNA | - | Deoxyribonucleic Acid |
| EDTA | - | Ethylenediaminetetraacetic acid |
| eIF | - | eukaryotic Translation Initiation Factor |
| ERK | - | Extracellular Signal Regulated Kinase |
| FBS | - | Fetal Bovine Serum |
| GDP | - | Guanine Di-phosphate |
| GTF | - | General Transcription Factor |
| GTP | - | Guanine Tri-phosphate |
| HEPES | - | N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] |
| Ig | - | Immunoglobulin |
| IRE | - | Internal Ribosome Entry Site |
| IRPE | - | Internal Ribosome Repositioning Element |
| K | - | Keratin |
| MAPK | - | Mitogen Activated Protein Kinase |
| MEK | - | MAPK/ ERK Kinase |
| MEM | - | Minimum Essential Medium |
| min | - | Minute(s) |
| MMLV-RT | - | Moloney Murine Leukemia Virus-Reverse Transcriptase |
| Mnk | - | MAPK-integrating Kinase |
| mRNA | - | Messenger RNA |

| | | |
|---|---|---|
| NCTCC | - | National Cell & Tissue Culture Centre |
| NE | - | Neuroendocrine |
| NSCLC | - | Non-Small Cell Lung Carcinoma |
| NSCLC-NE | - | Non-Small Cell Lung Carcinoma - Neuroendocrine |
| PBS A | - | Phosphate Buffered Saline A |
| PD98059 | - | Parke-Davis Compound |
| PHAS | - | Protein Heat and Acid Stable |
| PI-3K | - | Phosphatidyl Inositol 3-Kinase |
| PKC | - | Protein Kinase C |
| Pol | - | Polymerase |
| PVDF | - | Polyvinyl Diflouride |
| RA | - | Retinoic Acid |
| RNA | - | Ribonucleic Acid |
| RNase | - | Ribonuclease |
| Rnasin | - | Rnase inhibitor |
| rpm | - | Revolution(s) Per Minute |
| RT | | Room Temperature |
| RT-PCR | - | Reverse Transcriptase-Polymerase Chain Reaction |
| SB203580 | - | Smith-Cline Beecham Compound |
| SCLC | - | Small Cell Lung Carcinoma |
| SCLC-V | - | Variant Small Cell Lung Carcinoma |
| SDS | - | Sodium Dodecyl Sulphate |
| sec | - | Second(s) |
| SFM | - | Serum-Free Medium |
| SoS | - | Son of Sevenless Protein |
| TBS | - | Tris Buffered Saline |
| TCA | - | Trichloroacetic acid |
| TEMED | - | N, N, N', N'-Tetramethyl-Ethylenediamine |
| TF | - | Transcription Factor |
| Tris | - | Tris(hydroxymethyl)aminomethane |
| UTR | - | Untranslated Region |
| v/v | - | volume per volume |
| w/v | - | weight per volume |

Section 1.0

# *Introduction*

## 1.1 General Introduction

Strategies designed to treat lung cancers have been severely impaired by the paucity of knowledge regarding the biological mechanisms controlling the differentiation and development of both normal and disease states of the lung. Despite recent advances in modern medical science the use of anti-cancer strategies such as irradiation and chemotherapy have done little to increase the life expectancy of those diagnosed with lung cancer. The use of antisense and ribozyme technology to treat cancers is far from widespread application in the medical field (Scanlon and Kashani-Sabet, 1998). Such oligonucleotide approaches, using RNA sequences that bind to cellular RNAs to increase their sensitivity to RNases (antisense) or cleave them into functionally inactive RNAs (Ribozymes), have been severely impeded by instability and toxicity of the oligonucleotides, and difficulties in delivery of these drugs to target organs. An alternative approach is that of "Differentiation Therapy", designed to induce a more differentiated phenotype in tumours in an attempt to slow and even halt growth and progression of the cancer (Roth, 1992; Lotan, 1996). The differentiation strategy that has shown most promise to date in both *in-vitro* and *in-vivo* trials is that of the Vitamin-A/Retinoid based differentiation-inducing compounds (Gendimenico and Mezich, 1993; Lotan, 1996). However, for this or any of the other therapies to have any realistic chance of being effective, a better understanding of lung tumour biology is urgently required. Cancer has been referred to as a "disease of abnormal differentiation" (Sporn and Roberts, 1983) and as such is theoretically a prime target for such therapies.

In many cells, division results in a simple duplication of the parental phenotype. However, in some tissues the cells at the end of the differentiation pathway cannot divide (terminal differentiation) and are renewed by proliferation of a distinct sub-population, known as stem cells (Watt, 1991). These cells replenish both stem cell and daughter populations that can terminally differentiate (Figure 1.1, Section 1.4). A review by Emura (1997) highlighted the fact that understanding of lung stem cell biology lags behind that of organs such as the liver, skin, intestine and abdomenal mesothelium. This was attributed to the anatomical and functional complexity of the respiratory system, but data to date suggests that, analogous to other organs, there is only one type of epithelial stem cell throughout the lower respiratory tract. They are

multipotent for cell differentiation and able to yield lineage progenitors for ciliated, goblet, basal, Clara, neuroendocrine, alveolar typeI and alveolar typeII cells. In addition, Emura (1997) suggested the possibility of de-differentiation of related cells to replenish one another during situations such as damage to the lining of the lung. A novel cell line, DLKP isolated at the NCTCC (Law *et al.*, 1992) has been categorised as extremely poorly differentiated and consists of at least three subpopulations, termed SQ (Squamous), I (Intermediate) and M (Mesenchymal) (McBride *et al.*, 1998). These populations have shown the ability to interconvert and eventually, when cultured alone, replenish the parental phenotype. This, combined with the lack of expression of a number of differentiation-specific markers, has lead to the speculation that DLKP may represent a stem cell-like population. This has afforded a unique opportunity to study the process of lung cancer differentiation *in-vitro*, particularly the early stages of this process. Such studies will provide valuable insights into the mechanisms of early lung development, both in diseased and normal tissues, possibly identifying targets for therapeutic intervention and aiding in the design of strategies to treat lung cancers more effectively.

## 1.2 Keratins as markers of epithelial differentiation

A large proportion of the cytoplasm of vertebrate cells, normal and transformed, is occupied by components of the cytoskeleton, including actin, tubulin and the intermediate filaments (Moll *et al.,* 1982). They are formed in different cell types from different proteins of a multigene family or from different subunit polypeptides of a class of related proteins. By far the most striking differentiation specificity of composition has been observed in the intermediate-sized filaments. This class of filaments includes the desmin filaments typical of myogenic cells, the neurofilaments typical of neuronal cells, vimentins occur in mesenchymally derived cells and vascular smooth muscle cells, and the keratins occur in epithelial cells (Moll *et al.,* 1982, Hatzfeld and Franke, 1985; Daly *et al.,* 1998). Keratin Intermediate Filament (IF) proteins have three domains: a central alpha-helical rod domain of constant size that derives from common ancestors, and two end-domains of variable structure thought to be involved in tissue-specific functions (Blumenberg, 1988). The specificity of keratin expression patterns in epithelial cells has been used in prognostic and diagnostic situations as markers of both epithelial origin and state of differentiation in patients with small cell lung cancer (Bepler *et al.,* 1987; Broers *et al.,* 1988), and other tumour pathologies (Virtanen *et al.,* 1984; Trask *et al.,* 1990) to distinguish normal and tumour-derived epithelial cells. Keratins are thought to serve a structural function to protect the cell against environmental stresses and strains as for other filaments (Daly *et al.,* 1998), but their expression in human ovarian adenocarcinoma lines has been associated with altered sensitivity to various chemotherapeutic drugs (Parekh and Simpkins, 1995). Interestingly, in studies using a number of chemical differentiating agents the levels of mdr-1/Pgp (p-glycoprotein) increased and expression appears to correlate with the degree of differentiation (Mickley *et al.,* 1989). However, induction of these pumps is not always accompanied by expression of the multidrug-resistance phenotype, which may possibly be explained by changes in keratin expression during the differentiation of these cells. The human K8 mRNA encodes a nucleic acid-binding domain, suggesting that keratin filaments may bind to nucleic acid sequences and play a role in regulating DNA replication and gene transcription (Yamamoto *et al.,* 1990). It is also possible that they play a role in the regulation of translation of particular mRNAs through their localisation to regions within the cell, in a similar manner to the way in which polar

expression of developmental proteins *nos* and *bicoid* are regulated (Gavis *et al.*, 1992). Genetic disease states associated with loss of keratin regulation include the blistering phenotype of Epidermolysis Bullosa Simplex (EBS) (Oshima, 1992; Fuchs and Byrne, 1994) and development of dwarf phenotypes and diabetes in transgenic mice expressing the K8 gene (Casanova *et al.*, 1995).

The keratins (K) are divided into two categories; the acidic type I keratins are K9-20, while the more basic type II keratins are K1-8. Keratin filament formation is dependent on the pairing of partners from both groups to produce a proteolytically stable hetero-polymer filament (Kulesch *et al.*, 1989). Despite the fact that their function is relatively unknown, the pattern of expression of keratin filaments is specific to both epithelial origin and degree of differentiation (Tseng *et al.*, 1982). As described in "The Catalog of Human Cytokeratins" (Moll *et al.*, 1982), while K9-K11 are predominant in the epidermis, K12 has only been observed in the cornea. Cytokeratin 8 represents simple epithelia, and its normal partner, K18, shows the same tissue distribution (Trask *et al.*, 1990). K8 and K18 are the first keratins to appear during mouse development (Casanova *et al.*, 1995) and are thought to be the evolutionary ancestors of many of the present keratin forms (Blumenberg, 1988). Cytokeratin 19 is found in a broad range of epithelial tissues and is a major component of simple epithelia. K14 and K19 are known to be "promiscuous" in that they can partner Type II Keratins in the absence of their "usual" Type I partner to form stable filaments (Hatzfeld and Franke, 1985; Darmon, 1985; Lersch *et al.*, 1989). K19 lacks a variable terminal domain. This, combined with its promiscuity, means that K19 is thought to play a critical regulatory role by pairing with any one of the basic keratins without contributing a potentially harmful variable terminal domain, the region in which tissue-specific function of keratins resides (Blumenberg, 1988). It therefore acts to redress keratin imbalances. Keratins 7/8/18/19/20 have been associated with simple epithelia, while K4/5/17 are associated with stratified epithelia (Mobus *et al.*, 1994). Both classic and variant small cell lung cancers express K8 and K18/19, detectable by western blotting when immunocytochemical staining is weak (Elias *et al.*, 1988). Stem cell populations of the lung have been speculated to exist as pluripotent populations residing in tumours and cell lines (Trask *et al.*, 1990; Pfeifer *et al.*, 1991). The almost complete absence of keratin expression in DLKP, a novel

poorly differentiated NSCLC-NE/SCLC-variant cell line isolated at the NCTCC, has led to speculation that this cell line may represent a stem cell-like population.

### 1.2.1 Regulation of Keratin Expression

The regulation of keratin filament formation is complex and is controlled at multiple levels. Regulation of keratin expression has been reported at the transcriptional level (Roop *et al.*, 1988), involving AP-1 (Section 1.5.2.1) activation of transcription (Neznanov and Oshima, 1993) which is mediated by the ras signalling pathway (Pankov *et al.*, 1994). Relatively short sequences in the 5' upstream region of keratin genes can confer tissue-specific transcription (Blessing *et al.*, 1989; Neznanov and Oshima, 1993). In addition, histone and chromosomal insulation of keratin genes (Casanova *et al.*, 1995), labile inhibitors of transcription (Cremisi and Duprey, 1987), and post-transcriptional proteolysis (Kulesh *et al.*, 1989) have all been implicated in the cell-specific and developmental regulation of keratin filament formation. An important aspect to the proteolytic regulation of keratin filament formation, in which both partners of the pair are required for proteolytic stability and filament expression, is that it would appear that the expression of a type II keratin is sufficient to induce the expression of a type I partner (Giudice and Fuchs, 1987; Knapp and Franke, 1989; Lersch *et al.*, 1989, Rothnagel *et al.*, 1993). Type I keratin expression has been suggested to be dependent on accumulation of unpolymerised Type II keratin (Giudice and Fuchs, 1987) for proteolytic stability for overall filament formation. Type I proteolysis may form a universal regulatory element while specificity in Type II expression will therefore result in Type I induction and tissue-specific Intermediate Filament formation (Rothnagel *et al.*, 1993). Synthesis of both keratin types can be uncoupled and control of cytokeratin Intermediate Filament formation can occur at different levels (Knapp and Franke, 1989), strengthening this suggestion. There is substantial evidence for additional post-transcriptional regulatory mechanisms (Blouin *et al.*, 1991; Crowe *et al.*, 1993), including mRNA degradation (Paine *et al.*, 1992) and the suggestion that there is a possible block on the translation of certain keratin mRNAs, such as K8 (Tyner and Fuchs, 1984). This speculatively involves translational repression (Su *et al.*, 1994) and even masking of keratin mRNAs in epithelial squamous cell carcinomas (Winter and Schweizer, 1983).

## 1.3 Differentiation Therapy and Differentiating Agents

Cancer has been referred to as a "disease of abnormal differentiation" (Sporn and Roberts, 1983) and is theoretically a prime target for "Differentiation Therapy" (Lotan, 1996). The ultimate objective of this concept/strategy is not aimed at killing the tumour so much as to induce the cancer cells, whose growth rate and cell cycle have become deregulated, to commit to differentiate into more "normal" cells. A number of differentiating agents have shown promise in both laboratory and clinical trials, the majority of which are based upon vitamin derivatives such as Vitamin A and Vitamin $D_3$, while more toxic laboratory agents are used to delineate the actual mechanisms regulating the differentiation process *in-vitro*.

### 1.3.1 Retinoic Acid

The differentiation strategy that has shown most promise to date in both *in-vitro* and *in-vivo* trials is that of the Vitamin-A/Retinoid based differentiation-inducing compounds (Gendimenico and Mezich, 1993; Lotan, 1996). In clinical trials, topical application of Retinoic Acid (RA) was shown to reduce the formation of skin papillomas (Tenenbaum *et al.*, 1998). Retinoic Acid has shown very strong potential as a therapeutic in cases of APL (Acute Promyelocytic Leukaemia) (Asou *et al.*, 1998) and AML (Acute Myeloid Leukaemia) (Tallman, 1996) reducing the risk of relapse and increasing the chance of long-term survival (Takeshita *et al.*, 1995; Degos, 1997). Alternative approaches include the development of novel RA metabolism blocking agents that increase endogenous levels of RA by inhibiting its breakdown in cancer cells (Sciarra *et al.*, 1998), currently being applied to the treatment of prostate cancer.

Retinoids are chemical derivatives of Vitamin-A which are effective in modulating the differentiation of cells at physiological and non-toxic doses. Among the most commonly used are retinol, retinoic acid, and the synthetic retinoid N-(4-hydroxyphenyl), otherwise known as 4HPR (Sabichi *et al.*, 1998). These compounds act through common or similar mechanisms to influence the differentiation status of the cell. Retinoic Acid has been widely studied and its effects on cells are known to be mediated by a group of receptors that belong to the family of steroid hormone

nuclear receptors (Giguere *et al.*, 1987; Petkovic *et al.*, 1987). There are two classes of Retinoic Acid-binding nuclear receptor, the Retinoic Acid Receptors (RARs) and the Retinoid X Receptors (RXRs) (Xu *et al.*, 1997), to each of which belong three sub-species of receptor known as α, β, and γ. These nuclear receptors become internalised into the nucleus upon stimulation with RA to directly interact with genes containing sequences called RAREs (Retinoic Acid Response Elements), affecting their transcription. They are therefore transcriptional enhancers (Section 1.5.2).

The importance of RARs in the regulation of gene expression and the differentiation process has been shown using dominant-negative/truncated forms of these receptors, which inhibit differentiation and development and can promote features of malignancy (Aneskiebich and Fuchs, 1995, Saltou *et al.*, 1995). Mutant dominant negative RARs have been shown to inhibit skin development (Saltou *et al.*, 1995). Expression of RAR-β in gynecological cancers was able to inhibit their growth (Sabichi *et al.*, 1998) while suppression of RAR-β expression has been associated with development of NSCLC (Minna and Mangelsdorf, 1997; Xu *et al.*, 1997). Results to date suggest that RAR-β may be the main effector of RA-induced differentiation, while RAR-α and RAR-γ play roles in the induction of growth inhibition and apoptosis. RA has been observed to induce tissue transglutaminase expression and apoptosis in rat tracheobronchial epithelial cells (Zhang *et al.*, 1995) through a specific retinoid signalling pathway that involves RAR-α. The cells used in these studies lacked any RARβ mRNA expression, which suggests that these cells are incapable of a differentiation response to RA. Geradts et al (1993) showed that a number of human lung cancer lines, both NSCLC and SCLC, showed no detectable abnormalities in RAR-α gene structure but that in a high percentage there were obvious abnormalities in RAR-β, many failing to show RAR-β mRNA induction after treatment with RA. The RAR-β gene contains a RARE itself (Sucov *et al.*, 1990), resulting in autoregulation that has implicated RAR-β in amplifying the cellular response to low-level changes in RA concentrations. The loss of RAR-β is thought to be responsible for the failure of some tumours to respond to retinoid-mediated differentiation therapy, and has also been implicated in the poor state of differentiation and aggressive nature of some tumours. This agrees with the concept that, "cancer is a disease of abnormal differentiation" (Sporn and Roberts, 1983). RA

treatment of head and neck squamous cell carcinomas has been shown to increase the expression of all three RAR mRNAs, without any effect on the mRNAs of the RXRs (Zou *et al.,* 1994), suggesting that the RXRs do not harbour RAREs, known to exist in RAR-β.

While RARs directly influence gene activity in response to retinoic acid, the levels of retinoic acid in the cytoplasm and available to these receptors are thought to be regulated by cellular Retinoic Acid Binding Proteins (cRABPs). There have been two forms identified to date, cRABPI and cRABPII (Eller *et al.,* 1994), which are thought to directly interact with Retinoic Acid taken up by the cell to regulate its intra-cellular availability.

#### 1.3.1.1 Retinoic Acid affects Keratin Expression

Retinoic acid is a very potent regulator (both stimulatory and inhibitory) (Ivanyi *et al.,* 1993) of both keratin expression and differentiation of a wide range of cell types, including prostatic epithelial cells (Stellmach *et al.,* 1991; Peehl *et al.,* 1993), keratinizing epidermal cells (Kopan *et al.,* 1987), mammary carcinoma cells (Ivanyi *et al.,* 1993), neuroblastoma cells (Irving *et al.,* 1997), and variant small cell lung cancer cells (Doyle *et al.,* 1989). Interestingly, Kopan et al (1987) reported the appearance of type II (K1) expression in RA treated human epidermal cells that preceded the expression of their type I partner. This agrees with the hypothesis proposed in section 1.2.1 that type II keratins do indeed play a role in the regulation, stabilisation or induction of their type I partner. Vitamin A deficiency in culture has been shown to induce changes in a small cell lung cancer cell line to squamous cell type that could be reversed upon addition of retinoic acid to the medium (Terasaki *et al.,* 1987). In cultured human keratinocytes and in squamous cell carcinoma (SCC-13) cells of epidermal origin the expression of differentiation-specific K5, K6, K14 and K16 proteins is inhibited by RA (Stellmach *et al.,* 1991). In epidermal keratinocytes keratins 5, 6, 14, and 17 are suppressed and keratins 13 and 19 are induced (Gilfix *et al.,* 1985). A reduction in the levels of K18 mRNA was reported in the human NSCLC line, A549 upon exposure to RA (Ledinko and Costantino, 1990). In fact, it has been suggested that all keratins contain elements that make their expression

subject to regulation by cellular concentrations of RA (Gilfix *et al.*, 1985). The expression of K14 in differentiating basal stratified epithelia was shown to be directly regulated by interaction of nuclear RA- and hormone- receptors with a RARE (Retinoic Acid Response Element) in the K14 gene (Tomic *et al.*, 1990; Tomic-Canic *et al.*, 1992). A similar effect was reported for K19 expression in non-keratinizing oral epithelial subtypes (Hu and Gudas, 1994). Interestingly, this effect was attributed to a 3' enhancer in the K19 gene and K19 expression correlated with the levels of RAR-β mRNA. Transcriptional induction of K18 expression by RA in embryonal carcinoma (EC) and embryonic stem (ES) cells was attributed to the low levels of AP-1 (Section 1.5.2.1) and ETS complexes which increase during RA-induced differentiation of these cells (Pankov *et al.*, 1994). In fact, it is thought that the delayed induction of keratin expression observed during RA-induced differentiation may be partly due to the requirement for prior induction of AP-1 complex formation, stimulatory to the expression of keratins.

### 1.3.2 5'-BromodeoxyUridine (BrdU)

Bromo-deoxyuridine (BrdU) is a halogenated thymidine analogue that is known to influence the differentiation of cells. It is best referred to as a differentiation modulating agent since it has been shown to be a potent inducer of differentiation in some cell lines (Yen *et al.*, 1987; Sugimoto *et al.*, 1988; Valyi-Nagy *et al.*, 1993), while it can inhibit the differentiation of others (Seecoff and Dewhurst, 1976, Tapscott *et al.*, 1989; Lee *et al.*, 1992). BrdU competes with naturally occurring Thymidine for incorporation into DNA during replication and as such it, and other similar compounds, should be ideal candidates for anti-tumour agents, since they require cell division and DNA synthesis to exert their effects (Bick and Devine, 1977). While few clinical trials are based on the differentiation-modulating properties of this drug (Freeman, 1969; Ameye *et al.*, 1989), BrdU has been used widely as a radiosensitiser in an attempt to improve radiological treatments (Lawrence *et al.*, 1992; McGinn and Kinsella, 1993). Radiosensitisation trials to date include the treatment of malignant glioma (Vander *et al.*, 1990), ulcerative herpetic keratitis (van Bijsterveld *et al.*, 1989), malignant astrocytomas (Greenberg *et al.*, 1988) and malignant brain tumours (Matsutani *et al.*, 1988). More recently, BrdU has entered

clinical trials as a radiosensitiser in the treatment of pancreatic cancer (Robertson *et al.,* 1997), colorectal liver metastases (Robertson *et al.,* 1997) and cervical cancer (Eisbruch *et al.,* 1999), while studies in relation to malignant gliomas continue (Prados *et al.,* 1998). Administration of BrdU is normally by controlled perfusion (Doirion *et al.,* 1999), and has been used in combination with radiolabelled monoclonal antibodies (Buchsbaum *et al.,* 1994). While radiolabelled antibody approaches offer the potential of targeted chemotherapy, they are limited by low dose-relate deliverable. As such, the trials of Buchsbaum et al (1994) may offer a means of enhancing the efficacy of low dose radiolabelled monoclonal antibody approaches.

BrdU incorporates into DNA in a non-random fashion at sequences termed "fragile sites" (Hecht *et al.,* 1988; Sutherland, 1988; Sutherland, 1991). This explains the reproducibility of the effects observed with BrdU-induced differentiation. O'Neill and Stockdale (1973) developed a model for BrdU-induced modulation of differentiation that assumes that BrdU "sensitivity" resides on a single pair of chromosomes, suggesting the presence of a "master gene" or target through which BrdU exerts its effects. In this model, inhibition of differentiation occurs in a dominant fashion if approximately 30% or more of naturally occuring thymidine is replaced by BrdU in the readout strand of either chromosome. This sort of model agrees with the predicted mechanisms of action of a number of DNA-intercalating agents. BrdU substitution into DNA and intercalation of such agents may have similar effects, thought to be through direct DNA bending at either major or minor grooves, thereby altering promoter structure and availability to transcription factors. Intercalation of the antibiotics, elsamicin A or actinomycin D in the promoter of the c-*myc* gene induced a decrease in the level of transcription from this promoter (Vaquero and Portugal, 1998). However, relatively low levels of elsamicin incorporation actually induced an increase in c-myc transcription through the P1 promoter (section 1.5.2.2.1). Bromodeoxyuridine (BrdU) has been demonstrated to decrease c-*myc* expression at the transcriptional level in the leukaemic cell line, HL60 (Yen and Forbes, 1990) and in human melanoma lines (Valyi-Nagyi *et al.,* 1993). These results would appear to suggest that the c-*myc* promoter regions are particularly susceptible to modulation by agents that disrupt promoter structure either through Thymidine substitution (BrdU) or intercalation (Elsamicin). Alternatively, BrdU may directly influence the ability of

proteins to associate with DNA. In the *lac* operon, BrdU-substitution has been shown to result in increased binding of the *lac* repressor protein (Lin and Riggs, 1972), suggesting that BrdU may be capable of altering the binding of regulatory factors.

BrdU is considered by some scientists to be an inducer of pre-commitment to differentiation rather than an actual differentiation inducing agent. This was highlighted by the findings that BrdU treatment of HL60s for 24 hours, followed by treatment with Retinoic Acid resulted in a faster response to Retinoic Acid (RA) than the single addition of RA alone (Yen *et al.*, 1990). It would appear that BrdU can initiate some of the early changes induced by RA in HL60 differentiation, including early c-myc downregulation. However, the same author reported previously (Yen *et al.*, 1987) that pre-commitment to differentiation involves an early increase in c-myc levels in the same Leukaemic line, as induced by RA. This suggests that pre-commitment to differentiation in these cells involves increased expression of c-myc. It therefore appears that the true mechanisms of induction and commitment to differentiation remain unclear, even in individual cell types.

## 1.4 Lung Development and Cancer

While the processes behind differentiation, keratin expression patterns and development of the epidermis and some other organs are relatively well understood (Fuchs and Byrne, 1994), lung cell developmental biology lags behind. This is partly due to a failure to identify a stem cell of the lung. Essential properties of stem cells have been described as "immaturity to be able to generate a few lineages of partially differentiated progenitor cells for differentiation into mature cell types, lack of morphologically and functionally differentiated phenotypes, and a capacity for unlimited proliferative cycles and clonogenic growth" (Emura, 1997). Differentiated cells are thought to be produced, not directly from stem cells, but rather via a committed progenitor or transit amplifying population (Watt, 1991) (Figure 1.1). These cells continue and commit to differentiated cell types, allowing continual regeneration of the stem-cell population. The studies reviewed by Emura (1997) suggest the possibility of a single population of pluripotent stem cells in the lung with the additional possibility of de-differentiation of ciliated, secretory and basal cells to provide the lung with regenerative capacity. This has interesting parallels with the behaviour of the lung cell line, DLKP isolated here at the NCTCC (McBride *et al.*, 1998). Clones derived from this cell line exhibit the remarkable capacity to regenerate the mixed parental population over time.

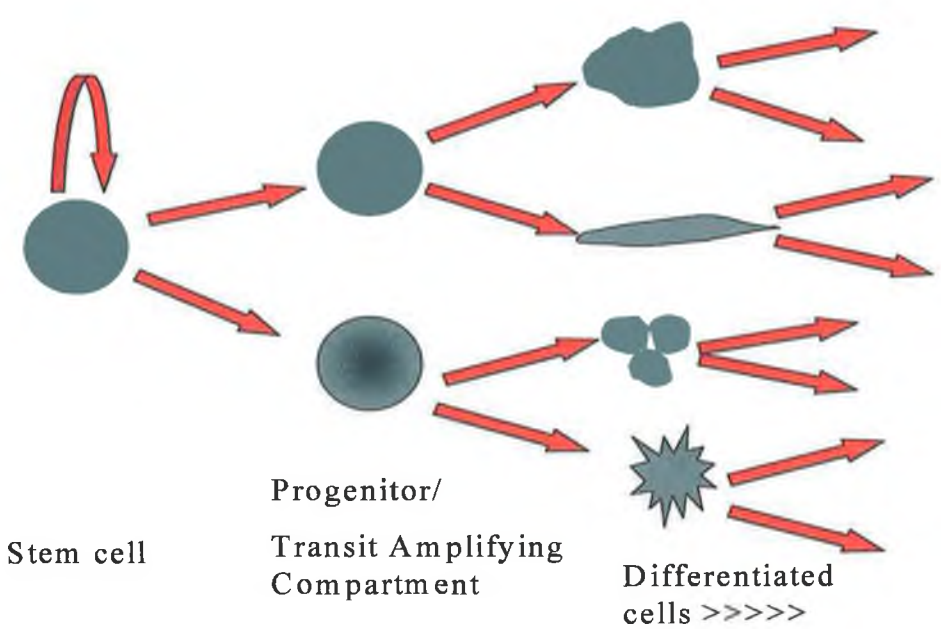



**Figure 1.1: Stem Cell regeneration and differentiation via progenitor cells.**

The onset and evolution of a cancer *in-vivo* is thought to occur via a process known as "field cancerization" (Sozzi *et al.*, 1995), which is a result of the progressive and cumulative loss of control of key growth regulatory factors. Loss of a key regulator (such as the DNA damage-repair regulator, p53) results in the loss of control of other key factors, which in turn results in further losses that rapidly spiral the cell out of control. Numerous proto-oncogenes, including both transcription and translation factors, have been identified as playing roles in the complex process of carcinogenesis. The complexity of the roles that these factors play in the regulation of gene expression is becoming obvious, and additional roles for these regulators in the control of other processes, such as differentiation and apoptosis ("programmed cell death") are now becoming evident.

## 1.5 Transcriptional Control of Gene Expression

Transcriptional control of gene expression during both proliferation and differentiation has been widely studied. Transcription factors such as MyoD and Myogenin have been shown to play critical roles in the regulation of muscle-specific differentiation (Weintraub, 1993; Buckingham, 1994). On the other hand, factors such as *c-fos*, *c-jun*, and c-myc have long been established as playing roles in the regulation of cellular proliferation, differentiation and transformation of a wide variety of cell types. Selective transcription of genes such as alcohol dehydrogenase (Adh) during development is known to occur through specific sequences in the promoter regions of genes that bind regulatory factors known as transcriptional enhancers (Novina and Roy, 1996). Despite this, the process of transcription and the mechanisms by which transcription factors regulate differentiation are still not fully understood. The process of transcription initiation is described in section 1.5.1. Some of the key factors involved in these processes are described in section 1.5.2.

### 1.5.1 Transcription Initiation

Transcription occurs in stages termed initiation, promoter clearance, elongation and termination. The main target for regulation appears to be the process of RNA polymerase binding and transcription initiation. RNA polymerase is the enzyme that "reads" the DNA code and converts its message into an RNA "message". This is then transported from the nucleus to the cytoplasm where it can be converted into protein by the translational apparatus of the cell (section 1.6). The three RNA polymerases lack intrinsic ability to interact specifically with DNA sequences but acquire specificity through interaction with cellular proteins called transcription factors. These place the RNA polymerase enzyme in the correct position on the DNA to begin transcription. There are three different RNA polymerase enzymes (Novina and Roy, 1996), which carry out distinct functions: RNA polymerase I (Pol I) transcribes ribosomal RNA (rRNA), RNA polymerase II (Pol II) transcribes protein-encoding messenger RNAs (mRNAs) and RNA polymerase III (Pol III) transcribes genes coding for amino acid transfer RNAs (tRNAs). The Pol II transcription (protein-encoding genes) process is the focus of this section.

### 1.5.1.1 The Process of Initiation; Pre-Initiation Complex Formation

The promoter region of eukaryotic protein-coding genes is arbitrarily divided into two segments: a core promoter region of around 50 nucleotides adjacent to the transcription start site and a more distal enhancer region (consisting of either positive or negative regulatory elements) (Figure 1.2). The two key genetic elements within the core promoter are the TATA box and/or the initiator (Inr) element. Core promoter structures contain combinations of these elements, termed $TATA^{+}$ $Inr^{-}$, $TATA^{+}$ $Inr^{+}$ and $TATA^{-}$ $Inr^{+}$ (reviewed; Novina and Roy, 1996). The majority of cellular promoters contain a TATA box. However, recently a growing number of genes are being identified that are termed "TATA-less" (do not contain a TATA motif) and appear to be predominantly housekeeping genes (Section 1.5.1.3).

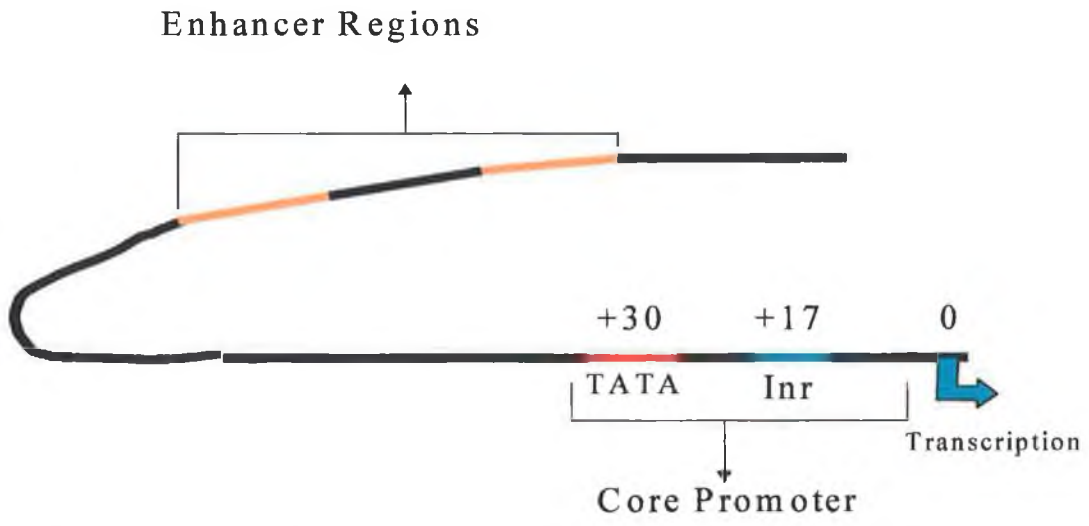



**Figure 1.2: Basic Structure of Gene Promoter Regions of DNA.** A core promoter consists of TATA (red) and Initiator (Inr) (Green) elements proximal to the start site. Distal Enhancer Regions (Orange) can have positive or negative effects on PIC formation at the core promoter region.

The basic process of transcription intiation involves the recruitment of RNA polymerase II to the transcriptional start site via tethering to various General Transcription Factors (GTFs) that recognise the promoter region and begin assembly of the "pre-intiation complex" (PIC), a giant complex estimated to consist of approximately 50 components adding up to a molecular weight of more than 3 MDa (3,000 kDa !!) (Halle and Meisterernst, 1996) (Figure 1.3). This occurs through the binding of a complex of factors called TFIID (Transcription Factor IID) to the TATA box in the vicinity of the start site (Roeder, 1991). The TBP (TATA-Binding Protein)

subunit of this complex provides the DNA-binding specificity and promoter recognition functions of this complex. This interaction is stabilised by TFIIA (Stargell and Struhl, 1996) and a "bridge" to the RNA polymerase holoenzyme (multi-subunit enzyme) is provided by TFIIB (Kornberg, 1996) and TFIIF. TFIIF is involved in both PIC formation and subsequent transcript elongation processes after initiation has occurred (decreasing "arrest" of elongation once transcription has begun) (Reines *et al.*, 1996). TFIIB spans 30 bases and so places the RNA polymerase upon the transcription start site, located 30 base pairs downstream of the TATA box. After formation of the PIC, two more general factors, TFIIE and TFIIH, are required for efficient intiation and promoter clearance. TFIIH provides a helicase function thought to be involved in the separation of the DNA strands around the transcription start site (Kornberg, 1996). The requirement for these two factors is directly related to the degree of supercoiling of the transcribing template, suggesting that the topological state of the template may determine minimum sets of general factors (Novina and Roy, 1996) required by specific genes for efficient expression.

Two models for transcription initiation exist at present (Koleske and Young, 1995) (Figure 1.3); a stepwise assembly of the RNA Pol II holoenzyme in which TFIID (via TBP) recognises the TATA box and this interaction is stabilised by TFIIA. TFIIB forms a "bridge" that places RNA Pol II directly upon the transcription start site and additional helicase functions of TFIIH unwind the promoter region to allow transcription to begin. Alternatively, a pre-formed holoenzyme is simply recruited to start sites in genes to which TFIID is already bound. These are termed the assembly and docking models, respectively (Sachs and Buratowski, 1997). RNA Pol II contains a long hepta-peptide repeat "tail", called the CTD (Carboxy-Terminal Domain) that interacts with numerous factors, such as GTFs and SRB (Supressor of Ribosome B) proteins, thought to be intermediates that interact in turn with transcriptional enhancers (Koleske and Young, 1995, Bjorklund and Kim, 1996). Phosphorylation of the CTD is thought to result in dissociation of attached factors to disassemble the PIC upon promoter clearance to begin the process of elongation, to transcribe the RNA message.

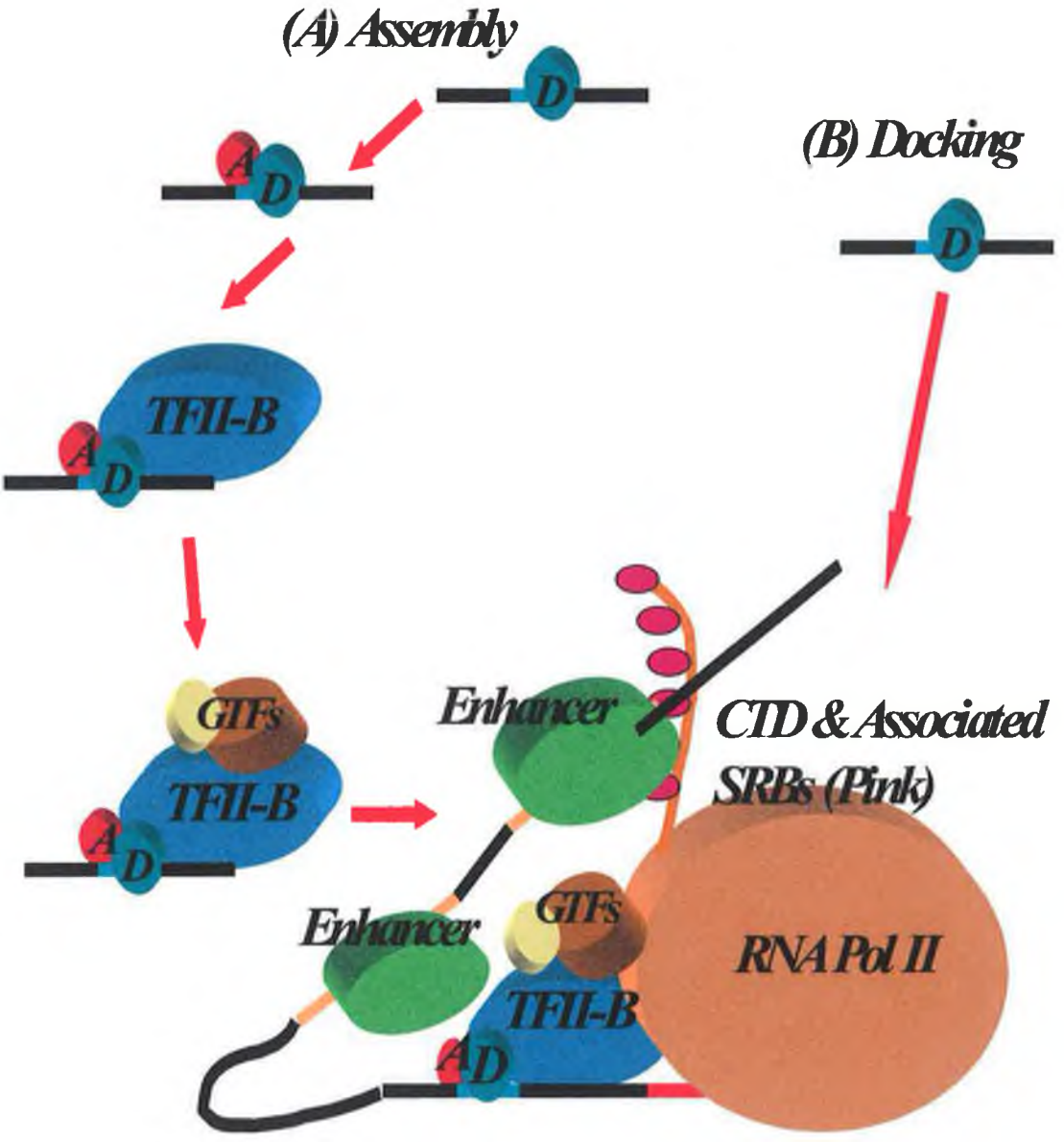



**Figure 1.3: Assembly (A) and Docking (B) Models of Transcription Pre-Initiation Complex Formation.** Transcription Factors bound to upstream Enhancer Elements (Orange Sequences) can interact with GTFs (General Transcription Factors) and CTD (Carboxy-Terminal Domain) tail-associated proteins (SRBs) to either positively or negatively regulate Complex Formation at the Promoter. The timing of their involvement in either model is unknown and as such is not included. A=TFIIA, D=TFIID.

All eukaryotic Pol II genes utilise this "basal apparatus" to initiate transcription. So how is the specificity in gene expression observed between different cell types and at different times during development achieved? Regulation is provided via enhancer sequences specific to the promoters of particular genes and the binding of regulatory co-factors to these sequences influence the rate of PIC formation.

#### 1.5.1.2 Transcriptional Enhancers

Every eukaryotic gene has a particular combination of positive and negative regulatory cis elements that are uniquely arranged as to number, type and spatial array upstream of the core promoter. These sequences, termed enhancer elements, are sites for sequence-specific transcription factors that either positively or negatively regulate PIC formation and control the cell-specific and temporal expression of that particular gene (Mitchell and Tjian, 1989). This is achieved by their ability to interact with one another and the basal transciption apparatus involved in PIC formation, and their activity and levels are regulated through signal transduction cascades (Karin and Smeal, 1992). Thus, extracellular mitogens (Section 1.6.4) control the growth and differentiation status of cells by directly influencing levels and activities of transcriptional enhancer molecules involved in gene-specific transcription.

The DNA binding activities of most transcription factors are localised to relatively small sub-regions consisting of 60-100 amino acids (Karin and Smeal, 1992). These form one of four structures known to date that are involved in DNA sequence recognition and binding: Zinc Fingers, Homeodomains, Leucine Zippers and Helix-Loop-Helix domains (figure 1.4). A common feature of all of these motifs is that they consist of a number of helical regions with different properties. A number of the helices place positively charged amino acids on the outer surfaces to contact DNA, while the remainder are known to be involved in contacting other transcription factors (review; Watson *et al.*, 1992c), allowing the enhancer to both bind DNA and interact with neighbouring factors. Additional domains, separate from DNA binding domains, have been identified as transcriptional activation domains and often factors have more than one of these. To date three primary types of activation domain are known (acidic, glutamine-rich, and proline-rich) and these activate transcription by directly contacting and stabilising general transcription factors, such as TFIID, and CTD-

associated proteins. Activators have additional domains that allow them to interact with other proteins. As such, the universal activator, GAL4 contains DNA- and Activation-Domains, while the specific activator, VP16, is unable to bind DNA and requires additional DNA-binding co-factors to function (Ptashne and Gann, 1990). It, therefore, can only function in cells containing these co-factors. Cellular expression of a particular gene depends on the complement of activator and repressor sequences (enhancer elements) present in its promoter and the relative levels of the respective binding factors (enhancer proteins) within a particular cell type at a particular time during development. Additionally, the complex interactions between various transcriptional enhancers themselves can result in altered activity of these factors, changing activators to repressors and vice-versa.

#### 1.5.1.3 Regulation of Gene Transcription from TATA-less promoters

In recent years there have been a growing number of reports of promoter elements that lack any discernible TATA box motif, originally thought to be a critical control point in the initiation process. Surprisingly, many of these, including creatine kinase, dihydrofolate reductase, cytochrome c oxidase and a number of ribosomal proteins (Azizkhan *et al.*, 1993; Basu *et al.*, 1993), are "housekeeping" genes. In the case of cytochrome c oxidase (COX), the –17 to +20 region of the promoter contains fused binding sites for two enhancer proteins, NF-E1 (also called YY1) and SP1 (Basu *et al.*, 1993). The transcription start site at position –8 to +9 is flanked by a 17 base initiator element (Inr). Mutation studies showed that basal transcription from this Inr element did not involve SP1 binding but was completely dependent on YY1 binding. The importance of SP1 sites in the regulation of TATA-less promoter initiation may actually depend more upon the presence of immediately adjacent YY1 binding. This is highlighted by the findings of Basu et al (1993) above, together with the demonstration that SP1 and YY1 physically interact to form a protein complex (Lee *et al.*, 1993; Seto *et al.*, 1993), suggesting a synergistic effect on TATA-less promoter activity in the presence of both factors. The role of this interaction with SP1 is still unclear, however, since SP1 actually counters YY1-mediated transcriptional activation of the TATA-less dihidrofolate reductase gene in *Drosophila* (Azizkhan *et al.*, 1993). In light of the importance of YY1 as both a transcriptional enhancer and

core element in TATA-less promoter formation, YY1 is discussed further in section 1.5.2.3.

#### 1.5.1.4 Differentiation and Development; Chromatin and Methylation in Gene Expression

An important mechanism by which gene expression is regulated during development involves the idea of "inaccessibility" of genes to the basal transcription machinery. Early developmental transcription is thought to be a "competitive" situation in which the transcriptional machinery of the cell compete with the chromatin for access to promoter elements (Prioleau *et al.,* 1994). Compartmentalisation of genes into chromatin, via histone proteins and nucleosomes, is thought to inhibit transcription until relieved by an appropriate activator signal. Supporting this concept, TBP cannot bind to the TATA box when DNA is complexed into nucleosomes, while activators such as SP1 and GAL4 retain DNA binding function (Stargell and Struhl, 1996). GAL4 was reported to be capable of displacing nucleosomes from the GAL1 promoter *in-vivo,* suggesting that one role of activators may be to open up chromatin to initiate transcription of developmentally repressed genes. Additionally, the CTD-tail of the RNA Pol II holoenzyme (Section 1.5.1.1) has been found to associate with members of a family of proteins, termed the SWI/SNF family, that can disrupt nucleosomes (Halle and Meisterernst, 1996).

Methylation of enhancer binding sites has been reported to affect the binding capacity and hence ability to activate gene expression of a number of transcriptional enhancers (Lamb *et al.,* 1991; Gatson and Fried, 1995). Therefore, methylation is thought to function in the silencing of gene expression during developmental processes. In addition, it has been suggested that methylation may play a role in carcinogenesis (Counts and Goodman, 1995) resulting either from the loss of methylation (hypomethylation) of promoter regions of oncogenes or hypermethylation of tumour supressor proteins.

### 1.5.2 Transcription Factors Central to Proliferation and Differentiation

While there are a whole host of transcription factors that have been identified in recent years suggested to play important roles in the regulation of processes such as proliferation and differentiation, a number of key factors appear more frequently than others. This suggests that they may play central roles in such processes on a more universal scale. The role that these factors play in the regulation of both basal and specific gene transcription is extremely complex. The promoter regions of many of these factors contain binding sites for the factors they regulate themselves, as well as binding sites for the protein they encode. This establishes very complex regulatory feedback loops that, at our present state of knowledge, appear dauntingly complex. While it is impossible to describe every transcription factor known to date, three key factors involved in proliferation, apoptosis and differentiation are discussed in greater depth below. Their interactions with many of the other well known enhancers are briefly described to give an impression of the complexity of the problem facing those attempting to solve the puzzle that is higher eukaryotic transcriptional regulation.

#### 1.5.2.1 The AP-1 Transcription Factor Complex

AP-1 (Activating Protein-1) consists of a family of protein initiation factors that hetero- and homo-dimerise to form transcriptionally active complexes. AP-1 was discovered as a transcription factor that mediates gene induction by the ester tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Karin, 1995) and hence the name TRE (TPA Response Element) for its recognition sequence. The main members of this family are *c-fos*, FosB, *c-jun*, JunB and JunD, as well as ATF (Activating Transcription Factor) (Angel and Karin, 1991; Karin *et al.*, 1997). Both Fos and Jun have been shown to possess DNA binding and leucine zipper regions, and contact DNA directly (Abate *et al.*, 1990). AP-1 activity is regulated through the relative ratios of hetero- and homo-dimer complexes, which exhibit differential activating potentials e.g. jun-jun dimers are more active than fos-jun in transactivating AP-1 sites (Smeal *et al.*, 1991) (Figure 1.4). The activity of fos and jun proteins is regulated by phosphorylation through MAP (Mitogen Activated Protein) kinases (Whitmarsh and Davis, 1996).

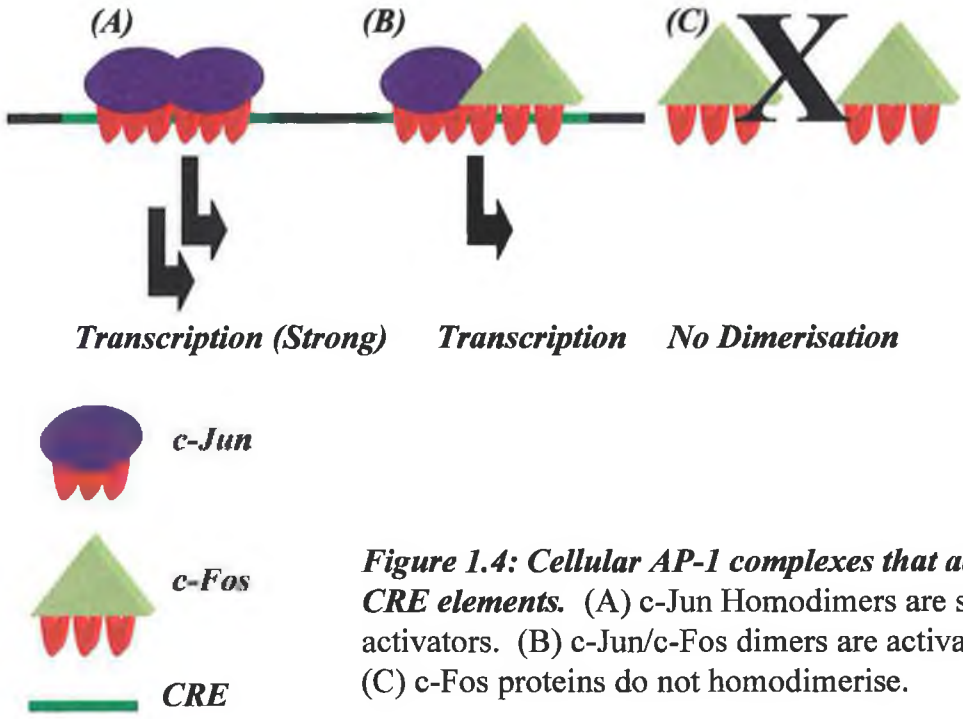



***Figure 1.4: Cellular AP-1 complexes that activate CRE elements.*** (A) c-Jun Homodimers are strong activators. (B) c-Jun/c-Fos dimers are activators. (C) c-Fos proteins do not homodimerise.

The reported roles of AP-1 in the regulation both proliferation and apoptosis appears contradictory, since thay are completely opposing processes. The decision to commit to apoptosis, differentiation or hyperproliferation probably depends on a whole host of other factors (activators) within the cell that will alter the response of genes to which AP1 binds. While both *c-fos* and *c-jun* are well established positive regulators of both proliferation and apoptosis, AP-1 has been implicated in the negative regulation of MyoD expression in dividing myoblasts (Pedraza-Alva *et al.,* 1994). This suggests that the growth stimulatory effects of AP-1 may include downregulation of differentiation-related regulatory factors, such as MyoD, through binding to negative-acting elements in the MyoD promoter region. Treatment of breast cancer cell lines with mitogens was shown to induce AP-1 activity, while treatment with the growth inhibitor and differentiating agent Retinoic Acid resulted in decreased AP-1 activity (van der Burg *et al.,* 1995). These reports suggest that growth stimulatory effects of AP-1 are downregulated during differentiation itself, while its activity may downregulate differentiation-related genes in actively dividing cells. However, in true cell-specific complexity, RA-induced differentiation of EC (Embryonal Carcinoma) and ES (Embryonal Stem) cells results in increased AP-1 expression (Pankov *et al.,* 1994), implying roles for AP-1 in the positive regulation of differentiation.

#### 1.5.2.1.1 The *c-fos* and *c-jun* proto-oncogenes of AP-1

c-Fos (Finkel osteogenic sarcoma) is a 42 kDa protein that belongs to the same family of bZIP (basic leucine zipper) transcription factors as *c-jun* (Curran, 1988; Karin, 1995). While a number of transcription factors have been shown to induce DNA bending around their binding sites to influence promoter structure, the ability of both Fos and Jun to induce such bends is dubious and presently in doubt (McGill and Fischer, 1998). However, DNA bending is probably only required by those factors, such as YY1 (section 1.5.2.3), that are central to the regulation of core promoter activity and transcription initiation. The regulatory function of both Jun and Fos probably lies in interactions with other enhancers and basal factors.

Key regulatory kinases control cell growth and differentiation through signalling pathways now known to influence the activity of both transcription and translation factors (cell signalling is briefly described in section 1.6.4.4). Oncogenic transformation by the Ha-Ras (Harvey-Ras) signalling kinase was shown to be mediated in part by the activation of *c-jun* and AP-1 activity (Binetruy *et al.*, 1991). Ras-mediated activation of both ERK/PKC and JNK (c-Jun N-terminal kinase) results in the phosphorylation of *c-jun* on Ser-73 and Ser-63 of the transactivation domain (Smeal *et al.*, 1991). Phosphorylation at these sites results in increased ability of *c-jun* to homo- and hetero-dimerise (Karin *et al.*, 1995), while phosphorylation next to its basic region inhibits its ability to homo-dimerise (Jun-Jun) but not to hetero-dimerise (Jun-Fos). This suggests that different signalling factors will activate different genes through the formation of different amounts of both hetero- and homo-dimers of *c-jun*. A similar mechanism of phosphorylation-mediated regulation of *c-fos* activity was reported by Deng and Karin (1994). The short sequence around the JNK phosphorylation site of *c-jun* containing Ser-63 and Ser-73, is conserved in *c-fos* (Derijard *et al.*, 1994; Davis, 1999) and is phosphorylated by a novel *c-fos* kinase, termed FRK (Fos-Regulating Kinase) (Deng and Karin, 1994). Activated Ras is thought, therefore, to activate three different kinases that carry its signal to the nucleus to induce AP-1 activity by altering both Fos and Jun phosphorylation levels and affinities for one another.

c-Fos expression occurs in a variety of different biological situations. While the basal levels of *c-fos* expression are very low in most cells, treatment with a variety of agents leads to a dramatic but transient induction of expression. Expression of *c-fos* can be induced throughout the cell cycle (Curran, 1988) in both quiescent and differentiating cells. Both viral (v-) and cellular (c-) Fos proteins can transform cells, despite their different carboxy terminals (Verma, 1986). However, to efficiently transform cells, *c-fos* requires the addition of a viral LTR (Long Terminal Repeat) sequence and the removal of a 3' non-coding element. The resulting loss of a 67 nucleotide region between the coding sequence and the poly(A) tail of the *c-fos* mRNA, absent in v-Fos, was suggested by Verma (1986) to be involved in an autoregulatory translational control of *c-fos* expression. While monocytic cell lines induced to differentiate were shown to express *c-fos* mRNA for at least 10 days, the *c-fos* protein was only detected for 120 min. The Fos protein was suggested to interact with this 67 nucleotide element and inhibit its own translation. Interestingly, the same mechanism of regulation has been proposed to regulate the levels of *c-jun* (Vig *et al.*, 1994) (Figure 1.5). Under normal conditions both *c-jun* and GR (Glucocorticoid Recpetor) are both transcriptionally active, through AP-1 sites in their 5' promoters. However, the *c-jun* mRNA is not translated because the *c-jun* protein interacts with the *c-jun* mRNA to inhibit its own translation. However, under mitogenic stimulation the Glucocorticoid Receptor is phosphorylated and its associated kinases dissociate. This allows interaction between GR and *c-jun* proteins, resulting in two interesting downstream effects on *c-jun* expression. Firstly, both *c-jun* and GR transcription are downregulated due to the lack of free *c-jun* available for AP-1 complex formation, now associated with the GR. Secondly, the lack of available *c-jun* results in a removal of the autoregulatory translational block on *c-jun*. This results in an increase in *c-jun* protein despite a decrease in *c-jun* mRNA (figure 1.5).

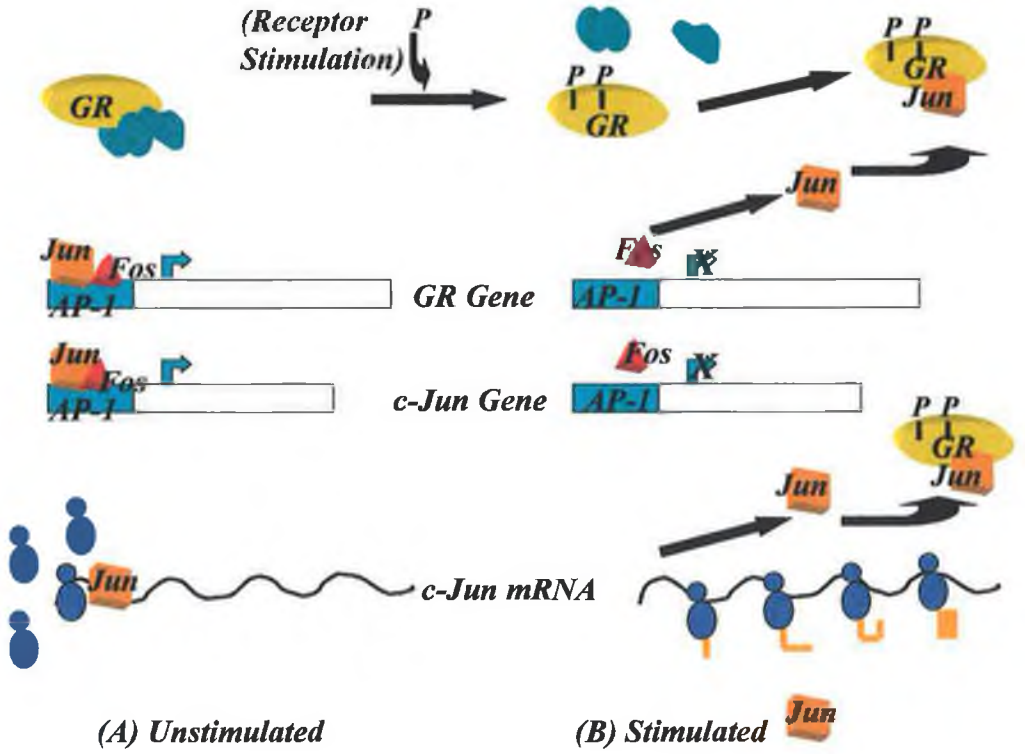



**Figure 1.5:** Translational Autoregulation of *c-jun* via association with the Glucocorticoid Receptor (GR). Mitogenic Stimulation results in association of *c-jun* with the GR, leading to dissociation of Fos/Jun AP-1 complexes. Despite decreased transcription, synthesis of the *c-jun* protein increases due to relief from autoregulation of *c-jun* translation.

While *c-fos* has been shown to play roles in oncogenic transformation (Verma, 1986; Ledwith *et al.,* 1990), elevated levels of *c-fos* are also observed during both differentiation (Hayashi *et al.,* 1998) and apoptosis (Colotta *et al.,* 1992; Smeyne *et al.,* 1993). *c-jun* on the other hand has been reported to inhibit receptor-mediated apoptosis (Shimizu *et al.,* 1996) in murine bone marrow cells while strong evidence for *c-jun* expression during human leukaemic lymphoblast apoptosis was provided by Zhou and Thompson (1996).

Clearly the roles played by the AP-1 complex and its components in proliferation, differentiation and development are complex. The outcome of elevated expression of either factor probably depends upon the presence of complementary factors within the cell. As such, responses will undoubtedly be cell-specific, and will even alter within

the same cell types depending upon the levels of complementary factors expressed at a particular time in development. Balsalobre and Jolicouer (1995) proposed that the effector genes for Fos proteins are likely to be different for different cellular processes. This undoubtedly applies to the majority of other transcription factors also. The complex and often seemingly contradictory roles and interactions between transcription factors is highlighted by the fact that both *c-fos* and c-Myc mRNAs are induced by the mitogenic signalling factor, PKC (Protein Kinase C) (Ran *et al.*, 1986). It would almost seem counter-productive then, that Fos/Jun has been reported to interact with a negative element in the c-*myc* promoter region (Takimoto *et al.*, 1989). This may, however, represent additional, as yet unknown complexities in the interactions and functions of enhancer proteins binding within individual genes.

#### 1.5.2.2 The c-*myc* proto-oncogene

First identified as the transforming gene of the avian myelocytomatosis virus (v-*myc*) (reviewed; Evan, 1990), the *myc* family of oncogenes must rank among the most widely studied of all proto-oncogenes. Despite this, there is a relative paucity of direct c-*myc* targets that have been identified to explain the capacity of this gene to induce transformation and malignancy (Ryan and Birnie, 1996). While no direct role for c-*myc* was found in some malignant conversions (DeBenedetti *et al.*, 1994), c-*myc* expression has been shown to be critical to transformation by both v-abl and BCR-ABL, as evidenced using dominant negative c-*myc* expression (Sawyers *et al.*, 1992). Genetic instability and abnormality is associated with lung cancers (Fong *et al.*, 1995) and c-*myc* abnormalities are frequently associated with carcinogenesis. c-*myc* activation has been shown to occur via gene amplification, chromosomal translocation, proviral insertion and retroviral transduction (Ryan and Birnie, 1997).

##### 1.5.2.2.1 c-Myc structure and Function

The c-*myc* gene is highly conserved, apart from its first exon, throughout vertebrate evolution. It first came to notice because of its homology to the viral oncogene, v-*myc*. While deregulated expression of c-*myc* has been associated with a variety of neoplasms, early studies indicated that introduction of the c-*myc* gene into normal fibroblasts was not sufficient to transform cells (reviewed: Evan *et al.*, 1990). The 5'

region of the c-*myc* gene contains four promoters, termed P0-P3. However, the two major promoters, P1 and P2 contribute 75%-90% and 10-25% of the cytoplasmic c-*myc* mRNAs, respectively (Ryan and Birnie, 1996; Nanbru *et al.,* 1997). The functional significance of these promoters remains a mystery. They may play roles in processes such as proliferation and differentiaion, or may simply represent evolutionary redundance of the P0 and P3 promoters. The c-Myc protein is a phosphoprotein, phosphorylated by casein kinase II (Hagiwara *et al.,* 1992) and DNA-PK (DNA-activated protein kinase) (Iijima *et al.,* 1992, Chibazakura *et al.,* 1997), and its expression is induced in response to serum and growth factor stimulation. c-Myc possesses a short cluster of basic amino acids that serve as nuclear localisation sequences (NLS) (Saphire *et al.,* 1998), in addition to DNA-binding leucine zipper motifs. The N-terminal region contains the transcriptional transactivation domain (Ryan and Birnie, 1996). There are two isoforms of the protein, c-Myc1 and c-Myc2 (Section 4.2.4.1.2), which differ by 20 amino acids in their N-terminal region (DeBenedetti, personal correspondence).

c-Myc exerts its effects through oligomerisation with other proteins (Figure 1.6), characteristic of other DNA-binding transcription factors (e.g. Jun and Fos, section 1.5.2.1.1). Originally thought to homodimerize, it is now known that this is untrue. Oncogenic activation of c-Myc requires heterodimerization with activating Max proteins (Amati *et al.,* 1993), which then bind DNA through basic-helix-loop-helix-leucine zipper motifs. Negative regulation of c-Myc activity occurs through interaction with another factor, termed Mad (Ryan and Birnie, 1996), which has no transactivating function but competes with Max for binding to the same region of the c-Myc protein. It is, therefore, a competitive inhibitor of c-Myc activation by Max. No initial sequence specificity of c-Myc binding was apparent, but it is now understood that c-Myc binds through a basic amino acid α-helix region (Fisher *et al.,* 1993) to what are termed myc-binding sequences or "E-box elements" (CACGTG). These sites require association of Max in addition to c-*myc* for activation (Ryan and Birnie, 1997). *In-vivo* activation of E-box containing genes by Myc/Max heterodimers, including an RNA helicase gene belonging to the DEAD-box family, has been demonstrated (Grandori *et al.,* 1996). c-Myc/Max complexes, active in transcription, appear to be dependent on the levels of c-Myc available within the cell

(Amati *et al.,* 1993), that is, Myc synthesis is rate-limiting for Myc-Max dimerisation and activity. Myc overexpression activates, while Max overexpression represses transcription through E-box sites. This is because Max/Max homodimers do not activate, and so compete with Myc/Max complexes when Max is overexpressed (Somer *et al.,* 1998). Max overexpressing lines show reduced expression of transiently transfected Myc-responsive genes (Zhang *et al.,* 1997), implying a role for Max expression in the regulation of processes such as differentiation.

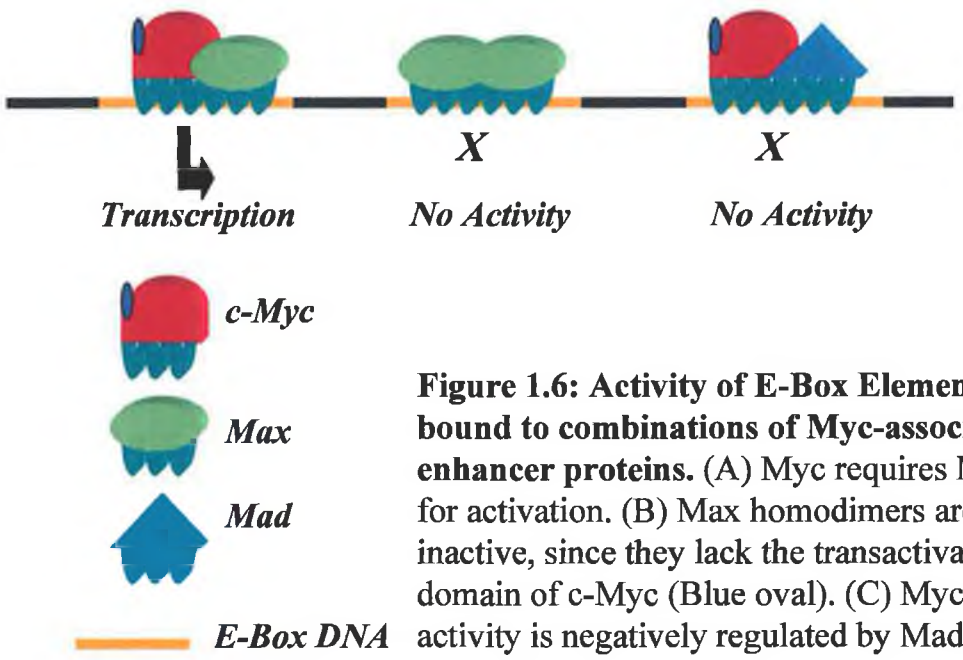



**Figure 1.6: Activity of E-Box Elements bound to combinations of Myc-associated enhancer proteins.** (A) Myc requires Max for activation. (B) Max homodimers are inactive, since they lack the transactivation domain of c-Myc (Blue oval). (C) Myc activity is negatively regulated by Mad.

Max appears to be extremely simple and is comprised of only 160 amino acids, 80 of which constitute the DNA-binding/dimerization domain (Cole, 1991), suggesting that transactivation of basal transcription occurs through the longer N-terminal region of the c-myc portion of the Myc/Max complex. This explains the lack of transactivation by Max homodimers and the findings that myc levels are rate limiting in the transactivation by Myc/Max heterodimeric complexes. Myc/Max, Max/Max and Mad/Myc complexes all bind to the Myc E-box with the same affinity (Somer *et al.,* 1998). Therefore, since the transactivation domain of these complexes lies in the Myc N-terminal, complexes lacking a Myc partner act as competitive inhibitors of Myc/Max transactivation.

However, c-Myc has also been shown to exhibit a degree of "dual functionality" in that it is capable of transcriptional repression, as well as activation (Antonson *et al.*, 1995), depending upon the position of the E-box relative to the transcription start site. As such, the role of c-*myc* in the regulation of cellular growth and proliferation should not be confined to a narrow view of transcriptional enhancement and stimulation of proliferation. Roles for c-myc in apoptosis and differentiation are evident, but as yet unclear. "It would be naïve to assume that the only transcriptional targets of c-*myc* are those involved in transformation" (Ryan and Birnie, 1997). Human bronchial epithelial cells transformed by overexpression of c-*raf*-1 and c-*myc* proto-oncogenes were capable of inducing multi-differentiated carcinomas in nude mice (Pfeifer *et al.*, 1991). This suggests that the role of c-*myc* in regulating differentiation may be cell-specific, and that downregulation of *myc* expression during differentiation (Yen and Forbes, 1990; Valy-Nagyi *et al.*, 1993) may not be a "universal" requirement of all cell types, as observed for AP-1 (Section 1.5.2.1). In addition, c-*myc* has been shown shown to play a role in the induction of apoptosis (Harrington *et al.*, 1994; Kohlhuber *et al.*, 1995).

c-Myc has been found to directly interact with a number of additional proteins, many of which are novel transcription factors in themselves (Figure 1.7). These interactions may form another level at which myc exerts its influence over the transcription process. A novel zinc-finger protein, termed Miz-1 (Myc interacting zinc-finger protein-1) has been identified that specifically interacts with Myc, but not with Max (Peukert *et al.*, 1997). Miz-1 is a transcription factor with potent anti-proliferative effects. Binding of Myc to Miz-1 inhibits the promoter activation activity of Miz-1, relieving the anti-proliferative effects of Miz-1 expression. Of note is the interaction between c-myc and the developmental regulator known as Yin-Yang 1 (YY1). YY1 regulates c-Myc levels, while association of c-Myc and YY1 proteins reduces the activity of both proteins. This may form the basis of an autoregulatory mechanism to control the levels/activity of these two proteins (Section 4.2.5). Such interactions with key transcription factors, regulating their activity, may play a significant role in the activity of c-Myc. This is particularly intriguing in light of the lack of direct transcriptional targets identified for c-Myc to date. A diagram of the known interactions between c-Myc and other enhancer proteins is shown in Figure 1.7 (reproduced from Ryan and Birnie, 1996):

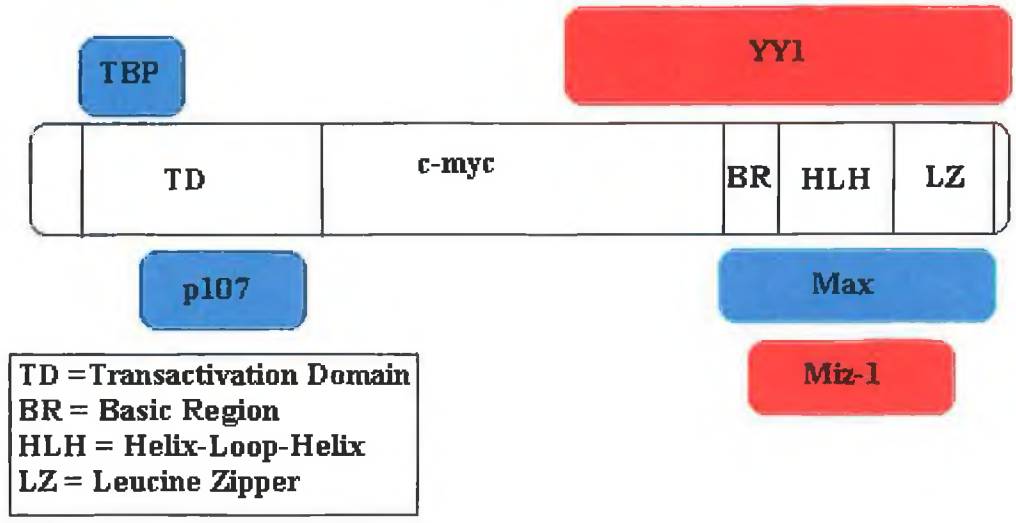



**Figure 1.7:** Regions of c-Myc interacting with other transcription factors. (Note: p107 is a member of the Retinoblastoma family of negative regulators. Its association with c-Myc inhibits the transactivation activity of c-Myc).

### 1.5.2.3 The Yin-Yang Transcription factor, YY1 (NF-E1, NF-δ, UCRBP, CF-1)

YY1 (Yin-Yang 1) is a developmentally important transcription factor, so-named because of its ability to act as both a transcriptional activator and repressor. It belongs to the GLI-Kruppel family of negative transcription factors (Licht *et al.,* 1990; Shi *et al.,* 1991), of which relatively few are known in eukaryotes. The YY1 gene was localised to chromosome 14 in humans (Yao *et al.,* 1998), although pseudogenes or additional YY1 genes have been suggested to exist (Zhu *et al.,* 1994). The promoter region of YY1 lacks consensus TATA or CCAAT boxes, but contains multiple SP-1 binding sites (Yao *et al.,* 1998), including a critical promoter region (Safrany and Perry, 1993). Four laboratories working independently cloned the YY1 gene in 1991, perhaps highlighting the universally important role of YY1 in transcriptional regulation:

1. Park and Atchison (1991) isolated a factor they termed NF-E1, which was capable of binding to both the immunoglobulin κ 3' enhancer and the immunoglobulin heavy-chain μE1 site, transcriptionally repressing and activating these promoters, respectively. The authors also reported that NF-E1 (Common Factor 1; CF1) was

capable of binding the c-*myc* promoter. The binding of CF1 was shown to be capable of activating transcription through a c-*myc* CF1 site (Riggs *et al.*, 1991). Overexpression of YY1 was shown to be a strong activator of murine c-*myc* expression, with mRNAs increasing from both the P1 and P2 promoters of the endogenous c-myc gene (Riggs *et al.*, 1993). These promoters account for the vast majority of c-*myc* transcript present in the cytoplasm [Section 1.5.2.2.1].

2. NF-δ was found to bind to and activate critical downstream promoter elements in the mouse ribosomal protein rpL30 and rpL32 genes (Hariharan *et al.*, 1991).
3. Flanagan et al (1991) isolated a negative transcription factor, UCRBP (UCR-Binding Protein) that bound to the upstream conserved region (UCR) of MMLV (Moloney Murine Leukemia Virus), downregulating promoter activity. A negative regulatory region in the HPV-18 (Human Papilloma Virus) was shown to bind YY1 with high affinity (Bauknert *et al.*, 1992) and mutation of the YY1 binding site leads to enhanced activity of the HPV-18 promoter. Many viruses that cause cancer have been found to have lost YY1 binding sites, which may be a means of escaping this negative regulation (Shrivastava and Calame, 1994).
4. Finally, YY1 was isolated and given its more widely used name by Shi et al (1991) when it was found to associate with the Adenovirus P5 promoter, activated by the viral E1A protein. In the absence of E1A this promoter is silenced by YY1, and only becomes activated in the presence of E1A. Both E1A and YY1 were found to share overlapping binding sites in the P5 promoter, but YY1 binding is not eliminated upon E1A binding, suggesting that competition for binding is not the means by which regulation occurs. E1A-mediated activation is speculated to involve unmasking regions of the YY1 N-terminal involved in activation but normally masked in the full-length protein (Lee *et al.*, 1994). (Figure 1.8).

Consensus activation and repression sequences for YY1 are shown below, although these are known to vary giving rise to changes in binding capacity of these sites for YY1 (Hyde-DeRuyscher *et al.*, 1995).

Activation: **CGGCCATCTTGNCTG**

Repression: **CCATNTTNNNA**

#### 1.5.2.3.1 The Structure and Function of YY1

There is evidence that YY1 is a phosphoprotein. Eight consensus phosphorylation sites are found in the deduced amino acid sequence and YY1 activity can be abolished through the use of phosphatases (Becker *et al.*, 1994). The amino acid sequence of the YY1 protein displays a number of unique properties to date, including acid rich domains similar to transcriptional activators, as well as Ala+Gly-rich and His rich sequences common to transcriptional repressors (Park and Atchison, 1991). The very unusual N-terminal region consists of 11 consecutive negatively charged amino acids and 12 consecutive histidines, thought to form two oppositely charged symmetrical helices separated by a highly flexible glycine-rich loop (Helix-Loop-Helix; HLH) (Hariharan *et al.*, 1991). These regions are speculated to be capable of forming an acidic activation domain that could be neutralised or modulated under certain conditions to allow interaction with polymerase II before and after transcription has commenced. The amino terminal transactivation domain requires amino acids 16-29 and 80-100 for maximal activity (Bushmeyer *et al.*, 1995).

The C-terminal contains four zinc fingers, characteristic of DNA-binding transcription factors, while the central region is largely unstructured, consisting of large loop and helix regions. The YY1 repression domain lies near the carboxy terminus and is embedded within the YY1 zinc finger region necessary for DNA-binding (Bushmeyer *et al.*, 1995). Particular importance has been placed upon zinc fingers 3 and 4 for repression activity.

The functional diversity of YY1 was conceivably attributed to its structural plasticity (Hariharan *et al.*, 1991). It is generally thought that repression of gene transcription is the usual function of YY1, with the activating N-terminal region being masked. Interaction with activating proteins, such as viral E1A, then releases the N-terminal region and converts YY1 to an activator of transcription through the same promoter (Figure 1.8). However, it has also been suggested that repression is not the intrinsic activity of YY1. Rather, YY1 acts to bend DNA (Natesan and Gilman, 1993) in a way that modulates the interaction of proteins bound to the two flanking regions. When the orientation of the YY1 binding site is reversed or the phasing of the sites is changed, YY1 becomes an activator of the same promoter (Natesan and Gilman, 1995). Rather

than bending two proteins away from one another, YY1 now bends them towards one another to bring them into closer contact and increase association. Therefore, YY1 will have distinct local effects on protein-DNA and protein-protein interactions depending upon the position and orientation of its binding site within the promoter, supporting a general role for YY1 in the building of highly organised promoter complexes. This is particularly important in the formation of promoter structures at TATA-less promoters (Section 1.5.1.3), since YY1 has been shown to bend DNA in a manner suitable to provide a site for transcription initiation (Kim and Shapiro, 1996). Both promoter orientation-dependent and co-factor-dependent activity of YY1 was also suggested in the human Interferon-γ promoter (Ye *et al.*, 1994). In this case, DNA-binding is a required function of YY1, while in other cases DNA-binding is not required for YY1 to exert its effects upon promoter formation and activity.

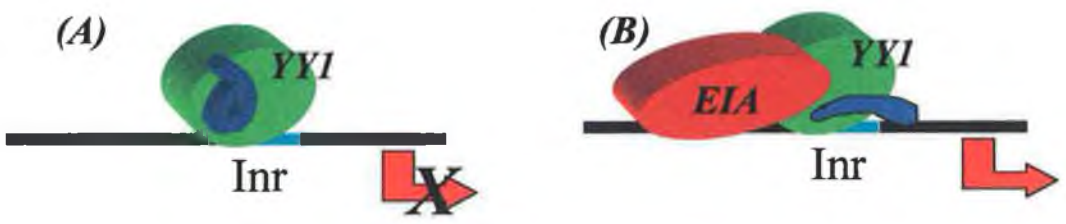



**Figure 1.8: Diagrammatic Representation of EIA-mediated activation of YY1 Transactivating Potential.** YY1 is a repressor of the P5 promoter (A), but in the presence of EIA, the N-terminal Activating Region (Blue) is unmasked and transcription is activated (B).

#### 1.5.2.3.2 Transcription Factors interact with YY1 to regulate its activity

A YY1 binding site in the *c-fos* promoter is required for adenovirus E1A activation of *c-fos* transcription (Gedrich and Engel, 1995). Rather unusually and almost paradoxically, repression by YY1 was also found to be independent of the presence of YY1 binding sites in *c-fos* reporter constructs (Zhou *et al.*, 1995). It was shown that YY1 repression was mediated through interaction of YY1 with CREB (cyclic AMP Response Element Binding) Proteins. Thus, within the *c-fos* promoter alone YY1 is known to interact with E1A and CREBPs to either increase or decrease transcription, respectively, and these functions can be both dependent and independent of the ability of YY1 to bind DNA. This has lead to claims that YY1 activity is regulated through

interactions with other proteins and that it must contain a C-terminal repression domain that is independent of its ability to bend DNA (Hyde-DeRuyscher *et al.*, 1995).

Numerous YY1-associated complexes appear to be targets for E1A activation (Shi *et al.*, 1991; Gedrich and Engel, 1995; Labrie *et al.*, 995). In fact, a major role of viral E1A may be the activation of genes normally repressed by YY1, including viral genes (Shrivastava and Calame, 1994). SP1 frequently acts as a regulator of YY1-associated complexes (Bennett *et al.*, 1999), particularly during TATA-less promoter complex formation (Section 1.5.1.3). "Bi-functionality" is evident in the ability of YY1 to simultaneously upregulate some genes while downregualting their antagonists. For example, transcription of the LDL (Low-Density Lipoprotein) receptor gene is inhibited by YY1 during high cholesterol (Bennett *et al.*, 1999), while that of Cholesterol Esterase is enhanced (Gauthier *et al.*, 1999). This was attributed to interactions of YY1 with SP1 in the cholesterol esterase promoter and with SRE-BP in the LDL promoter, inhibiting their function. The ability of YY1 to repress numerous SRE-BP (Serum Response Elemement-Binding Proteins) regulated genes has been associated with the displacement of Factor Y, a positive regulator of gene transcription (Ericsson *et al.*, 1999). Similar "bi-functionality" is evident during proliferation, in which YY1 upregulates c-*myc* gene transcription, correlating with cellular proliferation, and inhibits muscle actin expression, correlating with differentiation (Lee *et al.*, 1994). However, despite being shown to interact both *in-vitro* and *in-vivo* (Lee *et al.*, 1993; Seto eta l, 1993), both YY1 and SP-1 appear to function independently at the surf-1 promoter, where the YY1 binding site has been shown to be both necessary and sufficient to confer growth-factor inducibility in transcription of the Surf-1 gene (Cole and Gatson, 1997). Activation of transcription by YY1 independent of DNA-binding has been shown for the α-1 acid glycoprotein (AGP) promoter through functional interaction with a negative DNA-binding factor, termed Factor B (Lee and Lee, 1994). In the human GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor) promoter co-factors in addition to YY1 were required for activator function and promoter complex formation (Ye *et al.*, 1994), but in this case binding of YY1 to DNA is required (Ye *et al.*, 1996),

Additionally, YY1 has been suggested to participate in the stimulation of autonomously replicating human chromosome fragments through interaction with a replication-enhancing element, REE1 (Obuse *et al.,* 1998) and in the regulation of transposable elements of the genome (Becker *et al.,* 1993; Satyamoorthy *et al.,* 1993; Singer *et al.,* 1993). These elements are thought to be a major source of functional diversity allowing evolution to continue. Overall, YY1 appears to perform a multitude of tasks, many of which are influenced by its ability to bind to DNA and affect promoter structure and formation, as well as interact with numerous transcriptional enhancers to modify its own activity. The complexity of the regulatory effects of YY1 is highlighted by Bushmeyer et al (1995); "YY1 can either activate or repress some promoters depending on either promoter architecture or intracellular milieu". These unique properties suggest an unusual and complex role for YY1 in the regulation of gene expression.

#### 1.5.2.3.3 YY1 and TATA-less Transcription

As outlined in Section 1.5.1.3, YY1 is thought to play a central role in the formation of transcription initiation complexes at TATA-less promoters. Promotion of TATA-less transcription by YY1 was initially suggested by the *in-vitro* transcription experiments of Seto et al (1991) and Hahn et al (1992). YY1 has been shown to bend DNA and is thought to play a role in the formation of promoter structures for RNA pol II binding (Natesan and Gilman, 1995; Kim and Shapiro, 1996). In an *in-vitro* transcription reaction, supercoiled DNA templates could be transcribed in the presence of only YY1, TFIIB and RNA Pol II (Usheva and Shenk, 1994). Overall, YY1 is thought to be a key regulator of TATA-less promoter initiation, probably in all TATA-less promoters (Azizkhan *et al.,* 1993). Its ubiquitous expression is in agreement with the findings that many universally expressed housekeeping genes appear to lack any discernible TATA recognition sequence, including the YY1 gene itself (Yao *et al.,* 1998). In light of this, a report challenging the concept that TBP-mediated association of TFIID with the TATA-box is limiting in the rate of transcription initiation is of interest (Antoniou *et al.,* 1995). Altered transcription was only observed when TBP binding was drastically decreased in the promoter of the β-

globin gene. However, this promoter also contains an active YY1 binding site, the importance of which may have been overlooked by the authors.

The ability of YY1 to interact with TFIIB/D is also thought to be a means by which YY1 regulates TATA-less promoter formation, by-passing the requirement for TBP in these systems. Recently $TAF_{II}55$ (TATA-Binding Protein-Associated Factor), a subunit of TFIID, has been shown to interact directly with the largest subunit, $TAF_{II}230$ through its central region and with multiple activators – including SP1, YY1 and Adenoviral E1A – through a distinct amino-terminal domain (Chiang *et al.*, 1995). This subunit may form the "bridge" between transcriptional enhancers and the actual transcriptional components surrounding RNA polymerase II. It is possible that YY1 is part of, or is actually the "bridging unit", particularly in TATA-less promoters (since TAF55 is a basal unit, while YY1 appears to be "in-limbo" between enhancer and basal transcription factor, depending upon the promoter). The effects of SP1 on YY1-mediated transcription initiation, particularly from TATA-less promoters, may reside in its interaction with $TAF_{II}55/230$ to guide the initiation complex towards the Inr-associated YY1 to begin initiation.

Further evidence that YY1 plays a role transcription through TATA-less promoters has been provided by Gatson and Fried (1994), Cole and Gaston (1997), Johansson et al (1998) and Karantzoulis et al (1999). In addition, YY1 is thought to play a role in the downstream regulation of transcription (Last *et al.*, 1999). The majority of known transcriptional enhancers are upstream, since they would interfere with the actual transit of the RNA polymerase II if situated downstream, while YY1 appears to interact with many of the basal factors and may form part of the basal RNA holoenzyme in some circumstances.

#### 1.5.2.3.4 YY1 in Differentiation and Development

The unusual nature of the YY1 protein has led to speculation that it may play a key role in the regulation of differentiation and development. Both Chromatin structure and methylation are thought to be key mechanisms by which cells control specific gene transcription during differentiation. The Nuclear Matrix Protein-1 (NMP-1), a transcription factor which has been shown to associate with the nuclear matrix to

mediate gene-matrix interactions within the nucleus, has been shown to be none other than YY1 (Guo *et al.*, 1995). Sequences necessary for nuclear localisation and association with the nuclear matrix have been identified in the C-terminal region of the YY1 peptide (Bushmeyer and Atchison, 1998; McNeil *et al.*, 1998). Nuclear-matrix-associated transcription factors may affect gene regulation by mediating transient associations between DNA and the nuclear matrix, locally unravelling chromatin structure to allow the transcriptional machinery to access promoters and begin transcription, implying roles for YY1 in activating repressed genes during development.

Binding of YY1 to DNA during globin promoter formation is known to be methylation-sensitive (Satyamoorthy *et al.*, 1993; Yost *et al.*, 1993), which may imply a role for YY1 in tissue- and developmental-specific transcription of genes. A YY1 binding site is thought to function in the stage-specific expression of the fetal (gamma) globin gene (Zhu *et al.*, 1999). The human ε-globin gene is transcribed in erythroid cells only during the embryonic stages of development. A binding site for YY1, around nucleotide –269, was identified as critical in the formation of the ε-globin repressor complex (Raich *et al.*, 1995), forming part of the local regulatory elements suggested to be involved in the regulation of embryonic stage-specific expression of this gene. Processes such as these, resulting in the stage-specific switches in gene expression, are thought to be associated with methylation of CpG islands, which silence transcription of developmentally important genes and to which YY1 binding is sensitive.

In addition, levels of YY1 have been shown to decrease during differentiation of mouse myoblasts (Lee *et al.*, 1992). YY1 contains several peptide regions prone to proteolytic cleavage, raising the possibility that protease-mediated degradation events may contribute to diminished YY1 protein levels during myogenesis (Lee *et al.*, 1994). Two proteolytic pathways through which YY1 can be differentially targeted under different cell growth conditions have been identified (Walowitz *et al.*, 1998), identifying a role, at least partially, for protease calpain II (m-calpain). However, in serum starvation studies YY1 protein expression was lost only after 24 hours, despite the fact that YY1 transcript expression was lost within hours (Flanagan, 1995),

suggesting that the YY1 protein is relatively stable. This does not exclude the possibility that proteolytic regulation of YY1 levels may play a role in different processes.

Treatment of myoblasts with the differentiation modulating agent, BrdU results in inhibition of myogenesis, resulting in/from an increase in expression of YY1 and decreased α-actin levels (Lee *et al.*, 1992). Transfection of SRF (Serum Response Factor), which competes with YY1 for the regulation of α-actin gene transcription, could directly transactivate the actin promoter in BrdU-treated myoblasts. Both SRF and YY1 are ubiquitously expressed, suggesting that they may have antagonistic functions in regulating genes such as *c-fos*, α-actin and cardiac creatine kinase-M (Vincent *et al.*, 1993; Liu *et al.*, 1995) during development. High levels of YY1 in non-differentiated muscle cells down-regulate the dystrophin promoter, at least in part, by interfering with the spatial organisation of the promoter (Galvagni *et al.*, 1998). YY1 and a positive regulator of dystrophin, DPBF (dystrophin promoter bending factor), induce opposite bends in the CArG element of this promoter, suggesting that their binding induces alternative promoter structures to regulate muscle development.

## 1.6 Translational regulation of Gene Expression

Initial research into the control of gene expression in animal cells assumed that regulation would be mainly at the level of transcription, presuming that the cell would not expend valuable energy transcribing mRNAs that were not going to be translated into a functional protein. It has recently been realised, however, that regulation of gene expression at the level of translation is of critical importance to early embryonic development (Curtis *et al.*, 1995; Vassalli and Stutz, 1995), cell growth (DeBenedetti and Rhoads, 1990) and differentiation (Luis *et al.*, 1993; Beretta *et al.*, 1998). Translational regulation also allows cells to respond more rapidly to environmental stimuli such as nutritional changes (Wang *et al.*, 1998) and stresses such as viral infection (Clemens *et al.*, 1996) than would otherwise be permitted by the *de novo* response of transcription. During early embryogenesis many of the mRNAs produced are translationally repressed and sequestered in "masked", inactive but stable storage complexes called mRNPs (Messenger Ribonucleoproteins) (Vassalli and Stutz, 1995; Spirin, 1996). These allow a high rate of transcription in the early stages creating a stockpile of mRNA for use in later development. Translation has been shown to be essential for the generation of protein gradients and the graded distribution of proteins within a single cell, generating the asymmetries required for the formation of embryonic pattern (Gavis *et al.*, 1992; reviewed by Curtis *et al.*, 1995). It is now becoming evident that translational repression is an important mechanism employed by cells to closely regulate the temporal expression of differentiation-related genes such as LOX, Tra-2 and Lin-14 (section 4.2.2.1.1).

Eukaryotic mRNAs, unlike their Prokaryotic equivalents, have long runs of polyadenine residues, known as Poly(A) tails at their 3' ends and a 5' "Cap" consisting of methylated Guanine residues (Watson *et al.*, 1992). Neither feature is encoded on the DNA sequence but is added to the ends of transcribed RNAs as the transcript is processed (Figure 1.9).

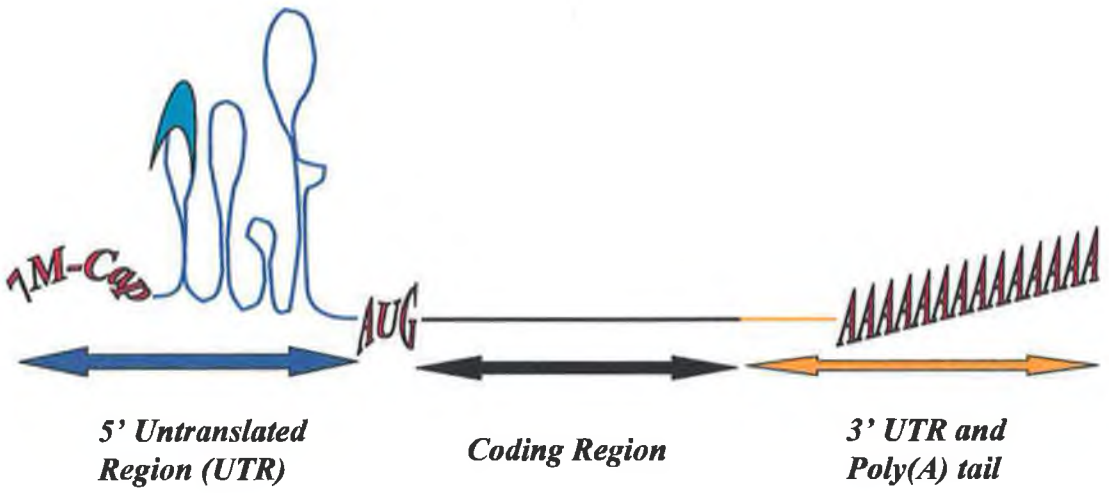



**Figure 1.9: Basic Structural Features of Eukaryotic the mRNA molecule.** (A) The Cap guides the ribosome onto the correct end of the mRNA. Complexity in the 5' UTR (Blue) and binding of repressor proteins (Green) inhibit the progress of the scanning PIC. (B) An in-context AUG (see fig. 1.11) determines the beginning of the coding region. (C) The 3' UTR (Orange) consists of elements that regulate the polyadenylation (AAA) of the 3' end of the mRNA.

### 1.6.1 The 3' Untranslated region

The 3' Untranslated region (UTR) of eukaryotic mRNAs consist of a string of non-coding sequences followed by a stretch of Adenine residues at the extreme 3' end, called a poly(A)-tail (Figure 1.9). The significance of the poly(A)-tail is only now beginning to be realised. The 3' UTR and poly(A)-tail, and their binding proteins are now thought to play a crucial role in the regulation of translation initiation, mRNA masking, mRNA degradation and storage, mRNA transport and localisation within the cell. It is now generally accepted that the 3' end of the mRNA interacts with the 5' end to influence events at the start of the transcript, laying to rest the old ideas of the mRNA being a one-dimensional, non-interacting molecule (Figure 1.10). In fact, the 3-D model of the structure of the mRNA is rapidly gaining support and it helps to explain the concepts of mRNPs, masking, and the very strong influence of the 3' UTR on initiation events at the extreme 5' end of the transcript. In agreement, several EM images have proven the existence of circular mRNAs.

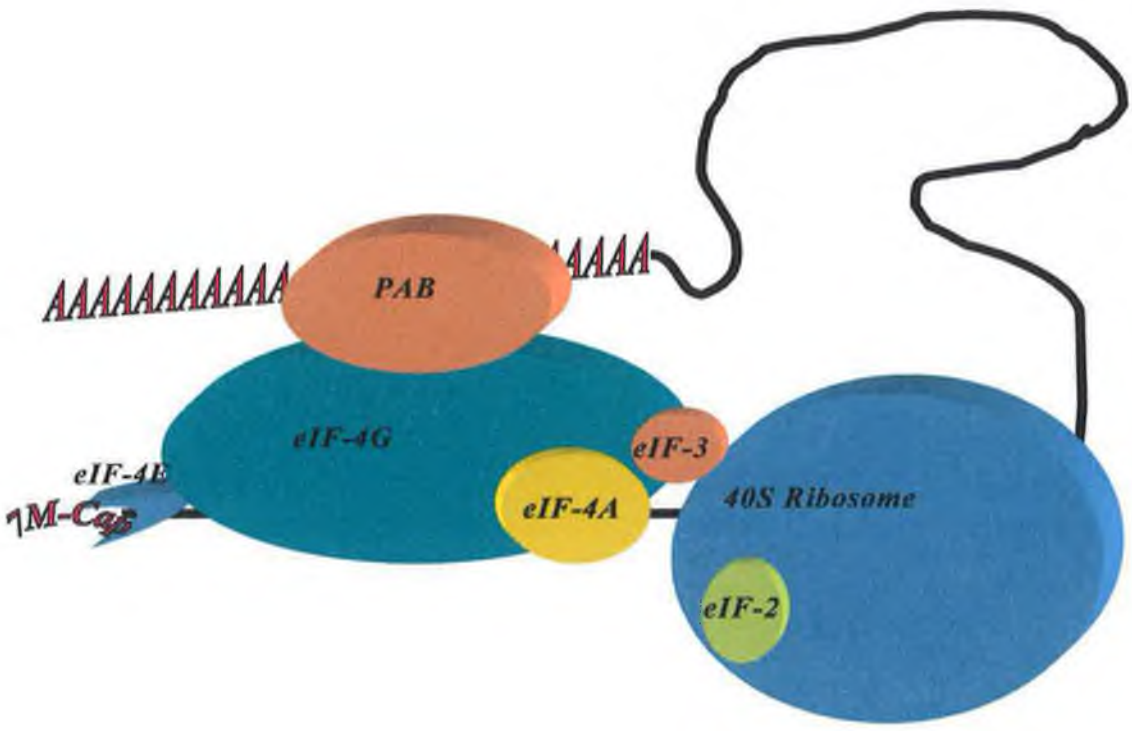



**Figure 1.10: Circularisation of eukaryotic mRNAs is thought to be due to interaction between 5'- and 3'-bound proteins.** Known to interact are eIF-4G and PAB (Poly(A)-binding protein) (Section 1.6.3.2.4.2).

The length of the poly(A)-tail influences the translation of many maternal mRNAs during early embryonic development. Repressed or dormant mRNAs are "awoken" through polyadenylation induced by developmental signals (Sonenberg, 1994; Spirin, 1996) which probaly recruits initiation factors to begin translation. The 3' UTR of maternal mRNAs is critical to the control of poly(A)-tail length and hence translational efficiency of the mRNA, as shown by the removal of the 3' UTR (Wormington, 1993). Dormant mRNAs have a short poly(A)-tail, mRNAs that are being silenced undergo a shortening in the length of the poly(A)-tail (deadenylation), mRNAs that are translationally recruited or activated undergo a poly(A)-tail lengthening, and it has been shown that a long poly(A)-tail is necessary for translation. Deadenylation is almost invariably coupled with translational repression (Wormington, 1993), with a large proportion of the cells mRNAs becoming deadenylated at maturation. The influence of the 3' UTR is thought to be due to the presence of cis-acting regulatory sequences that recruit factors that control the cytosolic deadenyaltion and readenylation of the mRNA (Vassali and Stutz, 1995), as

well as sequestering particular proteins to the mRNA involved in the regulation of translation initiation (Sonenberg, 1994; Tarun and Sachs, 1996; Fraser *et al.*, 1999).

### 1.6.2 The 5'-Untranslated Region ( 5'-UTR )

The 5'end of Eukaryotic mRNAs is blocked by the addition of a 7-Methyl-Gppp 'Cap' (7-M-Gppp) which consists of seven methylguanosine residues joined to mRNAs by triphosphate linkages added during the synthesis of the primary transcript (Figure 1.9). The Cap-site guides the ribosome onto the transcript via the Cap-binding protein, eIF-4E, from where it scans along the 5' UTR in search of an in-frame AUG start codon (Svitkin *et al.*, 1996). The 5' Cap-structure has been identified as playing roles, in addition to initiation, which include stability, splicing, 3' end processing and nucleocytoplasmic transport (Sonenberg and Gingras, 1998).

Once bound to the cap, "scanning" for the AUG initiator codon is regulated by the degree of secondary structure adopted by a particular mRNAs 5' UTR. Growth-related mRNAs tend to have long, complex 5' UTRs that impedes the progress of the scanning ribosome and renders these RNAs extremely susceptible to translational regulation (van der Velden and Thomas, 1999). The sequence context surrounding the AUG/CUG start site determines the rate of initiator codon recognition by the scanning ribosomal RNA (Section 1.6.3). In addition, the binding of mRNA-specific repressors has been found to play critical roles in the regulation of developmental proteins (reviews; Wormington, 1993; Sonenberg, 1994; Standart and Jackson, 1995), as well as in the rapid response of cells to environmental changes, such as the iron response (Bhasker *et al.*, 1993; Schalinske *et al.*, 1998). These are discussed further in section 4.2.3.3.1.

### 1.6.3 Translation Initiation

Initiation is the primary target for the control of translation, with the binding of the ribosomal pre-initiation complex to the mRNA and the scanning process being controlled through a number of mechanisms including RNA-binding repressors, modulation of the Initiation Factors involved (usually by phosphorylation), and the effects of secondary structure adopted by a particular mRNAs 5'-UTR.

The basic process of translation initiation involves the binding of the 40S Ribosomal subunit complexed with a charged initiator tRNA (tMet) to the 5' Cap from where it scans the 5' UTR in search of an in-frame AUG start codon. tRNAs (transfer RNAs) carry the Amino Acids to the actively translating ribosome during protein synthesis. The Eukaryotic signal to begin translation is an AUG codon in a particular context, and as such, all proteins begin with a Methionine (encoded by AUG and recognised by t-Met) that is later cleaved. The 40S ribosomal subunit is guided onto the correct region of the mRNA to begin translation by numerous Initiation Factors, known as eIFs (eukaryotic translation Initiation Factors), that catalyse various stages of the binding, scanning and initiation processes (reviews; Kaufman, 1994; Pain, 1996, Kleijn, 1998). The initiation complex binds to the mRNA 5'-UTR at the 7'-Methyl-Gppp Cap, guided by particular eIFs, from where it "scans" the 5' UTR in search of the initiator AUG codon. Recognition involves the Ribosomal mRNA and tRNA and the rate of initiation is influenced by the context of the bases surrounding a particular mRNAs AUG (Figure 1.11). The better the context of the bases surrounding the AUG initiator match those of the scanning complex RNA the slower the complex passes over the AUG codon, stalling the ribosome and increasing the rate of recognition of the initiator codon. It is primarily structural complexity in the 5' UTR that affects the rate of initiation due to the formation of secondary structures that can be quite inhibitory to the progress of the scanning 40S ribosomal complex.

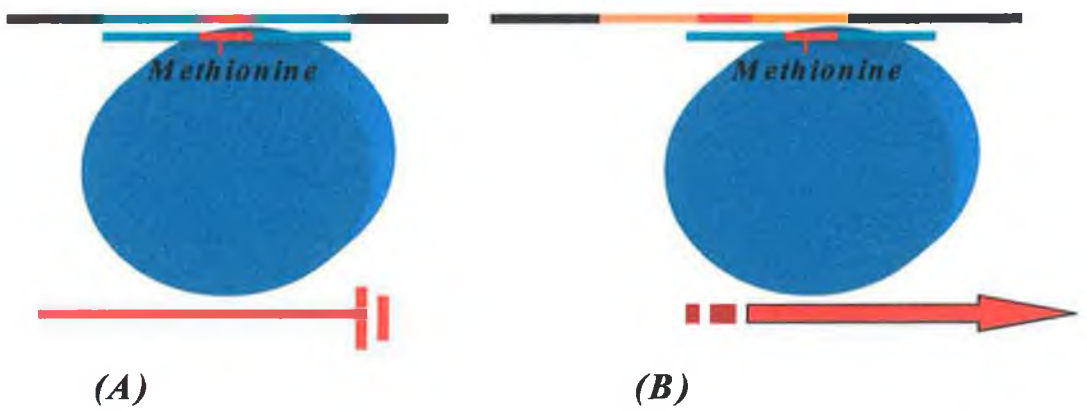



***Figure 1.11: Ribosomal Stalling and Start Codon Recognition.*** (A) A good context (green) match surrounding AUG Start codons results in Watson-Crick binding of RNAs, stalling of the ribosome and increased recogntion by the initiator Met-tRNA. (B) Poor contexts result in ribosomal "slipping", where the moving ribosome does not stall and recognises the AUG less frequently. Poor translation occurs in this case.

### 1.6.3.1 Pre-initiation complex formation and CAP-binding Events

The translation process is primarily regulated by the rate of Ribosome binding to the mRNA, which requires at least 13 different initiation factors known to date. The initiation factor eIF-2 binds Met-tRNA and forms a ternary complex with GTP, which then binds to the 40S Ribosomal subunit. This complex then associates with the 5'-end of the transcript via an interaction with another initiation factor, eIF-4F, which is the 5' Cap-binding complex, to form the 43S Pre-Initiation Complex (PIC). eIF-4F (section 1.6.4) consists of three primary subunits; eIF-4E, the cap binding protein, eIF-4G (p220), the scaffold protein upon which the eIF-4F complex is formed, and eIF-4A, the helicase that is involved in the unwinding or "melting" of 5'UTR structure to allow efficient Ribosome binding and scanning to occur. The PIC binds the 5' end of the mRNA at the Cap recognition site and begins to scan the 5'-UTR of the mRNA in the 3' direction in search of an AUG initiator codon in the appropriate context. The event of initiation is cyclic, in that once initiation at the AUG codon occurs the initiation factors dissociate and are recycled for use in another round of intiation (Jackson, 1998) (Figure 1.12).

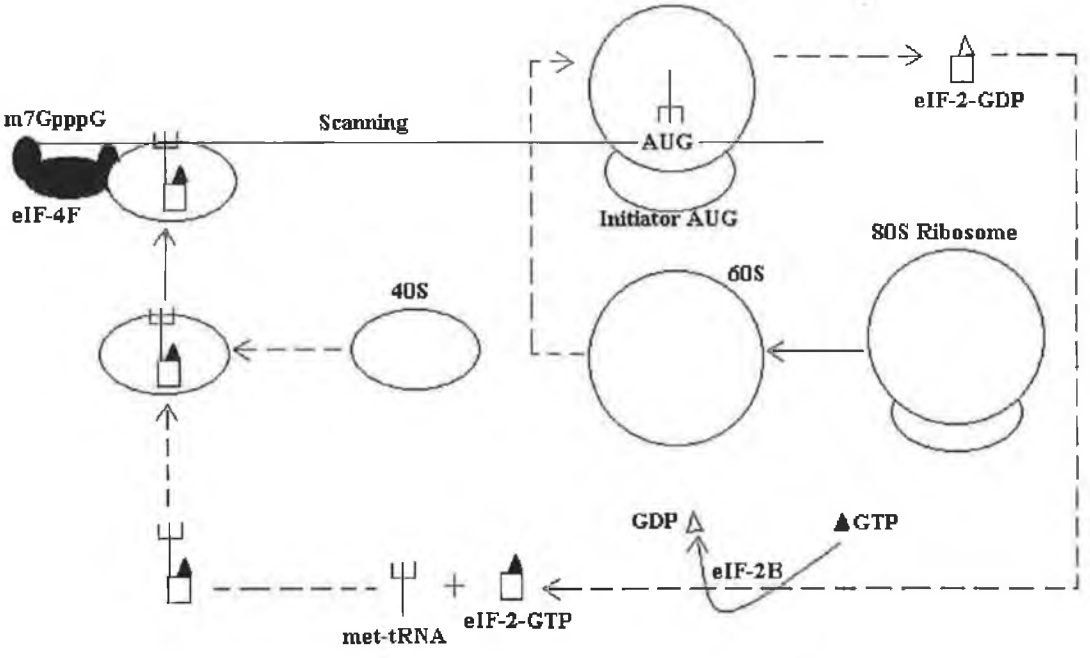



**Figure 1.12:** The process eukaryotic translation initiation is a "recycling" event.

### 1.6.3.2 The initiation Factors

With their individual roles the eukaryotic translation Initiation Factors (eIFs) act in concert to assemble the ribosome on the 5' end of the mRNA and begin the scanning process in search of the initiator AUG codon.

#### 1.6.3.2.1 eukaryotic Initiation Factor 1, eIF-1:

eIF-1 is essential to the transfer of Amino Acid-tRNA, as a tRNA-GTP-eIF2 complex, to the 40S ribosomal subunit (Pestova *et al.*, 1998, CSHL Abstracts) through interactions with eIF-2. eIF1/1A can destabilise abberant preinitiation complexes.

#### 1.6.3.2.2 eukaryotic Initiation Factor, eIF-2:

eIF-2 recruits the initiator tRNA (Met-tRNA) and conducts it as a Met tRNA-eIF2-GTP complex to the 40S Ribosomal subunit, to form the 43S pre-initiation complex (Colhurst *et al.*, 1987; Altman and Trachsel, 1993). Once the tRNA has released the charged Amino Acid upon AUG recognition, the eIF-2 polypeptide is released as a GDP bound inactive binary complex. The recycling of eIF-2 to begin another round of initiation requires that the bound GDP be exchanged for a molecule of GTP, which is catalysed by eIF-2B (Section 1.6.3.3.2.1). Within the cell, eIF-2B is present at lower molar concentrations than eIF-2, which forms a means for the regulation of eIF-2 activity mediated by eIF-2B. Phosphorylation of eIF-2, on the $\alpha$ subunits' Ser 51 residue (Mellor *et al.*, 1993) increases its affinity for eIF-2B thereby sequestering it in an inactive eIF2-GDP-eIF2B complex. The resultant reduction in the levels of free eIF-2B available to catalyse the recycling of eIF-2 reduces the rates of translation initiation (Oldfield *et al.*, 1994). Phosphorylation of the $\alpha$-subunit of eIF-2 and the subsequent down-regulation of translation has been associated with the mammalian eIF-2$\alpha$ kinases PKR and HRI, and the Yeast kinase GCN2 (Section 1.6.3.2.2.2). Glucose, which stimulates insulin protein synthesis levels, has been shown to stimulate the activity of eIF-2B (Gilligan *et al.*, 1996), increasing eIF-2 recycling.

eIF-2 is a complex of three polypeptide chains, α, β and γ, which are thought to remain associated throughout the process of initiation (Proud *et al.,* 1991). The α-subunit is the site for regulation of eIF-2 activity, via phosphorylation. The β-subunit is involved in the interaction of eIF-2 with eIF-2B (Section 1.6.3.2.2.1), which recycles eIF-2 via GDP-nucleotide exchange, and with eIF-5 (section 1.6.3.2.5), which catalyses GTP-hydrolysis to release the tRNA during initiation (Figure 1.13). The γ-subunit is the actual "carrier" of the GTP/GDP molecule (Asano *et al.,* 1998; Pavitt *et al.,* 1999).

eIF-2-mediated downregulation of translation is seen in response to viral infection, due to induction of PKR activity by viral ds-RNA, but recently roles have been discovered for eIF-2 phosphorylation in the regulation of cell growth and differentiation (Section 4.2.4.1.3).

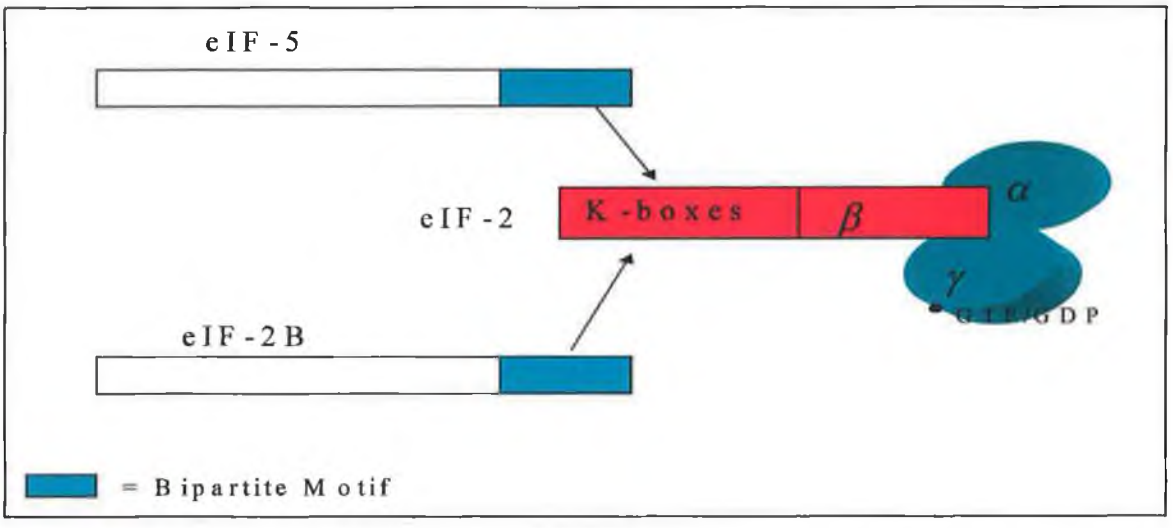



**Figure 1.13: Diagrammatic Representation of the Structure of the eIF-2 multi-unit Polypeptide and its interaction with eIF-5 and eIF-2B.** They bind eIF-2 by similar mechanisms (bi-partite motifs) and are both involved in nucleotide exchange in eIF-2, recycling eIF-2 (eIF-2B) and catalysing Methionine release (eIF-5) during initiation.

#### 1.6.3.2.2.1 eukaryotic Initiation Factor, eIF-2B

eIF-2B is frequently referred to as the GEF, or Guanine Nucleotide Exchange Factor, and is a complex of five polypeptide chains, termed α, β, γ, δ, and ε, in both mammalian cells and yeast (Oldfield and Proud, 1992, Oldfield *et al.,* 1994). It catalyses the guanine nucleotide (GTP/GDP) exchange reaction required to recycle eIF-2 bound as an inactive binary complex with GDP, into the active GTP-bound form capable of recruiting new molecules of initiator tRNA. In the absence of eIF-2B, nucleotide exchange in eIF-2 is extremely slow (Oldfield *et al.,* 1994). Composed of five non-identical subunits, eIF-2B is structurally complex compared with other exchange factors. eIF-2B can be physically and functionally divided into two parts (Figure 1.14): the regulatory subcomplex is composed of three homologous subunits (α, β, and γ) that responds to eIF-2α $Ser^{51}$ phosphorylation, while the functional nucleotide exchange/catalytic complex resides in the other two subunits (δ, ε) (Pavitt *et al.,* 1999). The eIF-2Bε subunit appears to possess the nucleotide exchange activity (Oldfield *et al.,* 1996). It has recently been speculated that eIF-2B, in conjunction with eIF-2α, are the key factors in the regulation of global protein synthesis (Kimball *et al.,* 1998; Kimball *et al.,* 1999). All eukaryotic mRNAs exhibit the same dependence for Met-tRNA for initiation, regulated by association with eIF-2α to become incorporated into the 43S PIC and ultimately into the translating ribosome.

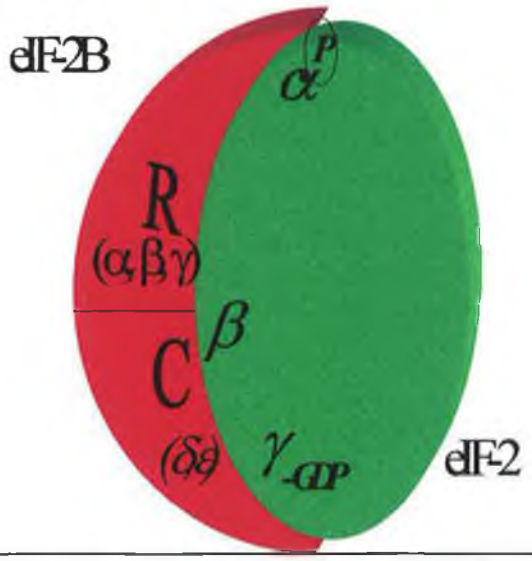



**Figure 1.14: Interactions between eIF-2 and its regulatory factor, eIF-2B.** R= Regulatory Region, C= Catalytic Region.

#### 1.6.3.2.2.2 The eIF2α Kinases

Phosphorylation of the eIF2α subunit of eIF2 is the best-characterised mechanism for the downregulation of protein synthesis (Review; Clemens *et al.,* 1996). This phosphorylation has been shown to involve a group of enzymes known as the eIF2α kinases. The eIF2α kinases possess catalytic domains that show 12 conserved subdomains characteristic of all eukaryotic Serine/Threonine kinases, together with similarities such as large insert sequences that distinguish them from other kinases (DeHaro *et al.,* 1993; Chen and London, 1995). The subdomain V homology within this class of kinases may indicate that this motif contains the substrate specificity domain. However, the non-catalytic regions are very different from one another, allowing different physiological signals to stimulate phosphorylation of eIF2 under different circumstances. For example, both HRI and PKR (discussed in the following paragraph) perform the same task of phosphorylating eIF-2α, via the subdomain V shared by this class of kinases. However, due to different non-catalytic regions, their activity is induced in response to different physiological signals (heme deficiency and viral infection, respectively) (DeHaro *et al.,* 1993).

Of the known eIF-2α kinases, HRI (Heme Regulated Inhibitor) is a mammalian Heme-Regulated Inhibitor kinase that is induced by heme deficiency (Chen and London, 1995). GCN2 is a yeast kinase that is activated by uncharged tRNA during conditions of Amino Acid starvation (DeHaro *et al.,* 1993), shutting down host protein synthesis at times when it is inappropriate. The best characterised of the kinases is the viral-induced PKR (Protein Kinase RNA-dependent/activated) protein. PKR is normally present in the cell in very low amounts, but it is rapidly induced to very high levels by the presence of interferons and is activated through interaction with dsRNA (Thomas and Samuel, 1992; Davis and Watson, 1996). PKR is one of the cells most critical defences against viral infection. Infected cells release interferons that induce neighbour cells to rapidly build up levels of PKR. When infected, dsRNA (a replication intermediate of many viruses) stimulates PKR activity, phosphorylating eIF-2 and inhibiting translation. Viral translation is "cap-independent" (Section 1.6.3.2.4.2) but still requires eIF-2 and the ribosome. Downregulation of eIF-2/2B activity by PKR therefore shuts down infected cells and also inhibits viral mRNA

translation. Strategies targetting eIF2α kinases are usually employed by viruses in an attempt to outwit the hosts defence mechanisms against infection (review; DeHaro *et al.*, 1993, Chen and London, 1995). These include virally encoded small RNAs with extensive secondary structures that bind to PKR, proteins that bind and sequester dsRNA, a protease that degrades PKR, and a protein that resembles a truncated version of the eIF2α substrate of PKR, acting as a decoy.

#### 1.6.3.2.3 eukaryotic Initition Factor 3, eIF-3

eIF-3 is composed of at least five subunits and strongly interacts with eIF-5 (Phan *et al.*, 1998). It promotes the binding of initiator tRNA to the Ribosome through interaction with eIF-2/5 and stabilises tRNA binding to the 40S subunit (Block *et al.*, 1998). It is thought to be involved in eIF-5/eIF-2 recruitment to the ribosome during PIC formation, the factors involved in AUG recognition. eIF-3 also interacts with eIF-4G (Section 1.6.3.2.4.2) and is thought to be the "bridge" between initiation factors and the ribosome itself.

#### 1.6.3.2.4 eIF-4; The Cap-binding Initiation Complex, eIF-4F

The eIF-4F initiation factor is a high-molecular-mass complex, the primary subunits being eIF-4A, eIF-4E, and eIF4G ( eIF4γ, p220 ). Evidence now points to a role for eIF4G in bringing together, in the correct orientation and in close proximity to the cap, the components necessary to unwind secondary structure in the mRNA and place the 40S Ribosomal subunit at the 5' end of the eukaryotic message.

##### 1.6.3.2.4.1 eukaryotic Initiation Factors, eIF-4A and eIF-4B

eIF4A is an ATP-hydrolysis-dependent RNA helicase that unwinds mRNA 5' structure to generate an efficient Ribosome binding site. eIF-4A is a prototype of a large family of RNA-helicases called the DEAD box family (Linder *et al.*, 1989). It plays a critical role in the initiation process and is required for mRNA-Ribosome binding both in its free form and as a subunit of eIF-4F, playing roles in both cap-dependent and cap-independent translation (Pause *et al.*, 1994; Sonenberg, 1996).

While dominant-negative eIF-4A inhibited translation *in-vitro*, addition of eIF-4A restored activity, the restoration being six-fold stronger for eIF-4F-associated eIF-4A. The activity of this enzyme has been shown to be weak without the presence of eIF4B, a subunit of eIF-4F that greatly enhances eIF-4A activity, possibly explaining the significant difference found by Pause et al (1994) between free and eIF-4F-associated eIF-4A in reactivating translation. eIF-4B requires eIF-4F formation at the cap, as well as ATP hydrolysis, for association with the PIC (Haghighat and Sonenberg, 1997).

#### 1.6.3.2.4.2 The "scaffold"; eIF-4G

eIF-4G is a 220 kDa protein that acts as a scaffold upon which the eIF-4F complex is formed and mediates the binding of the mRNA to the ribosome through interaction with 43S associated eIF-3 (Rau *et al.*, 1996). eIF-4G alone has no cap-binding function
(Haghighat, 1995). On the eIF-4G peptide, eIF-4E (the cap-binding protein) binds to the amino-terminal half and guides the complex onto the mRNA cap, while eIF-4A binds to the carboxy-terminal half (Sonenberg, 1996), forming the eIF-4F complex (Figure 1.15).

eIF-4G shares a common binding site with 4E-BPs (negative regulators of eIF-4E activity; Section 1.6.3.2.4.4) for association with eIF-4E, thereby establishing a competitive regulatory mechanism for eIF-4F complex formation (Rau *et al.*, 1996; Ptushkina *et al.*, 1998). Overexpression of eIF-4G has been shown to result in malignant transformation of NIH3T3 cells (Fukuchi-Shimogori *et al.*, 1997). While this occurred in the absence of increased levels of eIF-4E expression, it would seem logical to assume that the mechanism is simply through an increased competitive advantage over the 4E-BPs for association with eIF-4E, thereby increasing initiation events in eIF-4G overexpressing lines. eIF-4G has been shown to dramatically increase the binding affinity of eIF-4E for the cap structure (Haghighat and Sonenberg, 1997).

eIF-4G interacts with the Poly(A)-binding protein (PAB) resulting in increased translation (Tarun and Sachs, 1996). The exact sequence of events resulting in eIF-4F

complex formation and ribosome association are unknown, but are thought to occur as outlined in figure 1.15. Control of translation initiation is very reminiscent of its transcriptional counterpart, a fact highlighted by Sachs and Buratowski (1997) and may represent evolutionary "fine-tuning" of such complex regulatory mechanisms. PAB is thought to be involved in the recruitment of intitiation factors such as eIF-4G to the mRNA to allow them to interact with cap-bound eIF-4E. Its association with eIF-4G explains both the "circular" mRNA model and the seemingly strange influence of the 3' UTR on initiation events at the extreme 5' end of the transcript. PAB only associates with eIF-4G when complexed to poly(A) (Tarun and Sachs, 1996). eIF-4G-mediated association of the poly(A)-binding protein with eIF-4F is increased by serum stimulation (Fraser *et al.*, 1999) and enhances initiation of cellular capped mRNAs during growth stimulatory conditions.

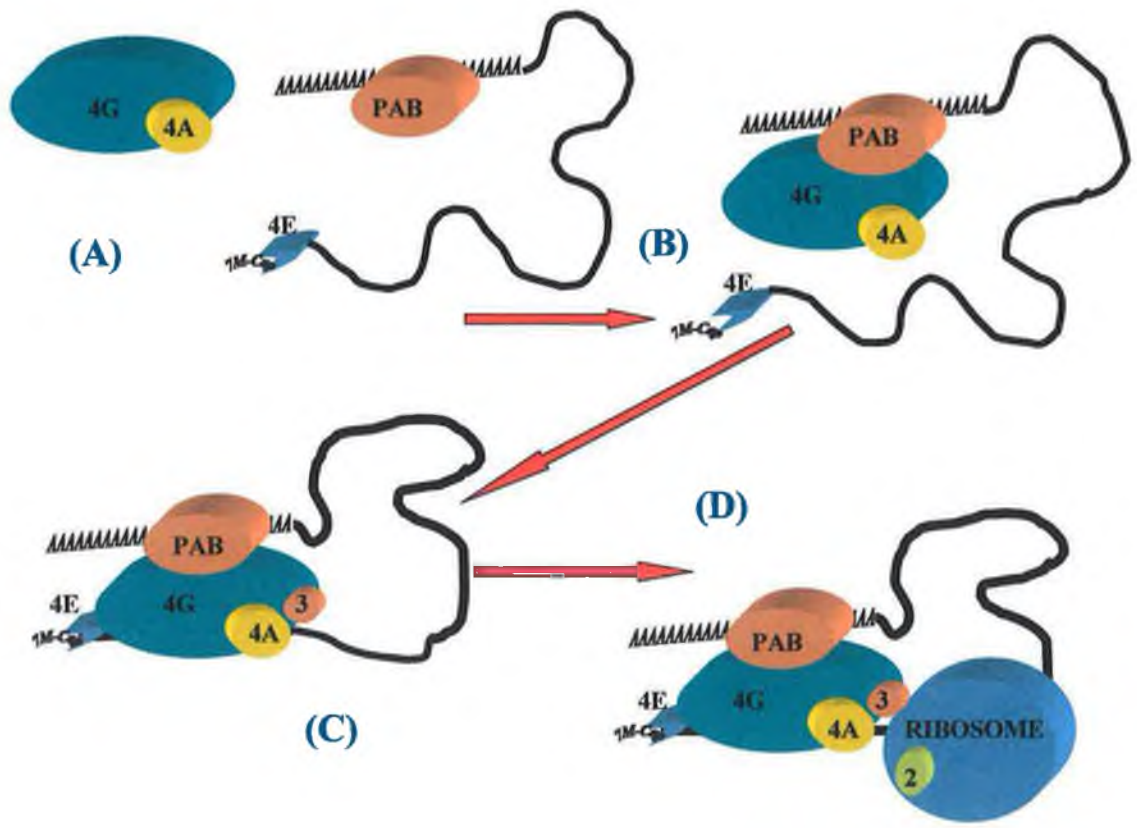



**Figure 1.15: Recruitment of eIF-4F factors to the mRNA by Poly(A)-binding protein (PAB):** (A) Poly(A)-associated PAB recruits eIF-4G (B) to bring it into the vicinity of cap-bound eIF-4E to form the eIF-4F complex (C) at the cap site. Additional eIFs that interact with eIF-4G then mediate the association of the ribosome with eIF-4F to begin scanning.

**Section 1.6.3.2.4.2.1 Viral IRES and eIF-4G:**

Viral infection results in cleavage of eIF-4G (Etchison and Smith, 1990; Huang and Schneider, 1991) and a reduction in cap-dependent translation (Haghighat *et al.,* 1997), since cleavage destroys eIF-4F complex formation (Figure 1.16). Viral mRNAs do not possess a cap and are translated via IRES (Internal Ribosome Entry Site) mechanisms, which are cap-independent. The C-terminal domain of eIF-4G is sufficient to support cap-independent translation in the absence of eIF-4E (Ohlmann *et al.,* 1996). eIF-4A, required for both cap-dependent and cap-independent translation in higher eukaryotes (Pause *et al.,* 1994; Sonenberg, 1996), remains associated with the C-terminal region of eIF-4G. eIF-4G cleavage during viral infection is mediated by virally encoded cysteine proteinases (L proteinases) and picarnovirus 2A protease (Lamphear *et al.,* 1993) that bisects the eIF-4G polypeptide shutting down host cap-dependent translation initiation and conferring selective advantage to viral mRNAs.

An alternative approach is observed in the case of the rotavirus RNA-binding protein NSP3, associated with the 3' end of the viral mRNA. NSP3 interacts with eIF-4G and evicts the poly(A)-binding protein from eIF-4F (Piron *et al.,* 1998), thereby downregulating host cell cap-dependent mRNA translation and "hijacking" eIF-4G.

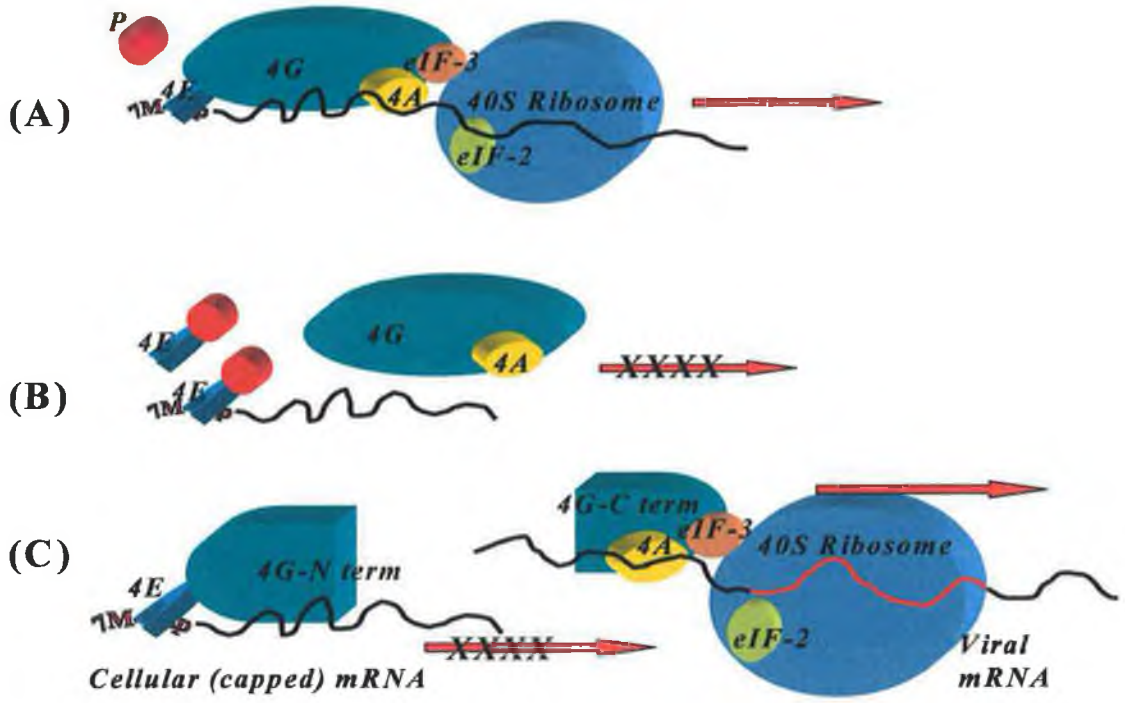



**Figure 1.16: eIF-4F complex formation is a target for downregulation of host protein synthesis by picarnoviruses.** (A) During growth 4E-Binding Proteins (4E-BPs) are phosphorylated, eIF-4F complexes form and translation proceeds. (B) During stress or apoptosis, 4E-BPs are dephosphorylated and inhibit eIF-4F formation. Translation is inhibited. (C) Viruses cleave eIF-4G. eIF-4F complexes do not form, but the C-terminal region, containing elements for helicase activity and ribosome binding, is sufficient for viral translation via IRES (Internal Ribosome Entry Sites) (Red Sequence) in the viral mRNA.

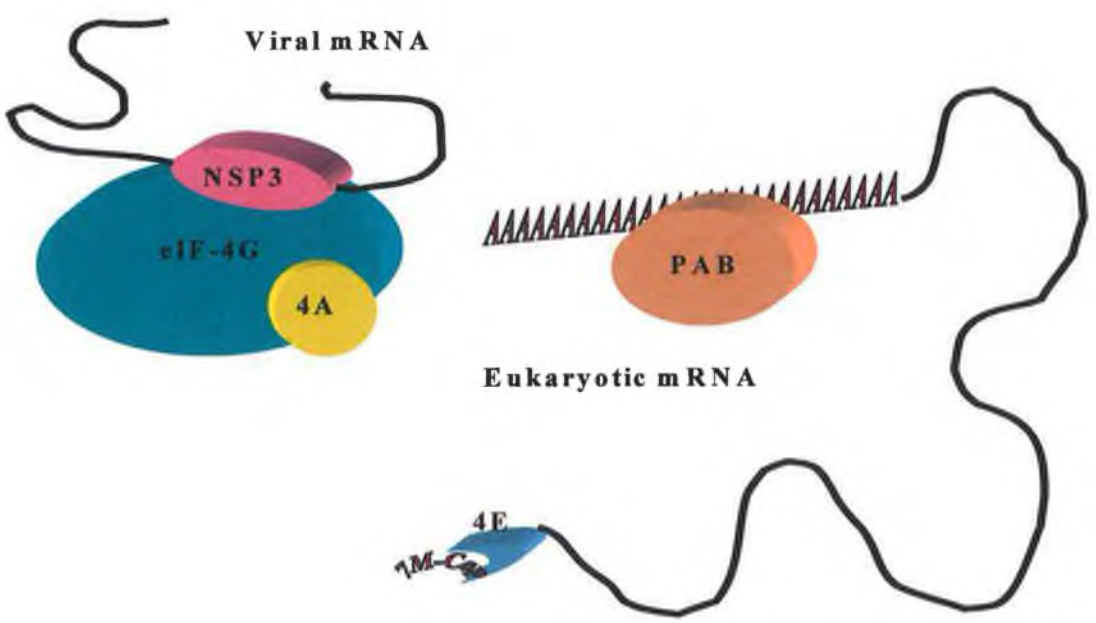



**Figure 1.17: An alternative mechanism for "hijacking" host translation employed by rotaviruses also targets eIF-4G.** Viral NSP3 competes for EIF-4G and evicts it from the PAB/4F complex, inhibiting host translation and allowing viral translation.

#### 1.6.3.2.4.3 eukaryotic Translation Initiation Factor, eIF-4E:

eIF4E, otherwise known as eIF4α or the small cap binding protein, binds directly to the 5' 7-Methyl-Gppp cap in an ATP-dependent manner, and is thought to be the first factor to interact with the mRNA to initiate translation. eIF-4E is a 25 kDa phospho-protein responsible for Cap-binding specificity in eIF-4F complexes during eukaryotic translation initiation events. eIF-4E consists of a single αβ domain which contains 8 antiparallel β strands forming a curved β sheet (Sonenberg and Gingras, 1998). This sheet is backed by three long α-helices. The mRNA cap-structure binds loosely to an hydrophobic pocket in the concave inner surface of eIF-4E, across which salt-bridges form after phosphorylation to "lock" the cap in place (Marcotrigiano *et al.*, 1998, CSHL abstracts), while the convex dorsal surface interacts in a mutually exclusive manner with either eIF-4G or the 4E-BPs [Sections 1.6.3.2.4.2 and 1.6.3.2.4.4]. Phosphorylation of eIF-4E occurs as part of the eIF-4F complex (Tauzon *et al.*, 1990) greatly enhancing and stabilising its association with the cap (Minich *et al.*, 1994; Joshi *et al.*, 1995).

eIF-4E is widely accepted as the limiting factor in translation initiation, particularly for mRNAs with complex 5' UTRs (section 1.6.5). It is present in molar levels significantly lower than that of other initiation factors (DeBenedetti and Rhoads, 1990; Sonenberg, 1996). It is the most specifically targeted mRNA-binding eIF and is an essential component of the cytoplasmic cap-binding complex. The cap-binding activity of the eIF-4E peptide is thought to reside in a highly evolutionarily conserved placement of tryptophan residues in both yeast and mammals (Altmann *et al.*, 1988). This factor therefore plays a critical role in the regulation of translation, particularly of specific mRNA species [Section 1.6.5], and the levels and activity of eIF-4E are critical to the control of cellular proliferation and differentiation (Jaramillo *et al.*, 1991). A rather novel and as-yet to be proven additional function for eIF-4E has been suggested, namely that it may play some part in the transport of mRNAs from the nucleus. The 5' Cap-structure is known to be involved in the process of nucleocytoplasmic transport (Sonenberg and Gingras, 1998), already thought to be the function of the novel eIF-4E homologue protein, eIF-4EHP (Rome *et al.*, 1998). In light of the Cap-binding specificity of eIF-4E and recent findings of localisation of a

fraction of eIF-4E to the nucleus (Pollard *et al.*, 1999), this additional role for eIF-4E is not implausible.

Frequently mammalian cells express at least two forms of this factor (Jaramillo *et al.*, 1991; Haghighat *et al.*, 1995). The gene(s) for eIF-4E is thought to lie on chromosome 4 in humans (Gao *et al.*, 1998). Gao et al. (1998) isolated two genes for eIF-4E from placental genomic libraries, in which case eIF-4E1 contained six introns but the other (eIF-4E2) was intronless. Subtle differences between the two genes were identified and both genes were reported to be differentially expressed in four human cell lines. A notable difference between the two genes was that the eIF-4E1 promoter contained c-myc-binding elements while that of eIF-4E2 did not, suggesting constitutive expression of the latter and inducible expression of the former. In fact, eIF-4E has been identified as one of the few targets for c-Myc induction (Rosenwald *et al.*, 1993; Jones *et al.*, 1996). The complexity of eIF-4E expression patterns in eukaryotic cells was highlighted by the findings that in Drosophila a single eIF-4E gene could code for three alternatively spliced mRNA transcripts, two of which resulted in expression of the same form of eIF-4E, while the other encoded an isoform differing at the amino-terminal sequence of the protein (Lavoie *et al.*, 1996). The three eIF-4E transcripts varied greatly in the lengths of their respective 5' UTRs, suggesting that each was subject to varying degrees of translational regulation themselves. This may reflect a means of autoregulating levels of eIF-4E expression during phases of hyper- and hypo-proliferation of cells.

#### 1.6.3.2.4.4 The 4E-Binding Proteins (4E-BPs)

The 4E-BPs are small, stable proteins (also called PHAS; Protein Heat- and Acid-Stable) (Lin *et al.*, 1994) that bind to the convex dorsal surface of eIF-4E. They inhibit translation by competing with eIF-4G for association with eIF-4E. They do not disrupt the binding of eIF-4E to the Cap, but interfere with the formation of the eIF-4F complex (Haghighat *et al.*, 1995) (Figure 1.16, Figure 1.19).

The 4E-BP family of phospho-proteins, of which there are three to date, conserve a central amino acid domain which contains the eIF-4E binding domain (Poulin *et al.*, 1998). They are phosphorylated along 5 phosphorylation sites clustered in the middle

of the protein, flanking the 4E-binding domain, and this degree of phosphorylation is thought to cause dissociation of the 4E-BPs from eIF-4E through electrostatic repulsion (Sonenberg and Gingras, 1998). The 4E-BPs are among the first proteins phosphorylated in response to Insulin and growth factors (Graves *et al.*, 1995; Lin *et al.*, 1995). Mitogenic stimulation results in a double activation of eIF-4E through the phosphorylation of eIF-4E-associated 4E-BP proteins, which results in their dissociation from the inactive eIF-4E-4E-BP complex (Poulin *et al.*, 1994) allowing eIF-4F formation and increased phosphorylation of eIF-4E itself (Tauzon *et al.*, 1990).

An unusual aspect of the 4E-BP family is that the structure only becomes ordered upon binding to eIF-4E. In the unbound form, 4E-BPs are structurally disordered, but once bound to the target through a conserved Yx4L region shared with eIF-4G, they become structured and can inhibit eIF-4E interaction with eIF-4G (Fletch *et al.*, 1998). They undergo what is called a disorder-to-order transition upon association with eIF-4E.

#### 1.6.3.2.4.5 eukaryotic Initiation Factor 5; eIF-5

eIF-5 is a monomeric phosphoprotein that interacts with the 40S initiation complex to promote the hydrolysis of ribosome-bound GTP, releasing GDP-eIF-2 (and $P_i$) (Si *et al.*, 1996) and allowing translation to begin once the AUG start codon has been recognised. This step is essential for the subsequent joining of the 60S ribosomal subunit to form a functional 80S Ribosomal complex that is active in peptidyl transferase activity. eIF-5 cannot hydrolyse GTP when in the free form. It has to be complexed with eIF2 in the 40S complex. eIF-5 interacts with the β-subunit of eIF2, through a C-terminal region between amino acids 315-340.

The interaction with eIF2 is thought to be mediated through sequences called Bipartite Motifs on eIF5 (Asano *et al.*, 1998), which interact with motifs in the β subunit of the eIF-2 protein, known as K-boxes (Figure 1.13; Section 1.6.3.2.2). Mutations in the K-box region decrease GDP/GTP exchange. Archaebacteria do not have bipartite motifs and k-boxes, and do not form complex initiation complexes. It is thought that

evolution has added these motifs to eukaryotic factors as "handles" to literally grab hold of other factors required for complex formation.

#### 1.6.3.2.4.6 eukaryotic Initiation Factor 6; eIF-6

eIF-6 prevents 40S binding directly to the 60S Ribosomal units (termed anti-association) (Si *et al.,* 1998, CSHL Abstracts). It is thought to mediate binding of the PIC to the 60S subunit to form the 80S ribosomal complex. It is a 26kDa protein that, *in-vivo,* associates only with free 60S ribosomes, and is involved in 60S biogenesis. eIF-6 is a ribosomal-associated protein and not a ribosomal protein itself.

### 1.6.4 Signalling pathways and translation initiation

It has long been accepted that the transmission of signals from the surface of the cell to the nucleus by protein kinases is the mechanism by which mitogenic signals act to stimulate cellular proliferation. Extracellular signals stimulate membrane receptors, which initiate a chain of phosphorylation that results in activation of downstream kinases that transport mitogenic signals to the nucleus. The final targets for phosphorylation and activation by this cascade are the functional proteins that affect gene transcription, the transcriptional enhancers (Marais and Marshall, 1996; Frost *et al.,* 1997; Sugden and Clerk, 1997). Therefore, different mitogens stimulate different receptors and as such activate different kinase cascades that have different effects on cellular proliferation and differentiation through activation of different sets of transcriptional enhancers. Both the activity and nuclear localisation of transcription factors such as c-Myc, *c-fos* and *c-jun* (Section 1.5.2) are regulated by signalling kinases. However, in recent years it has been realised that the role of kinases is not confined to the regulation of nuclear factors. Phosphorylation by kinases plays a critical role in regulating the activity of cytoplasmic translation factors, including a number of translation initiation and elongation factors (Proud and Denton, 1997).

Ras plays a central role in the regulation of numerous mitogen activated signalling pathways, as illustrated in Figure 1.18. Oncogenes such as src and ras were originally identified as retro-virus encoded genes that produced tumours, now known to be dominant mutated forms of host genes (proto-oncogenes) that had been picked up by

viruses (Cantley *et al.,* 1991). Ras was discovered as the cellular homologue of the transforming genes of the Harvey (H-Ras) and Kirsten (K-Ras) retroviruses (Bar-Sagi, 1989). The viral versions of the gene carried specific mutations (codons 12, 13 or 61) that prevented their proteins from becoming deactivated by dephosphorylation, resulting in uncontrolled growth stimulation and transformation of many cell types. In mammalian cells, ras genes encode 21-kDa GTP-binding proteins (Sjolander *et al.,* 1991). Activated Ras activates three distinct downstream MAPK (Mitogen Activated Protein Kinase) pathways, ERK, JNK and p38 (Vojtek and Der, 1998), two of which converge upon eIF-4E via the MAPK-integrating kinase, Mnk (Waskiewicz *et al.,* 1997). Mutated Ras proteins are incapable of becoming deactivated when a mitogenic signal has been removed, leading to uncontrolled stimulation of downstream kinases as if there was a continuous signal to proliferate emanating from the surface of the cell. The prevalence of Ras mutations in numerous cancer phenotypes, including those of lung tissues (Gazdar *et al.,* 1994) highlights the importance of signal transduction in the regulation of normal cellular proliferation and differentiation. There is a predominance of codon 12 K-Ras (Kirsten-Ras) mutations in non-small cell lung carcinoma (NSCLC), while there is a significant lack of Ras mutations in small cell lung carcinomas (SCLC) (Mitsudomi *et al.,* 1991), which compose an estimated 25% of all lung carcinomas (Woll *et al.,* 1991). The frequency of K-ras mutations in lung adenocarcinoma is estimated to be around 56% (Mills *et al.,* 1995). K-ras mutations have been suggested to be directly caused by exposure to carcinogens in tobacco smoke (Rodenhius and Slebos, 1992), perhaps explaining the prevalence of such mutations in lung carcinomas. Overall carcinogenesis of lung and other tissues is thought to be due to the accumulation of multiple chromosomal alterations (Testa *et al.,* 1992), in a process referred to as the "field cancerization" (Sozi *et al.,* 1995). However, Slebos et al (1989) suggest that simultaneous amplification of protooncogenes such as myc and activation of ras are rare events in NSCLC. While it has been suggested that activation of protooncogenes to oncogene status occurs through mutation, chromosomal translocation, gene amplification, or deregulation of transcription (Kern and Filderman, 1993), evidence now suggests a role for deregulated translation in these events (Saito *et al.,* 1983; West *et al.,* 1993). Downstream events induced by Ras activation include increased expression and/or activity of myc, fos and jun transcription factors, but in addition they are now known to increase protein synthesis rates (de Vries *et al.,* 1996).

It is noteworthy that the activity of eIF-4E is regulated by at least three major signalling pathways. eIF-4E is directly phosphorylated by ERK and p38, and indirectly by FRAP through dissociation of 4E-BPs (Graves *et al.,* 1995; Hara *et al.,* 1998). This highlights the importance of eIF-4E in the overall regulation of translation and cellular proliferation in response to mitogens. FRAP also stimulates the activity of eIF-4B and eIF-2B (Figure 1.18), the regulatory factors of eIF-4F helicase activity (eIF-4A) and initiator tRNA recruitment (eIF-2).

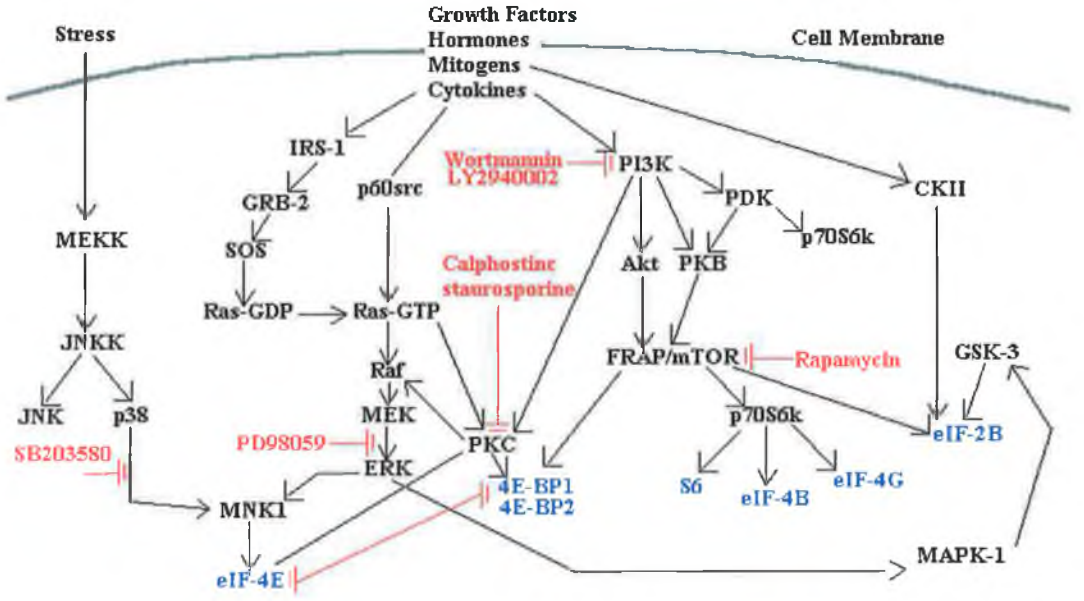



**Figure 1.18: Regulation of eIF activity by signalling cascades.** eIF-4E is a target for both p38 and ERK, via the integrating kinase, Mnk. In addition, its activity is regulated via FRAP/mTOR-mediated inactivation of 4E-BP activity. FRAP also activates eIF-2B and eIF-4B, the regulatory factors for eIF-2 and eIF-4, respectively.

Phosphorylation of eIF-4E results in increased cap-binding efficiency and increased translation (Minich *et al.,* 1994). Rychlik et al. (1987) suggested that $Ser^{53}$ was the active site for eIF-4E phosphorylation, later confirmed by the inability of $Ser^{53}$-mutants to transform cells (DeBenedetti and Rhoads, 1990). The altered protein failed to bind to the 43S initiation complex but was not inhibited in cap-binding affinity (Joshi-Barve *et al.,* 1990). However, disputing this without ruling out $Ser^{53}$ as one of

the sites, Kaufman et al (1993) illustrated the strong probability that there were additional phosphorylation sites for eIF-4E.

$Ser^{209}$ has now been illustrated to be the major phosphorylation site in mammalian eIF-4E (Flynn and Proud, 1995; Joshi *et al.*, 1995), that regulates its cap-binding efficiency. A novel kinase, termed Mnk (MAPK-integrating Kinase), has recently been discovered and is now thought to be the direct kinase for eIF-4E phosphorylation, which forms salt-bridges across the cap-binding site of eIF-4E to enhance its binding (Marcotrigiano *et al.*, 1998). Mnk was independently isolated by two groups. Fukanaga and Hunter (1997) isolated Mnk as a substrate for ERK and p38 kinases using a novel expression screening method for identifying protein kinase substrates. They showed that Mnk, a 47 kDa peptide, was expressed ubiquitously but at very low levels, and that the C-teminal contains the primary ERK phosphorylation site(s). Waskiewicz et al (1997) demonstrated that Mnk, of which there are two isoforms (Mnk1 and Mnk2), phosphorylates eIF-4E *in-vitro*. *In-vivo* inhibition of ERK and p38 resulted in inhibition of Mnk activity and eIF-4E phosphorylation. Mnk was suggested to define a convergence point between growth factor- and stress-induced protein kinase cascades (Figure 1.18). Mnk complexes more strongly with inactive than active ERK, suggeting that Mnk and ERK may dissociate after mitogen stimulation (Waskiewicz *et al.*, 1997), perhaps allowing Mnk to incorporate into the eIF-4F complex to phosphorylate eIF-4E. Mnk phosphorylates eIF-4E at its physiological site, $Ser^{209}$, and it has recently been demonstrated that Mnk associates with the C-terminus of eIF-4G (Pyronnet *et al.*, 1998), perhaps explaining the fact that eIF-4E is a better substrate for phosphorylation as part of the eIF-4F complex (Tauzon *et al.*, 1990). Despite at least five phosphorylated forms of the protein, two forms predominate, namely the non- and mono-phosphorylated ($Ser^{209}$) forms of eIF-4E (Flynn and Proud 1995; Sonenberg, 1996). It is now generally accepted that both $Ser^{53}$ and $Ser^{209}$ are phosphorylated in mammalian eIF-4E, probably playing distinct regulatory roles, and that $Ser^{209}$ is the major site detectable by isoelectric focusing. The yeast homologue of eIF-4E has been shown to be phosphorylated in different regions of the protein than the mammalian counterpart, at $Ser^{2}$ and $Ser^{15}$ in the N-terminal region (Zanchin and McCarthy, 1995).

A role for 4E-BP2 has been suggested in the developmental regulation of eIF-4E phosphorylation during human thymocyte maturation (Beretta *et al.*, 1998). However, this may not be a universal mechanism, as suggested by the lack of evidence of a role for either 4E-BP1 or 4E-BP2 in the regulation of eIF-4E phosphorylation in serum stimulated Xenopus Kidney cells (Fraser *et al.*, 1999). Phosphorylation of eIF-4E and 4E-BPs occurs through distinct kinase pathways (Diggle *et al.*, 1996; Wang *et al.*, 1998), namely the ERK/p38 and FRAP/mTOR pathways, respectively (Figure 1.18). This allows different physiological signals to regulate the activity of eIF-4E by different mechanisms.

Taken together a picture appears in which concomitant phosphorylation of $Ser^{53}$ and $Ser^{209}$ may occur, increasing both affinity for eIF-4G and the cap-structure, respectively. A sequence of activation might be suggested from the data known to date, but is purely hypothetical; FRAP-mediated phosphorylation of 4E-BPs results in dissociation from eIF-4E, eIF-4E phosphorylation at $Ser^{53}$ (either pre-existing, concomitant with 4E-BP phosphorylation or after) may mediate association of eIF-4E with eIF-4G to form the eIF-4F complex, resulting in phosphorylation at $Ser^{209}$ by eIF-4F-associated Mnk, stabilising Cap-bound eIF-4F (Figure 1.19). In agreement with this, eIF-4F-associated eIF-4E has been shown to be 85-100% phosphorylated, while "free" eIF-4E exists predominantly in the 50% phosphorylated form (Joshi-Barve *et al.*, 1992; Minich *et al.*, 1994).

It would appear paradoxical, therefore, that the stress kinase p38 is also capable of phosphorylating Mnk (Waskiewicz *et al.*, 1997) and eIF-4E. Cellular stresses such as arsenite and cytokines are capable of phosphorylating eIF-4E, and this phosphorylation is inhibited by the specific inhibitor of p38 kinase, SB203580 (Wang *et al.*, 1998). It has recently been speculated that phosphorylation of eIF-4E does not play a direct role in the stress response of the cell, but is rather a "preparation" by the cell for future recovery (McKendrick *et al.*, 1999). Arsenite, however, also increases eIF-2$\alpha$ subunit phosphorylation resulting in the observed decrease in global protein synthesis with this stress agent in these studies (Wang *et al.*, 1998). On the other hand, other stresses such as heat shock do not result in eIF-4E phosphorylation, due to dissociation of the eIF-4G/Mnk/eIF-4E complex and increased association between

eIF-4E and 4E-BP1 (Burley *et al.,* 1998, CSHL Abstracts; Cuesta *et al.,* 1998, CSHL Abstracts). Translation of Heat shock protein (hsp) mRNAs appears to be relatively cap-independent (Joshi-Barve *et al.,* 1992). Increased association of 4E-BP1 and eIF-4E was suggested by de Vries et al (1997) to play a role in the shut-down of general protein expression while retaining hsp synthesis during the heat-shock response in cells.

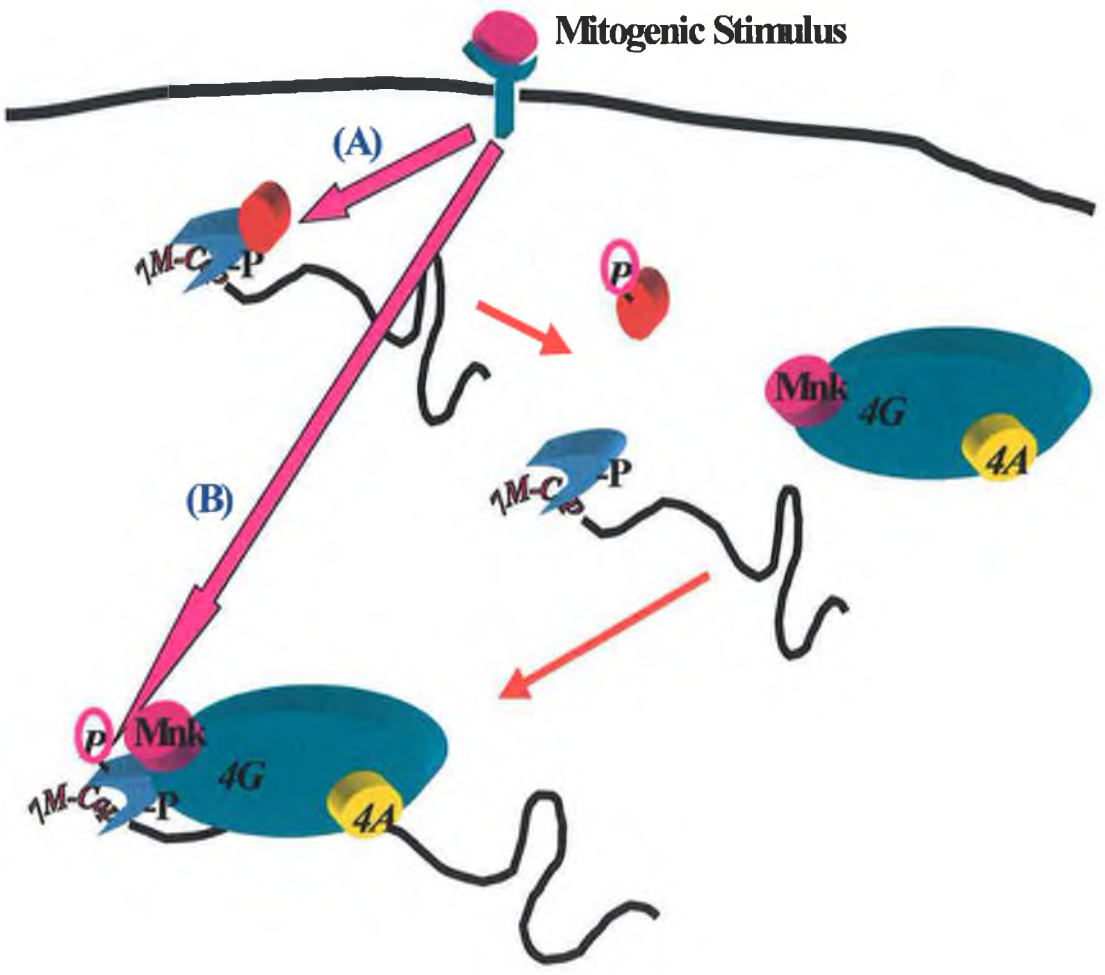



**Figure 1.19: Mitogenic Stimulation results in a double activation of eIF-4E and eIF-4F formation.** Initial events are thought to be the phosphorylation of the eIF-4E inhibitors, 4E-BPs by FRAP pathways (A). "free" eIF-4E is then available for association with eIF-4G. ERK/p38 kinase pathways stimulate eIF-4E phosphorylation through the eIF-4F associated kinase, Mnk (B), resulting in enhanced cap-binding of eIF-4F. Two points in this cascade as yet remain unclear: (1) eIF-4E is not definitely cap-associated prior to eIF-4F formation. (2) The timing and exact function of $Ser^{53}$ phosphorylation are unsure.

### 1.6.5 eIF-4E and selective translation of specific mRNAs

While eIF-4E is involved in the regulation of translation initiation in all cap-dependent eukaryotic events (all eukaryotic mRNAs are capped except for organellar mRNAs (Jaramillo *et al.*, 1991)), there are implications that eIF-2 and its regulatory factor, eIF-2B, are the critical factors in the regulation of global protein synthesis (Kimball *et al.*, 1998). Why then is eIF-4E still regarded as so critical in the regulation of the processes of cellular proliferation and the development of malignancy ? The answer lies in the fact that it regulates the primary initiation event of all cap-dependent mRNAs, the primary target for translational repression events, and more importantly in its ability to selectively increase the expression of growth-related mRNAs such as growth factors, receptor proteins and even transcription factors (review; Sonenberg and Gingras, 1998). This specificity in translational induction by eIF-4E is what has given this factor such status in the field of translation research in recent years.

Many growth-related mRNAs contain extremely complex, GC-rich 5' UTRs (Kevil *et al.*, 1995; Kevil *et al.*, 1996). The significance of this is only now being realised, as the role of eIF-4E and initiation of translation is becoming apparent. Such mRNAs are inefficiently translated and poorly compete for available eIF-4F, and as such have a high requirement for active eIF-4E to be translated efficiently. Elevated levels of eIF-4F, primarily regulated by the availability and activity of eIF-4E, result in dramatic and selective increases in the translation of these complex mRNAs. This is because simple, housekeeping mRNAs have short 5' UTRs and are naturally competitive for available eIF-4E. Therefore increased availability of eIF-4E/4F does not significantly enhance their translation. However, complex mRNAs can now compete for the excess eIF-4F, unwinding their secondary structure more efficiently. Increased rates of initiation due to excess eIF-4E prevent the secondary structure reforming in the 5' UTR, resulting in more frequent and efficient initiation (polysomal initiation). This results in very significant increases in translation of these mRNAs, as illustrated in figure 1.20.

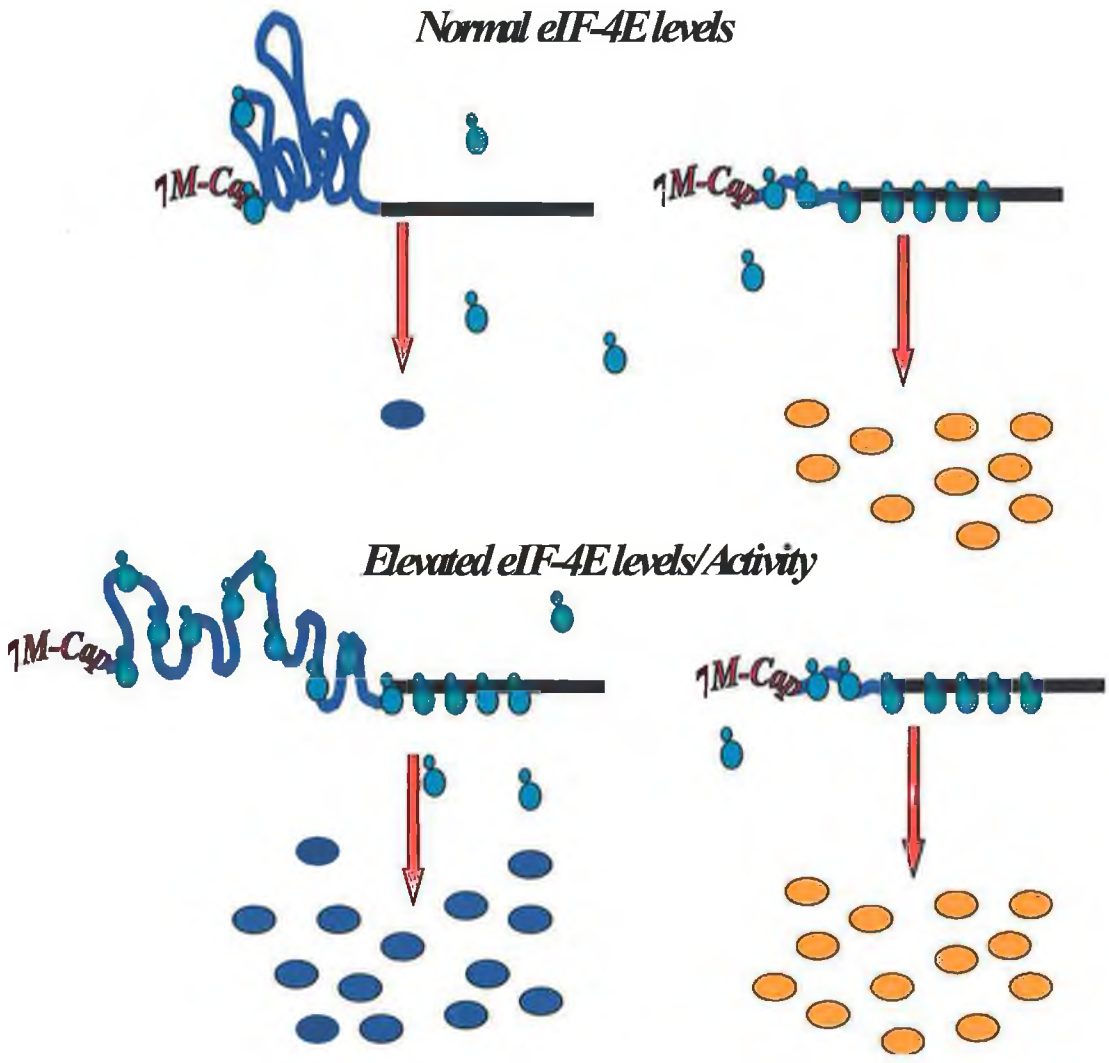



**Figure 1.20: Selective Translation of Complex mRNAs by during increased eIF-4E levels/activity.** (Left) Complex mRNAs are normally inefficiently translated. Elevated eIF-4E (bottom) unwinds complex mRNA structures and increases initiation rates. This maintains the unwound state of the complex mRNA resulting in more frequent and efficient initiation. (Right) Simple mRNAs are translated efficiently and are relatively unaffected by elevated eIF-4E (bottom).

Selective translation by by eIF-4E has been reported for numerous growth-related mRNAs with complex 5' UTRs. A 10-fold increase in c-*myc* translation was observed in serum-stimulated EBV (Epstein-Barr Virus)-immortalised B-cells (West *et al.*, 1998). Overall protein synthesis rates only increased 3-fold, suggesting that c-*myc* was being selectively translated. Overexpression of eIF-4E in CHO cells results in a selective increase in VPF (Vaso Permeability Factor) expression (Kevil *et al.*, 1996), exhibiting as much as 130-fold increase in secreted VPF protein. A role for eIF-4E in

the regulation of cell growth was also shown by its ability to regulate the expression of other complex mRNAs, such as cyclin $D_1$ (Rosenwald *et al.*, 1995) and Ornithine Decarboxylase (Rousseau *et al.*, 1996; Shantz *et al.*, 1996). A role in cell cycle progression may be implied by the fact that cyclin $D_1$ is an initiator of this process (Won *et al.*, 1992) and the fact that eIF-4E is a major MAPK target, a kinase shown to be required for $G_2$ to M-phase transition during the mitotic cycle (Abrieu *et al.*, 1997). A direct link between Mek1, an upstream kinase of Mnk, and the cell cycle machinery has also been established (Greulich and Erickson, 1998). Rapamycin, an inhibitor of FRAP kinase activity, causes increased binding of 4E-BP1 to eIF-4E but has only a small effect on total protein synthesis in many cell types. This is probably due mainly to marked inhibition of the translation of specific mRNAs that have a high requirement for eIF-4E, such as myc, rather than effects on global translation (Vries *et al.*, 1997).

#### 1.6.5.1 eIF-4E: a new oncogene?

The ability of eIF-4E to selectively and dramatically increase the translation of specific growth-related mRNAs (Section 1.6.5) has highlighted it as a critical factor in the regulation of cellular proliferation. eIF-4E is among the few known targets for transcriptional activation by c-Myc (Rosenwald *et al.*, 1993; Jones *et al.*, 1996; Gao *et al.*, 1998) and it is now suspected that eIF-4E may be a major effector of c-myc activity. In fact, eIF-4E is now considered to be an oncogene in its own right and has been associated with numerous cancers. To date, eIF-4E has been identified as overexpressed in head and neck, and breast tumours (DeFatta *et al.*, 1999). eIF-4E-mediated selective synthesis of factors such as VPF (Kevil *et al.*, 1996) and VEGF (Vascular Enodothelial Growth Factor) is thought to be an additional factor in explaining the role of eIF-4E in malignancy (Scott *et al.*, 1998). Such dramatic and selective increases in expression of VPF might contribute to the ability of a tumour to form blood vessels at points of metastasis, allowing enhanced invasion by tumours overexpressing eIF-4E. Overexpression of eIF-4E in HeLa cells results in aberrant growth and morphology (DeBenedetti and Rhoads, 1990), while in NIH3T3 and Rat2 fibroblasts, eIF-4E overexpression caused tumorigenic transformation (Lazaris-Karatzas *et al.*, 1990). CHO cells transformed with eIF-4E display increased c-myc expression but only become tumorigenic upon transplantation to mice in the presence

of co-transfected Max (DeBenedetti *et al.*, 1994), inferring a role for c-myc in transformation of cells by eIF-4E. However, DeBenedetti et al. (1994) stated that they could not find a role for c-myc in transformation, nor could they determine whether its increased expression was a cause or consequence of the transformed phenotype. It is therefore possible to suggest that the transforming ability of c-myc may lie, at least to some significant degree, in its ability to induce eIF-4E expression. This statement is not as wild as it seems taken in light of the relative lack of c-myc targets identified, combined with the ability of eIF-4E to selectively regulate the expression of growth related transcripts, including c-myc itself. One important biological function of c-myc may be to increase cell growth by increasing the expression of eIF-4E and eIF-2$\alpha$ (Rosenwald *et al.*, 1993). In addition, an RNA helicase from the DEAD-box family, possibly eIF-4A, has been shown to be c-myc inducible (Grandori *et al.*, 1996), adding to the idea that c-Myc may be a key regulator of the translational capacity of the cell.

West et al. (1995) showed that c-myc levels were increased in Blooms syndrome cell lines and that this increase was at the translational level. While it was suggested that this translational increase was unrelated to eIF-4E levels, which remained unchanged in control and BS cells, notably eIF-4E levels but not phosphorylation were examined in these studies. It was suggested that aberrant translational control of this proto-oncogene may be a factor in the cancer predisposition of BS individuals, with increasing evidence that translational control plays a pivotal role in the normal regulation of the c-myc gene. Perhaps overlooked by the authors was an additional translation-based mechanism by which cancers can overexpress c-myc, reported by Saito et al (1983). c-Myc expression is elevated in a form non-Hodgkins Lymphoma and involves a translocation of the c-Myc gene. The translocated gene is expressed, but possesses a significantly simplified 5' UTR due to the loss of exon 1 during the translocation. The translocated c-Myc, therefore, escapes the requirement of the "normal" c-Myc mRNA for elevated levels of eIF-4E, and increases its ability to compete for available eIF-4F resulting in high levels of c-Myc expression.

## 1.7 Aims of Thesis:

Immunocytochemical and Northern Blotting analysis by Dr. Shirley McBride (NCTCC) suggested that treatment of the lung cancer cell lines, DLKP and A549, with 5'-Bromo-2-deoxyuridine (BrdU) induces keratin expression at a post-transcriptional level. However, for publication, clearer immunocytochemical analysis was required, along with the development of quantitative methods (western blotting/ immunoprecipitation) for the determination of keratin levels in both treated and untreated cells; performing these experiments formed the starting point for the project described in this thesis.

Due to the lack of detectable keratin expression in DLKP by immunocytochemistry and the induction of keratin expression in only 10-15% of DLKP cells upon exposure to BrdU, immunoprecipitation techniques rather than western blotting alone were required. Problems of antibody breakdown and background interference were overcome through the adaptation of a biotinylation-based procedure, modified to detect low level keratin expression.

The mechanism by which BrdU induced keratin expression in DLKP was completely unknown. The question was approached from a number of angles:

1. Does DLKP regulate keratin synthesis at the translational level?
2. Does BrdU influence this translational mechanism in any way and how?
3. Are there any key factors induced by BrdU that may explain its ability to influence the differentiation status of DLKP?

To address the question of translational repression in DLKP, *in-vitro* translation procedures needed to be developed. After optimising conditions and determining the most suitable system for our needs, cytoplasmic extracts from DLKP were prepared by modifying a technique for the isolation of cytoplasmic RNAs. This procedure was used to ensure the stability of RNAs added to these sytems for further investigation. The basic principle of these experiments was to see if an extract from DLKP could inhibit the translation of keratin synthesis *in-vitro*, which would provide strong evidence for translational repression.

To address our second question, the effect of BrdU on translation initiation factors was examined, to identify any possible changes that might explain the ability of BrdU to increase keratin protein levels without altering keratin gene transcription. We suspected that BrdU-induced changes in the translational capacity of cells might be involved in the post-transcriptional induction of keratin expression in the lung epithelial cell lines studied.

To investigate the mechanism by which BrdU influences the differentiation of epithelial lung cancer cells, and more particularly how BrdU could influence the translational capacity of cells, an unexpected effect for a DNA-interacting drug, key regulators of eIF-4E function were investigated in BrdU-treated cells. Regulators of both eIF-4E levels (transcriptional enhancers) and activity (kinases) were examined.

cDNAs coding for key proteins identified by studies using BrdU were transfected into DLKP to assess their ability to induce simple epithelial differentiation in this poorly differentiated cell line. It was hoped that compiling results from BrdU-treated cells and transfections would allow us to develop a model for the regulation of K8 and K18 synthesis in our lung cell line models, with possible implications for understanding the early stages of lung development as well as aspects of de-differentiation in lung cancer. Such models are severely lacking in lung biology.

Additional studies were carried out using the vitamin-A derivative, Retinoic Acid (RA) in order to assess the ability of physiological differentiating agents to affect the differentiation status of DLKP.

Section 2.0

# *Materials & Methods*

## 2.1 WATER

Ultrapure water was used in the preparation of all media and solutions. This water was purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12 - 18 MΩ/cm resistance.

## 2.2 GLASSWARE

Solutions pertaining to cell culture and maintenance were prepared and stored in sterile glass bottles. Bottles (and lids) and all other glassware used for any cell-related work were prepared as follows:- all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1 hour. Following scrubbing and several rinses in tap water, the bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving.

## 2.3 STERILISATION

Water, glassware and all thermostable solutions were sterilised by autoclaving at $121^0$C for 20 minutes (min) under pressure of 1bar. Thermolabile solutions were filtered through a 0.22μm sterile filter (Millipore, millex-gv, SLGV-025BS). Low protein-binding filters were used for all protein-containing solutions.

## 2.4 MEDIA PREPARATION

Medium was routinely prepared and sterility checked by Joe Carey. The basal media used during routine cell culture were prepared according to the formulations shown in Table 2.4.1. 10x media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7.45 - 7.55 using sterile 1.5M NaOH and 1.5M HCl. The media were then filtered through sterile 0.22μm bell filters (Gelman, 121-

58) and stored in 500ml sterile bottles at $4^0$C. Sterility checks were carried out on each 500ml bottle of medium as described in Section 2.5.6.

The basal media were stored at $4^0$C up to their expiry dates as specified on each individual 10x medium container. Prior to use, 100ml aliquots of basal media were supplemented with 2mM L-glutamine (Gibco, 25030-024) and 6% foetal calf serum (Sigma, F-7524 Batch # 78H3355) and this was used as routine culture medium. This was stored for up to 2 weeks at $4^0$C, after which time, fresh culture medium was prepared.

**Table 2.4.1** Preparation of basal media

| | **DMEM** (Gibco, 12501-029) | **Hams F12** (Gibco, 21700-109) |
|---|---|---|
| **10X Medium** | 500ml | Powder |
| **Ultrapure $H_20$** | 4300ml | 4700ml |
| **1M HEPES*** Sigma , H-9136 | 100ml | 100ml |
| **7.5% $NaHCO_3$** BDH, 30151 | 45ml | 45ml |

* HEPES = N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

For most cell lines, ATCC (Ham's F12/ DMEM (1:1)) supplemented with 6% FCS, 1% Sodium Pyruvate and 2mM L-glutamine was routinely used.

## 2.5 CELL LINES

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work which involved toxic compounds was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet. Each cell line was assigned specific media and waste bottles. Only one cell line was worked with at a time in the cabinet which was allowed to clear for 15min between different cell lines. The cabinet itself was cleaned each week with industrial detergents (Virkon, Antec. International; TEGO, TH.Goldschmidt Ltd.), as were the incubators. The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2.5.1. Lines were maintained in 25cm$^2$ flasks (Costar; 3050) or 75cm$^2$ flasks (Costar; 3075) at 37$^0$C and fed every two to three days.

### 2.5.1 Subculture of Adherent Lines

During routine subculturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment.

Waste medium was removed from the flasks and rinsed with a pre-warmed (37$^0$C) trypsin/EDTA (TV) solution (0.25% trypsin (Gibco, 25090-028), 0.01% EDTA (Sigma, EDS) solution in PBS A (Oxoid, BR14a)). The purpose of this was to remove any naturally occurring trypsin inhibitor which would be present in residual serum. Fresh TV was then placed on the cells (2ml/25cm$^2$ flask or 4ml/75cm$^2$ flask) and the flasks incubated at 37$^0$C until the cells were seen to have detached (5-10 min). The trypsin was deactivated by addition of a equal volume of growth medium (*i.e.* containing 6% serum). The entire solution was transferred to a 30ml sterile universal tube (Sterilin; 128a) and centrifuged at 1,000 rpm for 5 min. The resulting cell pellet was resuspended in pre-warmed (37$^0$C) fresh growth medium, counted (Section 2.5.3) and used to re-seed a flask at the required cell density or to set up an assay.

### 2.5.2 Subculture of suspension cells

Cell lines growing in suspension did not require enzymatic detachment. The cell suspension was removed to a sterile universal and centrifuged at 1000rpm for 5 min. The resulting cell pellet was resuspended in pre-warmed ($37^0$C) fresh growth medium, counted (Section 2.5.3) and used to re-seed a flask at the required cell density or to set up an assay.

**Table 2.5.1** Cell lines used during the course of this study

| Cell line | Basal medium | Cell type | Source |
|---|---|---|---|
| DLKP (and subpopulations SQ/I/M) | ATCC[2] | Human poorly-differentiated lung carcinoma | Dr. Geraldine Grant, NCTCC |
| HL60* | RPMI-1640[1] | Human leukaemic line | ATCC[2] |
| A549 | ATCC[2] | Human lung adenocarcinoma | ATCC[2] |

* These cells grow in suspension

[1] RPMI-1640 (Gibco, 52400-025) supplemented with 10% FCS and 2mM L-glutamine

[2] ATCC = American Type Culture Collection

### 2.5.3 Cell Counting

Cell counting and viability determinations were carried out using a trypan blue (Gibco, 15250-012) dye exclusion technique.

An aliquot of trypan blue was added to a sample from a single cell suspension in a ratio of 1:5. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the

dilution factor being taken into account, and final cell numbers were multiplied by $10^4$ to determine the number of cells per ml. The volume occupied by the chamber is 0.1cm x 0.1cm x 0.01cm *i.e.* 0.0001cm$^3$. Therefore cell number x $10^4$ is equivalent to cells per ml. Non-viable cells were those that stained blue while viable cells excluded the trypan blue dye and remained unstained.

### 2.5.4 Cell Freezing

To allow long term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below -180$^0$C. Once frozen properly, such stocks should last indefinitely.

Cells to be frozen were harvested in the log phase of growth (*i.e.* actively growing and approximately 50 - 70% confluent) and counted as described in Sections 2.5.3. Pelleted cells were re-suspended in serum. An equal volume of a DMSO/serum (1:9, v/v) was slowly added dropwise to the cell suspension to give a final concentration of at least 5x10$^6$ cells/ml. This step was very important as DMSO is toxic to cells. When added slowly the cells had a period of time to adapt to the presence of the DMSO, otherwise cells may have lysed. The suspension was then aliquoted into cryovials (Greiner, 122 278) which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -80$^0$C). After 2.5 to 3.5 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required.

### 2.5.5 Cell Thawing

Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution which is toxic at room temperature. The suspension was centrifuged at 1,000 rpm. for 5 min, the DMSO-containing supernatant removed and the pellet re-suspended in fresh growth medium. A viability count was carried out (Section 2.5.3) to determine the efficacy of the freezing/ thawing procedures. Thawed cells were placed into tissue culture flasks with the appropriate volume of medium (5ml/25cm$^2$ flask and 10ml/75cm$^2$ flask) and allowed to attach overnight. After 24 hours, the cells

were re-fed with fresh medium to remove any residual traces of DMSO.

### 2.5.6 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture. Samples of basal media were inoculated into Columbia (Oxoid, CM331) blood agar plates, Sabauraud (Oxoid, CM217) dextrose and Thioglycollate (Oxoid, CM173) broths which detect most contaminants including bacteria, fungus and yeast. Growth media (*i.e.* supplemented with serum and L-glutamine) were sterility checked at least 2 days prior to use by incubating samples at $37^0$C, which were subsequently examined for turbidity and other indications of contamination.

## 2.6 *MYCOPLASMA* ANALYSIS

*Mycoplasma* examinations were carried out routinely (at least every 3 months) on all cell lines used in this study. These analyses were performed by Mary Heenan and Michael Henry.

### 2.6.1 Indirect Staining Procedure

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells. These cells were incubated with supernatant from test cell lines and then examined for *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present. A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells.

NRK cells were seeded onto sterile coverslips in sterile Petri dishes at a cell density of $2x10^3$ cells per ml and allowed to attach over night at $37^0$C in a 5% $CO_2$, humidified incubator. 1ml of cell-free (cleared by centrifugation at 1,000 rpm for 5 min) supernatant from each test cell line was then inoculated onto a NRK Petri dish and incubated as before until the cells reached 20 - 50% confluency (4 - 5 days). After

this time, the waste medium was removed from the Petri dishes, the coverslips washed twice with sterile PBS A, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid:methanol-1:3) for 10 min. The fixative was then removed and after air drying, the coverslips were washed twice in deionised water and stained with 2ml of Hoechst 33258 stain (BDH)(50ng/ml) for 10 min.

From this point on, work was carried out in the dark to limit quenching of the fluorescent stain.

The coverslips were rinsed three times in PBS. They were then mounted in 50% (v/v) glycerol in 0.05M citric acid and 0.1M disodium phosphate and examined using a fluorescent microscope with a UV filter.

### 2.6.2 Direct Staining

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid, CM403) - supplemented with 16% serum, 0.002% DNA (BDH; 42026), 2mg/ml fungizone (Gibco,15290-026), $2x10^3$ units penicillin (Sigma, Pen-3) and 10ml of a 25% (w/v) yeast extract solution - to optimise growth of any contaminants and incubated at $37^0C$ for 48 hours. Samples of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had also been supplemented as above and the plates incubated for 3 weeks at $37^0C$ in a $CO_2$ environment. The plates were viewed microscopically at least every 7 days; the appearance of small, "fried egg" -shaped colonies is indicative of a mycoplasma infection.

## 2.7 DIFFERENTIATION STUDIES – Bromodeoxyuridine (BrdU) and Retinoic Acid (RA)

Differentiation studies were carried out using 5-bromodeoxyuridine (BrdU) (Sigma, B5002) or Retinoic Acid (Sigma, R2625). BrdU powder was reconstituted in UHP water to a stock concentration of 10mM and the resultant solution was filter sterilised through a sterile 0.22μm filter, aliquoted into sterile Eppendorfs and stored at -20$^0$C for up to 1 year. Retinoic acid powder was reconstituted in 95% EtOH in sterile UHP to a stock concentration of 2mM and the resultant solution was filter sterilised through a sterile 0.22μM filter. RA stocks were aliquoted into sterile cryovials to prevent loss due to evaporation, and stored at –80$^0$C for up to 1 year.

### 2.7.1 Differentiation Assays:

For immunocytochemical analysis (Section 2.8), cells were plated onto 6-well plates (Costar, 3516) at densities of 1x10$^4$ cells per well. 1 ml of medium was sufficient for each well. The cells were allowed to attach and form colonies by incubating at 37$^0$C, 5% $CO_2$ for 24 hours. The plates were covered with parafilm to prevent contamination. 1 ml fresh medium containing either 2x BrdU (20μM) or 2X RA (40μM) was then added to each well. Plates were wrapped in aluminium foil because of the light-sensitive nature of BrdU-treated cells and the RA compound, and incubated for up to 7 days. Medium was replaced every 3-4 days over the course of the assay. All waste medium was retained for disposal by incineration. At the end of the assay the cells were fixed with methanol as described in Section 2.8.1. Immunocytochemistry/fluorescence was then carried out using a range of antibodies as described in Section 2.8.2.

For additional analytical techniques (western blotting, immunoprecipitation, iso-electric focusing, PCR and Northern blotting), cells were inoculated into 75cm$^2$ flasks at a density of 1x10$^5$ cells per flask and allowed to attach and form colonies. BrdU- or RA-containing medium, at a concentration of 10μM or 20μM, respectively, was then added to the cells after 24 hours. The medium was replaced with fresh, BrdU- or

RA-containing medium every 3-4 days. The cells were then harvested by trypsinisation, washed in sterile PBS A, counted, pelleted and stored at -80$^0$C until required. For RNA extraction (section 2.14), pellets were lysed in tri-reagent and stored at -80$^0$C.

For HL60 suspension cultures, 75-cm$^2$ flasks were inoculated with 1 x 10$^6$ cells in 10 ml medium and incubated for 24 hours. 2.5 ml 5X BrdU-containing medium was then added to the cells. Medium was replaced every 3-4 days by centrifugation and resuspension of the resultant cell pellet in 10-15 ml fresh, 10μM BrdU-containing medium. Cells were then harvested by centrifugation, washed in sterile PBS A and stored at –80$^0$C until required. No RA studies were performed on these cells.

## 2.8 IMMUNOCYTOCHEMISTRY

### 2.8.1 Fixation of cells

For fixation, medium was removed from 6-wells plates, cells were rinsed 3 times with PBS A and then incubated at -20$^0$C for 7 minutes using ice-cold methanol. The methanol was then removed from the cells, which were allowed to dry at 37$^0$C for a few minutes and then stored at -20$^0$C until required.

### 2.8.2 Immunocytochemical procedure

The avidin-biotin complex (ABC) technique combined with the vector red visualisation (Vector Laboratories, SK-5100) procedure was used in all immunocytochemistry experiments. The ABC method involves application of a biotin-labelled secondary antibody to cells probed with a primary antibody, followed by the addition of avidin-biotin-alkaline phosphatase complex which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains alkaline phosphatase (AP) molecules. The AP enzyme then reacts with a Vector Red solution to give an insoluble, red-coloured precipitate. The formation of this red-coloured precipitate is indicative of primary antibody reactivity.

The procedure used is as follows:

Cell preparations (6-well tissue culture plates) which had been previously fixed in methanol and frozen at -20$^0$C were allowed to thaw and equilibrate at room temperature. A grease pen (DAKO, S2002) was used to encircle cells in tissue culture plates to retain the various solutions involved. The cells were equilibrated in Tris-buffered saline (TBS) (0.05M Tris/HCl, 0.15M NaCl, pH 7.6) for 5 minutes. The slides were then incubated for 20 minutes at room temperature (RT) with either normal rabbit (DAKO, X092) or goat (DAKO, X0907) serum diluted 1:5 in TBS to block non-specific binding, depending upon the host source of the primary antibody in question. This was then removed and 25-30µl of optimally-diluted primary antibody (Table 2.8.1) was placed on the cells. The slides and tissue-culture plates

were placed on a tray containing moistened tissue paper and incubated at $37^0$C for 2 hours or $4^0$C overnight. The primary antibodies used in the study are listed in Table 2.8.1. The slides were then rinsed in TBS/ 0.1% Tween (Sigma, P-1379) for 5min x3 times, and then incubated for 30 min with a suitable biotinylated secondary antibody (rabbit anti-mouse immunoglobulins (DAKO, E354); goat anti-rabbit (DAKO, E0432) diluted 1:300 in TBS. The slides were rinsed as before and incubated with strepABComplex/Alkaline Phosphatase (AP) (DAKO, K377) for 30 min at RT, after which they were rinsed again in TBS/ 0.1% Tween for 5min x3 times. The cells were then incubated with a Vector Red solution (DAKO, S3000) for 10-15 min. Excess Vector Red solution was then rinsed off with UHP water, allowed to dry and samples mounted using a commercial mounting solution (DAKO, S3023).

### 2.8.3 Immunofluorescence

Immunofluorescence was performed using a similar approach to that described in 2.14 above. Cell preparations (6-well tissue culture plates) which had been previously fixed in methanol and frozen at $-20^0$C were allowed to thaw and equilibrate at room temperature. A grease pen (DAKO, S2002) was used to encircle cells in tissue culture plates to contain the various solutions involved. The cells were equilibrated in Tris-buffered saline (TBS) (0.05M Tris/HCl, 0.15M NaCl, pH 7.6) for 5 minutes. The slides were then incubated for 20 minutes at room temperature (RT) with normal rabbit/goat serum (DAKO, X092/Dako, X0907) (depending upon the primary in question) diluted 1:5 in TBS to block non-specific binding. This was then removed and 25-30µl of optimally-diluted primary antibody was placed on the cells and incubated on a tray containing moistened tissue paper at $4^0$C overnight. The following day the slides were then rinsed in TBS/ 0.1% Tween (Sigma, P-1379) for 5min x3 times. All subsequent manipulations were performed in a darkened room, and incubations were performed in trays covered in tinfoil as a precaution to minimise "quenching" of fluorescence by exposure to light for extended periods. Cells were incubated for 60 min with TRITC-labelled rabbit anti-mouse immunoglobulins (Sigma, T-5393) diluted 1:160 in TBS/0.1%Tween or FITC-labelled goat anti-rabbit immunoglobulin (Sigma, F-6005). The slides were then rinsed in TBS/ 0.1% Tween (Sigma, P-1379), x3 in 15 min., air-dried and mounted in fluorescent mounting

medium (DAKO, S3023). Antibody reactivity was determined by UV-excitation of the conjugated secondary antibodies through the appropriate filters (Rhodamine; TRITC and Ultra-violet; FITC). Co-fluorescent studies were performed by co-incubation of antibodies from different hosts (Keratin 8 and eIF-4E, table 2.8.1) and detected using a mixture of both TRITC-labelled mouse and FITC-labelled rabbit secondary antibodies, visualised through different filters.

**Table 2.8.1** Primary antibodies used for immunocytochemistry/immunoflourescence

| **Antibody** | **Dilution/ Concentration** | **Supplier** | **Catalogue no.** |
|---|---|---|---|
| Keratin 8 (M) | 1/200 | Sigma | C-5301 |
| Keratin 18 (M) | 1/800 | Sigma | C-8541 |
| eIF-4E (M) | 1/250 | Transduction Laboratories | E27620 |
| eIF-4G (γ) (M) | 1/150 | Transduction Laboratories | E46520 |
| eIF-4E (R) | 1/1000 | Simon Morley | X |
| eIF-4G (R) | 1/1000 | Simon Morley | X |

Nomenclature: (M) = Mouse-anti-human IgG
(R) = Rabbit-anti- human IgG
P-eIF4E = Phospho-specific anti-eIF-4E antibody
X = Antibody was a gift

## 2.9 WESTERN BLOT ANALYSIS

Proteins for western blot analysis were separated by SDS-polyacylamide gel electrophoresis (SDS-PAGE).

### 2.9.1 Sample preparation

Cell pellets (Section 2.7.1) were lysed in TG lysis buffer (20mM Tris-HCl pH 8, 10% glycerol, 1% TritonX-100, 1.5mM $MgCl_2$, 2mM EDTA, 137mM NaCl, 1mM $Na_3VO_4$, 1mM Pefabloc (Boehringer, 84500920-22), and 1X Protease inhibitor cocktail (Boehringer, 1697498) for 20 min on ice. The extracts were either used immediately for western blot analysis or snap frozen in liquid nitrogen and stored at $-80^0$C. Alternatively, cells were lysed by resuspension in boiling loading buffer (2.5ml 1.25M-Tris/HCl, 1.0g SDS, 5.8ml glycerol and 0.1% bromophenol blue (Sigma, B8026) made up to 25ml with distilled water) and incubated at $100^0$C for 2-3 min., cooled to room temperature and used immediately for western blot analysis.

### 2.9.2 Gel electrophoresis

Resolving and stacking gels were prepared as outlined in Table 2.9.1 and poured into clean 10cm x 8cm gel cassettes which consisted of 1 glass and 1 aluminium plate, separated by 0.75cm plastic spacers. The resolving gel was poured first and allowed to set. The stacking gel was then poured and a comb was placed into the stacking gel in order to create wells for sample loading. Once set, the gels could be used immediately or wrapped in aluminium foil and stored at $4^0$C for 24 hours.

Before samples were loaded onto the stacking gels, equal cell numbers ($2x10^4$ cells per lane) were lysed in 2x loading buffer (Section 2.9.1). The samples were then loaded alongside molecular weight colour protein markers (Sigma, C-3437). The gels were run at 250V, 45mA for approximately 1.5 hours (until the protein was run at least half way into the gel as judged by the migration of colour markers during the electrophoretic process). An exception was that of eIF-4G, a 220 kDa protein that required electrophoresis for approximately 2.5-3.5 hours at 350V, 63 mA on 7.5% gels (apparatus was cooled on ice during the process and all running buffers were pre-

chilled by incubation at -20$^0$C for approximately 30-60 min). To resolve different forms of 4E-BP1, samples were separated on 15-20% polyacrylamide gels. All gels were made from a stock of Acrylamide (details below). Sample calculations for two different percentage gels are shown in table 2.9.1.

**Table 2.9.1** Preparation of electrophoresis gels

| **Components** | **Resolving gel (7.5%)** | **Resolving gel (12%)** | **Stacking gel** |
|---|---|---|---|
| Acrylamide stock* | 3.75ml | 6ml | 0.8ml |
| Ultrapure water | 8.0ml | 5.75ml | 3.6ml |
| 1.875M-Tris/HCl, pH 8.8 | 3.0ml | 3.0ml | - |
| 1.25M-Tris/HCl, pH 6.8 | - | - | 0.5ml |
| 10% SDS (Sigma, L-4509) | 150µl | 150µl | 50µl |
| 10% APS (Sigma, A-1433) | 60µl | 60µl | 17µl |
| TEMED (Sigma, T-8133) | 10µl | 10µl | 6µl |

* Acrylamide stock = 29.1g acrylamide (Pharmacia, 17-1300-02) and 0.9g NN'-methylene bis-acrylamide (Sigma, N-7256) made up to 100ml with distilled water

### 2.9.3 Western blotting

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine (Sigma, G-7126) pH 8.3-8.5 without adjusting) for 20 min. Proteins in gels were transferred onto Hybond ECL nitrocellulose (Amersham, RPN 2020D) or PVDF (Polyvinyl diflouride) (Boehringer, 1722026) membranes by semi-dry electroblotting. Six sheets of Whatman 3mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus. Excess air was removed from between the filters by moving a glass pipette over the filter paper. Nitrocellulose or PVDF (pre-activated in methanol for 1-2 min. and washed in UHP for 5 min), cut to the same size of the gel, was soaked in transfer buffer and placed over the filter paper, making sure there were no air bubbles. The acrylamide gel was placed over the nitrocellulose and six more sheets of presoaked filter paper were placed on top of the gel. Excess air was again

removed by rolling the pipette over the filter paper. The proteins were transferred from the gel to the nitrocellulose/PVDF at a current of 0.34mA at 15V for 20-30 min. depending upon the size of the protein.

eIF-4G, due to its size (220 kDa), required transfer for 2 hours at 0.8mA and 20-25 V. Semi-dry transfer set-up was as follows, protocol obtained from Dr. Simon Morley (Sussex, UK):

**1.** Anode buffer 1: (4 sheets of filter paper; squeeze dry)
33.35g Tris, 200ml Methanol in 1 L.
**2.** Anode buffer 2: (2 sheets of filter paper; squeeze dry)
3.03g Tris, 200ml Methanol in 1 L.
**3.** PVDF membrane (pre-activated in methanol as before).
**4.** Polyacrylamide gel.
**5.** Cathode Buffer: (4 sheets of filter paper; squeeze dry)
3.03g Tris, 5.25g 6-amino-n-hexanoic acid (Sigma, A-2504), 200ml Methanol in 1 L.

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus to ensure even exposure of the membrane blot to all reagents.

The nitrocellulose/PVDF membranes were blocked for 2 hours at room temperature with fresh filtered 5% non-fat dried milk (Cadburys; Marvel skimmed milk) in TBS/ 0.1% Tween, pH 7.4.

After blocking, the membranes were rinsed with TBS/0.1% Tween and incubated with primary antibody overnight at $4^0$C. Primary antibodies used are listed in table 2.9.2. The following day the primary antibody was removed and the membranes rinsed 3 times with TBS/ 0.1% Tween. The membranes were incubated in 1/1000 dilution of a suitable HRP-labelled secondary antibody (Mouse; Sigma, A-6782 or Rabbit; Sigma, A-4914) in TBS/0.1% Tween for 1 hour at room temperature (R.T.). The secondary was then removed and blots were washed for 15 min in TBS/0.1%Tween. Bound antibody was detected using enhanced chemiluminescence (ECL) (Section 2.9.4).

**Table 2.9.2:** Antibodies used for western blot analysis

| **Antibody** | **Dilution/ Concentration** | **Supplier** | **Catalogue no.** |
|---|---|---|---|
| Keratin 8 (M) | 1/400 | Sigma | C-5301 |
| Keratin 18 (M) | 1/800 | Sigma | C-8541 |
| eIF-4E (M) | 1/500 | Transduction Laboratories | E27620 |
| c-myc (M) | 1/500 | Santa Cruz | SC-040 |
| eIF-4E (R) | 1/10,000 | Simon Morley | X |
| eIF-4G (R) | 1/10,000 | Simon Morley | X |
| 4E-BP1 (R) | 1/2,500 | Nahum Sonenberg | X |
| YY1 (R) | 1/500 | Santa Cruz | SC-281 |
| eIF-2α (M) | 1/1000 | Simon Morley | X |
| ERK-1 (M) | 1/500 | Pharmingen | 13621A |
| P-ERK (M) | 1/1000 | John Lyons | X |
| RAR-α | 1/1000 | Santa Cruz | Sc-551 |
| RAR-β | 1/1000 | Santa Cruz | SC-552 |
| CRABPI | 1/1000 | Affiniti, UK | MA3-813 |

Nomenclature: (M) = Mouse anti-human IgG.

(R) = Rabbit anti-human IgG.

P-ERK = Phospho-specific anti-ERK antibody.

X = Antibody was a gift.

### 2.9.4 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions.

After blots were washed in TBS/0.1% Tween x3 times for 5 min, a sheet of parafilm was flattened over a smooth surface, *e.g.* a glass plate, making sure all air bubbles were removed. The membrane was then placed on the parafilm, and excess fluid removed. 1.5ml of ECL detection reagent 1 and 1.5ml of reagent 2 were mixed and covered over the membrane. Charges on the parafilm ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane wrapped in cling film. The membrane was exposed to autoradiographic film (Kodak; X-OMAT S, 500 9907) in an autoradiographic cassette for various times, depending upon the strength of the signal obtained. The autoradiographic film was then developed.

The exposed film was developed for 5min in developer (Kodak, LX24), diluted 1:6.5 in water. The film was briefly immersed in water and transferred to a Fixer solution (Kodak, FX-40) diluted 1:5 in water, for 5min. The film was transferred to water for 5 min and then air-dried.

## 2.10 IMMUNOPRECIPITATION

Immunoprecipitation was carried out using a cellular labelling and immunoprecipitation kit (Boehringer Mannheim, 1647652) according to manufacturer's instruction.

### 2.10.1 Sample preparation

Cells were treated with BrdU or RA as described in Section 2.7.1, harvested, pelleted at a known cell number and stored at -80$^0$C until required.

Pre-chilled lysis buffer was added to the cells which were then sonicated (10 pulses) on ice and incubated at 4$^0$C for 30min. The lysate was then centrifuged at 13000rpm in a microfuge for 10 min at 4$^0$C. The supernatant was transferred to a fresh Eppendorf and labelled with 25µl of biotin-7-NHS stock solution for 15 min at RT, as described in the manual accompanying the kit. The reaction was stopped by adding 50µl of stop solution (50mM $NH_4Cl$) and incubating for 15 min at 4$^0$C on a rotating belly-dancer (Stovall).

### 2.10.2 Sample immunoprecipitation

The lysates were pre-cleared using 50µl of Protein A or Protein G beads per ml of sample for 3 hours to completely remove proteins that may bind to the agarose beads and result in non-specific binding. The samples were then centrifuged at 13000rpm in a microfuge for 20 seconds to pellet the beads. The supernatants were collected in fresh eppendorfs to which an equivalent to 10µg of either K8 or K18 antibody was added. Samples were then rocked overnight at 4$^0$C. 50µl of protein A (Boehringer Mannheim, 1719408) or protein G (Boehringer Mannheim, 1719416) agarose beads, depending on the binding specificity of the Ab to be tested (see Table 2.10.1), were then added to the samples. The precipitated protein was then allowed to bind to the beads by incubating the mixture on a belly-dancer overnight at 4$^0$C. Controls included both K8/18 antibodies in lysis buffer and irrelevant antibodies (EGF-R) to illustrate the specificity of keratin immunoprecipitation.

The complexes were then collected by brief centrifugation at 13000rpm in a microfuge for 20 seconds. The supernatant was carefully removed. The protein-bead complexes were washed with a number of different stringency buffers. Samples were washed in 1ml of wash buffer 1 (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% NP-40, 100μg/ml PMSF, 1μg/ml each of leupeptin and aprotonin) on a rocking apparatus for 20 min. The complex was then pelleted by centrifugation as before and the supernatant carefully removed and discarded. A second wash in buffer 1 was performed, followed by washes (x2) with 1ml of wash buffer 2 (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% NP-40) in a similar manner to the first wash. A third wash was carried out in buffer 3 (50mM Tris-HCl, 500mM NaCl and 0.1% NP40).

**Table 2.10.1** Agarose beads chosen to precipitate the required proteins

| **Antibody used** | **Isotype of Ab** | **Avidity to Protein-A** | **Avidity to Protein-G** | **Beads chosen** |
|---|---|---|---|---|
| Keratin 8 | Mouse IgG1 | + | ++++ | Protein-G |
| Keratin 18 | Mouse IgG1 | + | ++++ | Protein-G |

After the final wash, 60 μl 5x loading buffer was added to the pellets. The proteins were then denatured by heating to $100^0$C for 3 min. Equal volumes were loaded and separated on 12% polyacrylamide gels, as described in Section 2.9.

The proteins were then transferred to nitrocellulose/PVDF membrane as described previously in Section 2.9.3 and were blocked overnight at $4^0$C in 5% Marvel in TBS/ 0.1% Tween. The following day, membranes were then rinsed briefly with PBS A and incubated for 1 hour in anti-biotin secondary antibody (mouse monoclonal anti-biotin IgG (Sigma, A0185) diluted 1:4000 in TBS). The precipitated proteins were then visualised using ECL as described in Section 2.9.4, or supersignal ECL (Pierce, 34075) for shorter timepoints in differentiation studies.

## 2.11 VERTICAL SLAB ISOELECTRIC FOCUSING (IEF) FOR eIF-4E

Vertical slab IEF is more convenient than older methods for the determination of the phosphorylation status of proteins, allowing samples to be run and separated vertically alongside each other for direct comparison. The procedure was developed in the laboratories of Prof. Chris Proud (Dundee, UK) and Dr. Simon Morley (Sussex, UK).

The procedure was carried out in a Bio-Rad Protean II mini-gel apparatus (0.75 mm spacers) (Biorad, 165-4998).

### 2.11.1 IEF Gel preparation

Gels were poured as described by the manufacturer (Biorad). The gels were as follows:

Incomplete stock solution (Filter sterilised through 0.22μM filter and stored at $4^0$C):

- 42.8 ml UHP
- 4.86 g acrylamide
- 274.3 mg bis-acrylamide
- 1.71 g CHAPS (Sigma, C-9426)

Working gels were then prepared from the stored incomplete stock solution as follows:

- 3.5 ml incomplete gel mix
- 3.24 g Urea
- 0.45 ml ampholines (Pharmacia, wide range pH 3.5-10, (80-1125-87)
- 20 μl 10% APS
- 10 μl TEMED

Gels were covered loosely in cling film while setting, which improves the quality of wells formed (avoiding drafts).

### 2.11.2 Sample Buffer Preparation

7X sample buffers were prepared and stored in aliquots at $-20^0$C:

- 21% (v/v) ampholines (as for IEF gel)
- 14% (v/v) β-mercaptoethanol
- 35% (w/v) CHAPS
- 30% deionised water

For working IEF buffer, 7X sample buffers were thawed:

- 143 µl 7X sample buffer
- 0.54 g Urea
- 1.1 ml UHP.

Samples, in TG lysis buffer, were mixed with an equal volume of working IEF buffer and analysed as in section 2.11.4. Alternatively, cell pellets were lysed directly in IEF working buffer.

### 2.11.3 Running Buffers

Both anode and cathode buffers were prepared and chilled at $-20^0$C before use.

The outer chamber of the IEF apparatus was filled with Cathode Buffer:
0.05 M Histidine (Sigma, H-8000) in UHP.

The inner chamber of the IEF apparatus was filled with Anode Buffer:
0.01 M Glutamic Acid (Sigma, G-1251) in UHP.

### 2.11.4 The IEF procedure

Due to the very high voltages used in this procedure the apparatus was surrounded with ice to avoid overheating of the gels. This is not completely necessary, but is used as a precaution and to improve the resolution of bands.

1. Once the gel was set the combs were removed and wells were washed out thoroughly with UHP.
2. 30 µl working IEF buffer was added to each well.
3. Each well was then carefully overlaid with 10µl 6M Urea.
4. An overlay of 0.01M glutamic acid was then applied, and the chambers filled with cathode and anode buffers as described in section 2.11.3.
5. Gels were then prefocused for 1 hr on **reverse polarity**.

   20 min. at 200 V.

   20 min. at 300 V.

   20 min. at 400V.
6. After prefocusing, the wells were washed out thoroughly with UHP and the samples were loaded. A control lane consisting of rabbit reticulocyte lysate (see section 2.19) in IEF buffer was used as a means of orientating the gel (runs as a brown smear) for transfer and as a positive control for eIF-4E.
7. Wells were then overlaid with 10µl 6M Urea and glutamic acid as for prefocusing.
8. Focusing was carried out as follows, all on **reverse polarity**:

   20 min. at 500 V.

   20 min. at 550 V.

   20 min. at 600 V.

   20 min. at 650 V.

   20 min. at 700 V.

   20 min. at 750 V.

   20 min. at 1,000 V.
9. After focusing the gel is gently "floated" off the plates into transfer buffer and subjected to standard transfer and antibody detection as described for western blotting (section 2.9.3) using anti-eIF-4E antibody (Table 2.9.2). Focusing separated the phosphorylated and non-phosphorylated forms of the protein.

## 2.12 SERUM STARVATION AND KINASE ACTIVATION STUDIES

Cells were innoculated at 5 x $10^5$ cells in 5 ml fresh medium in 25 $cm^2$ flasks (Costar, 3050) and allowed to grow for 48 hours. Flasks were then washed x3 times in Serum Free Medium (SFM) and incubated for 48 hours in SFM. Cells were then pre-incubated for 60 min in the presence of specific kinase inhibitors (Table 2.12.1) (see Figure 4.10; Section 4.3.2) before re-stimulation with medium containing 10% serum.

**Table 2.12.1:** Kinase Inhibitors for Serum Starvation Studies

| **Inhibitor** | **Target Kinase** | **Concentration** | **Source** | **Cat. No.** |
|---|---|---|---|---|
| PD98059 (MW=267.29) | MEK-ERK activation | 50μM | John Lyons Onyx Pharm. | X |
| SB203580 (MW=*) | p38 kinase | 30nM | John Lyons Onyx Pharm. | X |
| Rapamycin (MW=914.2) | FRAP/mTOR | 20nM | Calbiochem | CN 681675 |
| Wortmannin (MW=428.4) | PI-3K | 100nM | Calbiochem | CN 553210 |

Nomenclature: X = Inhibitor was a kind gift of Dr. John Lyons.

* = MW unavailable. Supplied as a 10mM stock solution.

Samples were harvested by washing x3 times in chilled, sterile PBS A, followed by incubation in 650 μl ice-cold TG lysis buffer (section 2.9.1) for 20 min. Samples were snap frozen in liq. $N_2$ and stored at $-80^0$C. Protein concentrations of samples were determined using the BCA micro-assay described in section 2.13.

The effect of serum stimulation on ERK activity was determined by western blotting (Section 2.9) using phospho-specific antibodies (Table 2.9.1). Even loading of ERK protein in lanes was shown using ordinary ERK antibody, allowing the relative levels of ERK phosphorylation to be determined. The effects of specific kinase inhibitors on the phosphorylation of eIF-4E were determined using IsoElectric Focusing, as described in section 2.11.

## 2.13 BCA Micro-assay for protein concentration

A 1/10 dilution of samples was made in sterile UHP. All standards were diluted in 0.2% Triton X-100 (final concentration of Triton X-100 in diluted samples to be measured) to ensure that the same amount of triton was present in samples and standards. This was only a precaution since the BCA assay is relatively insensitive to triton concentrations below 1%.

A working BCA reagent mixture (Pierce, 23235) was made by combining 25 parts Solution A with 24 parts Solution B, mixing and then adding 1 part Solution C. 120µl samples and standards were added to the wells of a 96-well plate, to which 120 µl working BCA mix was added. The plate was gently agitated to mix the samples and incubated for 30 min at $37^0$C. After ensuring no air-bubbles were present, samples were read at 595 nm in a plate reader. From a standard curve, the protein content of each sample was determned.

## 2.14 RNA EXTRACTION

For all procedures using RNA, most glassware, solutions and plastics were treated with 0.1% diethyl pyrocarbonate (DEPC) before use, a strong inhibitor of RNase activity.

RNA was extracted from cells as follows:

Cells were trypsinised, washed once with PBS A and the sample was counted. Approximately $10^8$ cells were pelletted and lysed using 1ml of TRI REAGENT™ (Sigma, T-9424). The samples were allowed to stand for 5 min at RT to allow complete dissociation of nucleoprotein complexes and then snap-frozen in liq. $N_2$ and stored at $-80^0$C.

When thawed, samples were allowed to stand for 5 min before 0.2ml of chlorofom was added per ml of TRI REAGENT™ used. Samples were then shaken vigorously for 15 sec and allowed to stand for 15 min at RT. Samples were then centrifuged at 13000rpm in a microfuge for 15 min at $4^0$C. This step separated the mixture into 3 phases; the RNA was contained in the colourless upper aqueous layer. This layer was then transferred to a fresh Eppendorf and 0.5ml of isopropanol was added. The sample was mixed and allowed to stand at RT for 10 min before being centrifuged at 13000rpm in a microfuge for 10 min at $4^0$C. The RNA formed a precipitate at the bottom of the tube. The supernatant was removed and the pellet was washed with 1ml of 75% ethanol and centrifuged at $4^0$C for 5-10 min at 8500rpm. The supernatant was removed and the pellet was briefly allowed to air-dry. 20-30µl of DEP-C water was then added to the RNA to resuspend the pellet.

Concentrations of RNA in samples were calculated by determining OD at 260nm and 280nm and using the following formula:-

$$OD_{260nm} \times \text{Dilution factor} \times 40 = \mu g/ml \text{ RNA}$$

The purity of the RNA extraction was calculated by determining its OD at 260nm and 280nm. An $A_{260nm} : A_{280nm}$ ratio of 2 is indicative of pure RNA. Only those samples with ratios between 1.7 and 2.1 were used.

## 2.15 REVERSE TRANSCRIPTASE REACTION

Reverse transcriptase (RT) reactions were carried out in laminar flow cabinets using micropipettes which were specifically allocated to this work.

cDNA was formed using the following procedure:-

- 1µl oligo $(dT)^{12-18}$ primers (1µg/µl) (Promega; C1101)
- 1µl total RNA (1µg/µl) (section 2.14)
- 3µl water

were mixed in a 0.5ml Eppendorf (Eppendorf, 0030 121.023), heated to $70^0$C for 10 min and then chilled on ice. To this, the following were added:-

- 4µl of a 5x buffer (250mM-Tris/HCl pH 8.3, 375mM-KCl and 15mM-$MgCl_2$)
- 2µl DTT (100mM) (Gibco; 510-8025 SA)
- 1µl RNasin (40U/µl) (Promega; N2511)
- 1µl dNTPs (10mM of each dNTP)
- 6µl water
- 1µl Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (40,000U/µl) (Gibco; 510-8025 SA).

The solutions were mixed and the RT reaction was carried out by incubating the Eppendorfs at $37^0$C for 1 hour. The MMLV-RT enzyme was then inactivated by heating to $95^0$C for 2 min. The cDNA was stored at $-20^0$C until required for use in PCR reactions as outlined in Section 2.16.

## 2.16 POLYMERASE CHAIN REACTION

A standardised polymerase chain reaction (PCR) procedure was followed in this study. The Eppendorf tubes used (Eppendorf, 0030 121 023) and the sterile water were DEPC-treated. All reagents had been aliquoted and were stored at $-20^0$C and all reactions were carried out in a laminar flow cabinet.

Each PCR tube contained the following:-

- 24.5µl water
- 5µl 10x buffer* (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100)

- 3µl 25mM-$MgCl_2$*
- 8µl dNTPs (1.25mM each of dATP, dCTP, dGTP and dTTP) (Promega; U1240)
- 1µl each of first and second strand target primers (250ng/µl)
- 1µl each of first and second strand endogenous control primer (250ng/µl) (β-actin)
- 0.5µl of 5U/µl *Taq* DNA polymerase enzyme*
- 5µl cDNA

*(Promega; N1862)

A drop of autoclaved mineral oil was placed in each reaction tube to prevent evaporation and the DNA was amplified by PCR as follows:

$95^0$C for 1.5 min - to denature double-stranded DNA

30 cycles: $95^0$C for 1.5 min. - denature
$55^0$C for 1 min - anneal
$72^0$C for 3 min. - extend
$72^0$C for 7 min. - extend

The reaction tubes were then stored at $4^0$C until analysed by gel electrophoresis as described in Section 2.17.

Primers were: K8/18 (McBride *et al.*, 1999), eIF-4E (designed by Dr. Noel Daly, NCTCC), c-myc (NicAomhlimh, R., PhD thesis, 1997) and β-actin (NicAomhlimh, R., PhD thesis, 1997).

## 2.17 ELECTROPHORESIS OF PCR PRODUCTS

A 3% agarose gel (NuSieve, GTG) was prepared in TBE buffer (5.4g Tris, 2.75g boric acid, 2ml 0.5M-EDTA pH 8.0 in 500ml water) and melted in a microwave oven. After allowing to cool, 0.003% of a 10mg/ml ethidium bromide solution was added to the gel which was then poured into an electrophoresis apparatus (BioRad). Combs were placed in the gel to form wells and the gel was allowed to set.

10µl loading buffer (50% glycerol, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue, 1mM EDTA) was added to 50µl PCR samples and 20µl was run on the gel at 80-90mV for approximately 2 hours. When the dye front was seen to have migrated the required distance, the gel was removed from the apparatus and examined on a UV-transilluminator and photographed.

## 2.18 NORTHERN BLOT ANALYSIS OF RNA LEVELS

Standard Northern Blotting techniques were used to examine cellular RNA levels. The RNA samples to be analysed were first separated by Formaldehyde-Agarose gel Electrophoresis

### 2.18.1 Formaldehyde-Agarose gel Electrophoresis

A 100ml 1% agarose gel was prepeared by dissolving 1g of agarose in 73.4 ml of sterile distilled water (SDW). The gel was then cooled to around 60°C and 10ml of 10X MOPS buffer (0.25M MOPS, 0.05M Na acetate, 0.01 EDTA, pH 7.0) was added along with 16.6 ml formaldehyde and mixed well before pouring. The running buffer for the gel was 1X MOPS containing 12.9 ml formaldehyde/300ml. RNA samples were diluted to the same concentration to allow equal loading volumes. The RNA samples were mixed with RNA loading buffer (2.9µl 10X MOPS, 5µl formaldehyde, 14.3 µl formamide, 1.43µl tracking buffer) and heated to 65°C for 15min, placed on ice and loaded onto the gel. The RNA samples were run on the gels at 75mV for 2 hours alongside RNA size markers (Promega). The gels were washed in 3 changes of sterile UHP over 30 minutes.

### 2.18.2 Northern Blotting

A sheet of Hybond-N (Amersham) was cut to the same size as the RNA gel. A tray or glass dish was half filled with the transfer buffer (20X SSC (8.823 % (w/v) tri-sodium citrate, 17.532 % (w/v) NaCl, pH 7-8)). A platform was made to stand in the tray above the level of the transfer buffer and a wick (3MM filter paper) was placed over the platform into the transfer buffer. The RNA gel was placed loading side down on the wick platform without trapping air bubbles. The Hybond-N was placed on top of the RNA gel and three sheets of 3MM filter paper placed upon the Hybond-N. A stack of absorbent tissue paper over 5cm high was placed on top of the filter paper and finally a glass plate with a 750g weight were placed on top of the paper stack. The transfer was carried out overnight. After blotting, the transfer apparatus was dismantled and the gel loading tracks were marked on the Hybond-N to allow land identification. The nucleic acid was fixed to the membrane by baking at 80°C for 2 hours and stored until use between two sheets of dry filter paper.

After blotting, the gel was rehydrated in a 1 μg/ml EtBr solution. The gel was than viewed under a U.V. lamp. The efficiency of RNA transfer to the membrane could then be assessed by looking for remaining traces of 28 and 16S ribosomal bands. The lane on the gel containing the RNA markers was removed from the gel before blotting and stained with EtBr alongside the blotted gel. The position of the RNA markers were photographed and used as a reference to size bands on the developed Northern Blots.

### 2.18.3 Radioactive Labelling of Probes

All DNA probes were labelled with [$\alpha$-$^{32}$P]dCTP (Amersham) using the Prime-a-gene labelling kit (Promega : U1100) according to the supplied protocol.

To test the percentage incorporation of nucleotides into the DNA probes the following protocol was carried out. 1 μl out of the 50μl reaction mix was diluted 1 in100 with water. 1μl of the diluted probe was then blotted onto four 1cm$^2$ pieces of filter paper and air dried. Two of these pieces of filter paper were washed twice for 10 minutes in

10% Tri-chloro Acetic Acid (Riedel-del Haen: UN-No-1839), rinsed in 100% ethanol and air dried. Then the counts on the two washed and unwashed pieces of filter were measured using a scintillation counter. The filter paper was placed in scintillation counter tubes with 10ml of scintillation fluid (Ecolite) and the Counts per minute (CPM) read. The CPM of the washed pieces of filter paper as a percentage of the unwashed pieces of filter paper gave the percentage incorporation of oligonculeotides into the probe.

### 2.18.4 Hybridisation of labelled probes to RNA membranes

The baked Hybond-N membranes with the mRNA samples were prehybridised overnight at 65°C in 10ml of hybridisation buffer (In 100ml : 43ml 1 M Sodium phosphate pH 7.2, 33 ml 20% Sodium Dodecyl Sulphate (SDS), 20 ml 5% BSA, 4ml 0.5 M EDTA) per membrane. The hybridisation was carried out in glass hybridisation tubes in a hybridisation oven. The appropriate probe was heated to 94°C for 3 min before addition to 10ml of preheated (65°C) hybridisation buffer. Sufficient probe was used to give $3x10^{6}$ CPM/ml hybridisation buffer. The pre-hybridisation buffer was discarded from the hybridisation tubes and replaced with the fresh hybridisation buffer containing the probe. Hybridisation was carried out at 65°C overnight. The membranes were then washed at 65°C for 5 min in 2X SSC, followed by 2 x 15 min washes in 0.5X SSC, 0.1% SDS and 2 x 15 min washes in 0.1X SSC, 0.1% SDS. The membranes were wrapped in cling film and exposed to X-ray film at -80°C for the desired length of time (typically 24h to 5 days).

Probes used were: K8/18 (McBride *et al.*, 1999); eIF-4E (made by Dr. Noel Daly, NCTCC).

## 2.19 *IN-VITRO* TRANSLATION

*In-vitro* Translation (IVT) was performed using commercially available Wheat Germ Extract (WG) and Rabbit Reticulocyte Lysate Systems (RRLs) (Promega, L-4330) and RRLs (Boehringer, 1103-032). Wheat Germ (WG) Systems proved unsuitable for use and so the procedure described below is for RRL (Boehringer) systems only.

1. "Master-mixes" were made for all reactions to avoid differences between individual reactions. Translation mixtures were made as follows (per reaction):
   - 2 µl Translation reaction mixture (vial 2 and 3) (Amino Acids)
   - 1 µl Potassium Acetate (vial 4)
   - 1.5 µl Magnesium Acetate (vial 5)
   - 8.5 µl Nuclease free UHP (vial 8)
   - 2 µl RNA (stock of 0.5 µg/µl in nuclease free UHP)

   Total Volume 15 µl. For control reaction not containing RNA, 2µl nuclease free UHP (vial 8) was added to maintain constant volumes.
2. Reactions were mixed thoroughly. Rabbit Reticulocyte Lysate aliqouts were then thawed quickly, as recommended by the manufacturer, mixed thoroughly and added to the reaction mixtures:
   - 10µl Rabbit Reticulocyte Lysate (vial 1)

   Total volume 25µl
3. Reactions were mixed again, centrifuged briefly in a microfuge tube and incubated for 60 min. in a water-bath at $30^0$C.
4. Reactions were stopped by placing on ice and then subjected to Western blotting analysis (section 2.9) to determine protein synthesis from exogenous RNA added to the systems (RNA was prepared as described in section 2.14).

### 2.19.1 Translation Inhibition Studies

To determine whether or not inhibitors of translation were present in the poorly differentiated cell line, DLKP, cellular extracts were added to IVT reactions.

### 2.19.1.1 Preparation of Cellular Extracts

1. Approximately $10^8$ cells were harvested and washed in ice-cold PBS.
2. The cells were carefully resuspended in 375 µl ice-cold lysis buffer (50 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1000U/ml RNAsin (Sigma, R-2520), made up in DEPC-treated UHP and filter sterilised.
3. The cell suspension was incubated on ice for 5 min. and cell lysis was observed microscopically (nuclear lysis, as expected, did not occur).
4. The suspension was then transferred to an eppendorff and centrifuged at 2,000 rpm for 10 min to remove nuclei.
5. The supernatant was carefully removed to a fresh eppendorff and stored in aliquots at $-80^0$C.

Since these preparations are based on a simple method for isolation of cytoplasmic RNA, and included RNase inhibitors, they were deemed suitable for translation inhibition studies.

### 2.19.1.2 IVT using cytoplasmic extracts

Standard IVT reactions were used, as described in section 2.19 above.

1. 50µl Cytoplasmic extract was thawed on ice and combined with an additional 60U RNAsin for 2-3 min. 5µl extracts were used for inhibitor studies.
2. 2µl total RNA (0.5µg/µl) was added to this and incubated for 20-30 min at $30^0$C or $4^0$C (works using both temps) ("inhibition study mixture"). A water control, containing DEPC UHP in place of extract was included.
3. IVT reaction mixtures were prepared during this incubation period. They were as described in section 2.19 except 1.5 µl of water was used in place of 8.5 µl to allow for the volume of the inhibition study mix (7µl) to be added.
4. After incubation of the inhibition study mixes and controls, IVT mixes from step 3 were added, and samples were subjected to IVT and western blotting analysis as described in section 2.19 and 2.9, respectively.

## 2.20 OVEREXPRESSION STUDIES

eIF-4E and YY1 cDNAs were kind gifts of Prof. Arrigo DeBenedetti (Louisiana, USA) and Dr. Finian Martin (Dublin, Ireland), respectively. Both plasmids were obtained as a culture of pre-transformed cells resistant to geneticin (GEN) and ampicillin (AMP).

### 2.20.1 Plasmid Preparation

Cultures were streaked on LB agar containing 50μg/ml Geneticin (Sigma, G9516) and 50μg/ml Ampicillin and incubated at 37$^0$C overnight. From these, a single colony was inoculated into 10ml of LB AMP/GEN (50μg/ml each) and grown overnight. A 2ml sample of this suspension was then added to 200ml of TB AMP/GEN 50μg/ml and left to grow overnight at 37°C for large-scale isolation of plasmid from transformed cells. The following day the cells were pelleted and pZ523 spin columns (5 Prime → 3 Prime Inc.; 5-523523) were used to isolate the plasmid according to the manufacturer's instructions. This procedure involved lysing the pellet in 20ml of an ice-cold solution containing 50mM glucose, 25mM Tris-Cl, 10mM EDTA, pH8.0 and 5mg/ml lysozyme (Sigma; L6876) at room temperature for 10-15min. 40ml of a 0.2N NaOH and 1.0% SDS solution was gently mixed with the lysate until the suspension became clear and then incubated on ice for 10min. 30ml of 3M K-Acetate, pH5.2 was added to the above and mixed gently until a flocculent precipitate appeared at which stage the mixture was stored on ice for at least 10min. The sample was centrifuged at 35,000g. for 1h at 4°C after which the supernatant was recovered and added to 0.6 volume of 100% Isopropanol, mixed gently and left at room temperature for 20-30min. The suspension was then centrifuged at 35,000g. for 30min at 20°C after which the supernatant was discarded and the pellet washed in ice-cold 70% ethanol and resuspended in 5ml of TE, pH8.0. To remove any contaminating RNA the plasmid solution was treated with RNase Plus (5 Prime → 3 Prime Inc.; 5-461036) (to a final dilution of 1:250) for 30min at 37°C followed by phenol:chloroform:isoamyl alcohol extraction. 10M ammonium acetate was added to the aqueous phase to a final concentration of 2.0M and 0.6 volume of 100%

Isopropanol was added to the sample, mixed and stored at room temperature for 20-30min. The sample was centrifuged at maximum speed in an epifuge and the DNA pellet was washed in 70% ethanol and resuspended in 3.6ml of 10mM Tris-HCl, 1mM EDTA, and 1.0M NaCl, pH8.0. 1.8ml of this sample was loaded into one of two pZ523 columns (following the manufacturer's instructions) and the column effluent was precipitated with 0.6 volume 100% Isopropanol, as described previously. The DNA was pelleted at maximum speed in an epifuge, washed in 70% ethanol and resuspended in TE. The DNA concentration was determined by measuring the $OD_{260nm}$.

### 2.20.2 Lipofectin Transfection of attached mammalian cells

On the day prior to transfections, cells to be transfected were plated from a single cell suspension and seeded into 25cm$^2$ flasks at 3x10$^5$ cells per flask. On the day of the transfection, the plasmids to be transfected were prepared along with the lipid transfection reagents according to the manufacturers protocols (Lipofectin - GibcoBRL ; 18292-011). The cells were transfected for four hours in the absence of serum after which the media was supplemented with 10% serum overnight. The following morning flasks were washed with serum-containing medium and re-fed. Selection began 12-24 hours after re-feeding. For all transfections the cells were incubated at 37$^0$C.

### 2.20.3 Selection of Transfected cells

After transfection, cells that had taken up the plasmid were selected by feeding the cells with media containing geneticin (Sigma; G9516) - the plasmids used had a geneticin-resistant marker, therefore, only those cells containing the plasmid will survive treatment with geneticin. 2 days after transfection the flask of cells was fed with 200μg/ml geneticin in complete media. The concentration of geneticin was increased step-wise evey 2 days to a final concentration of 800μg/ml. Untransfected control flasks died off after 4-5 days. From transfected cells, frozen stocks were made and cells were prepared for immunocytochemical (Section 2.8) and western blot (Section 2.9) analysis.

Section 3.0

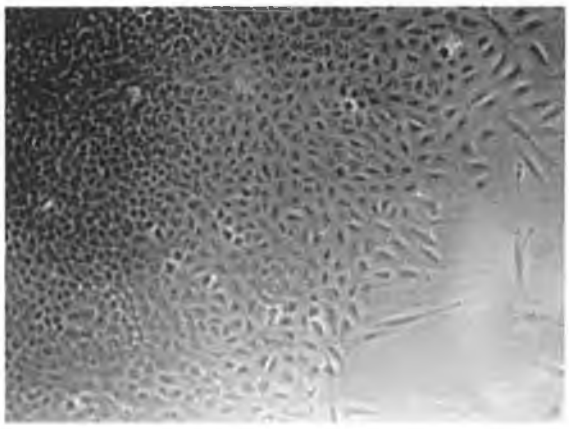
# *Results*

## Section 3.1 Bromodeoxyuridine and Keratin Expression:

### 3.1.1 Morphological changes in BrdU-treated cells:

Figures 3.1.1.1 and 3.1.1.2 show the morphological changes induced in BrdU-treated A549 and DLKP. Both cell types exhibit distinct changes in morphology, with BrdU-treated cells appearing flattened and significantly larger than untreated cells, attributable to the induction of attachment factors and changes in cytoskeletal protein expression.

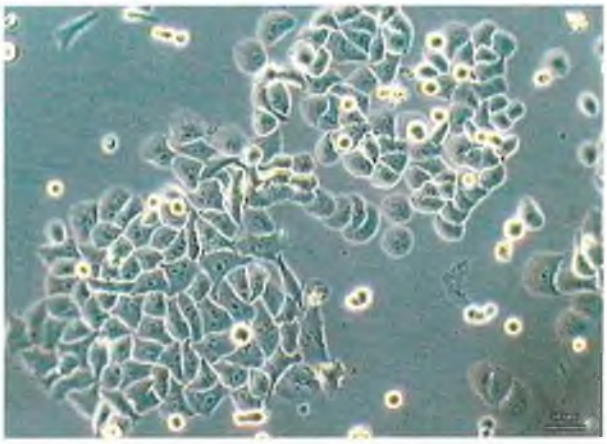
To determine whether or not BrdU induces a terminal differentiation in these cells 7 day-old treatments of DLKP were washed in fresh medium to remove residual BrdU and re-fed in the absence of BrdU. Figure 3.1.1.3 illustrates the gradual reversal of the morphology adopted by BrdU-treated DLKP in the absence of continued exposure to BrdU. By days 10-14 of removal of BrdU cells retain a degree of morphological change characteristic of differentiated cells, maintaining an increased cell flattening and surface area. At this stage it was noted that the growth rates and medium consumption remained below that of untreated cells but progressively increased. By day 30, cells had reverted to a morphology more characteristic of untreated cells. Continued treatment of cells with BrdU results in maintenance of the differentiated phenotype (figure 3.1.1.3) and very slow cell division and growth rates (flasks only required passaging every 5 weeks or more) over a period of four months. These results suggest that BrdU is not killing or selecting for responsive cells.



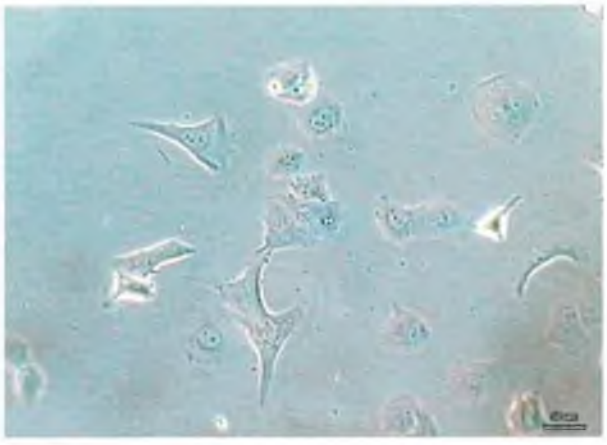
(a)

(b)

**Figure 3.1.1.1 Morphological changes in A549 upon exposure to BrdU.**
(a) Untreated A549, (b) 10μM BrdU-treated A549, grown for 7 days. Magnification x20.



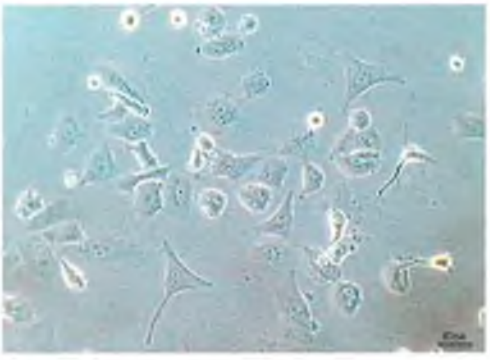
(a)



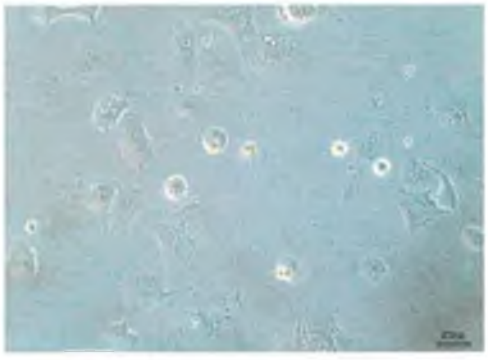
(b)

**Figure 3.1.1.2 Morphological changes in DLKP upon exposure to BrdU.**
(a) Untreated DLKP, (b) 10μM BrdU-treated DLKP, grown for 7 days. Image Analysis revealed, on average, a 2-fold increase in diameter of cells upon treatment with BrdU. Size Bar (bottom left) = 50 μm. Magnification x20.

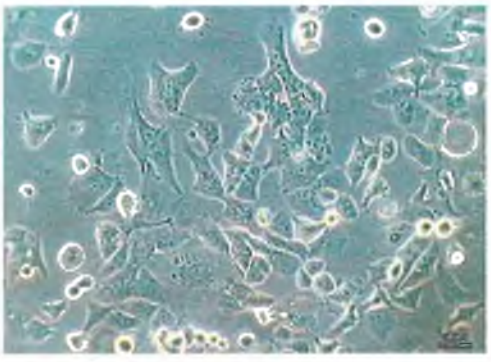
**A.1** 7 (0) Day



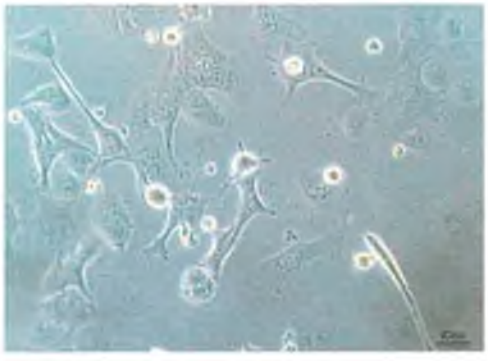
**B.1** 7 (0) Day



**A.2** 17 (10) Day

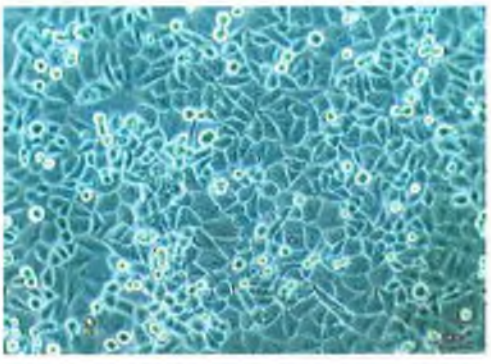


**B.2** 17 (10) Day



**A.3** 37 (30) Day

**Continued Exposure**



**B.3** 37 (30) Day

**Discontinued Exposure**

**Figure 3.1.1.3: Removal of BrdU from differentiated DLKP cells.** Cells were treated for 7 days with 10μM BrdU. Subsequently cells were re-fed with medium lacking BrdU. Days are numbered to include the original 7-day BrdU exposure (numbers in brackets represent days after removal of stimulation with BrdU). Magnification x20. **(A)** Continued exposure to BrdU maintains and even enhances the differentiation of DLKP, as assessed by morphology (A.1 - A.3). **(B)** Removal of BrdU results in a gradual reversion of DLKP to a morphology more characteristic of the parental population (B.1 - B.3).

### 3.1.2 Growth Profiles of BrdU-treated Cells

Figure 3.1.2 illustrates the growth inhibitory effects of 10μM BrdU on the epithelial cell lines studied. Both A549 and DLKP exhibit a strong inhibition of growth upon exposure to BrdU.

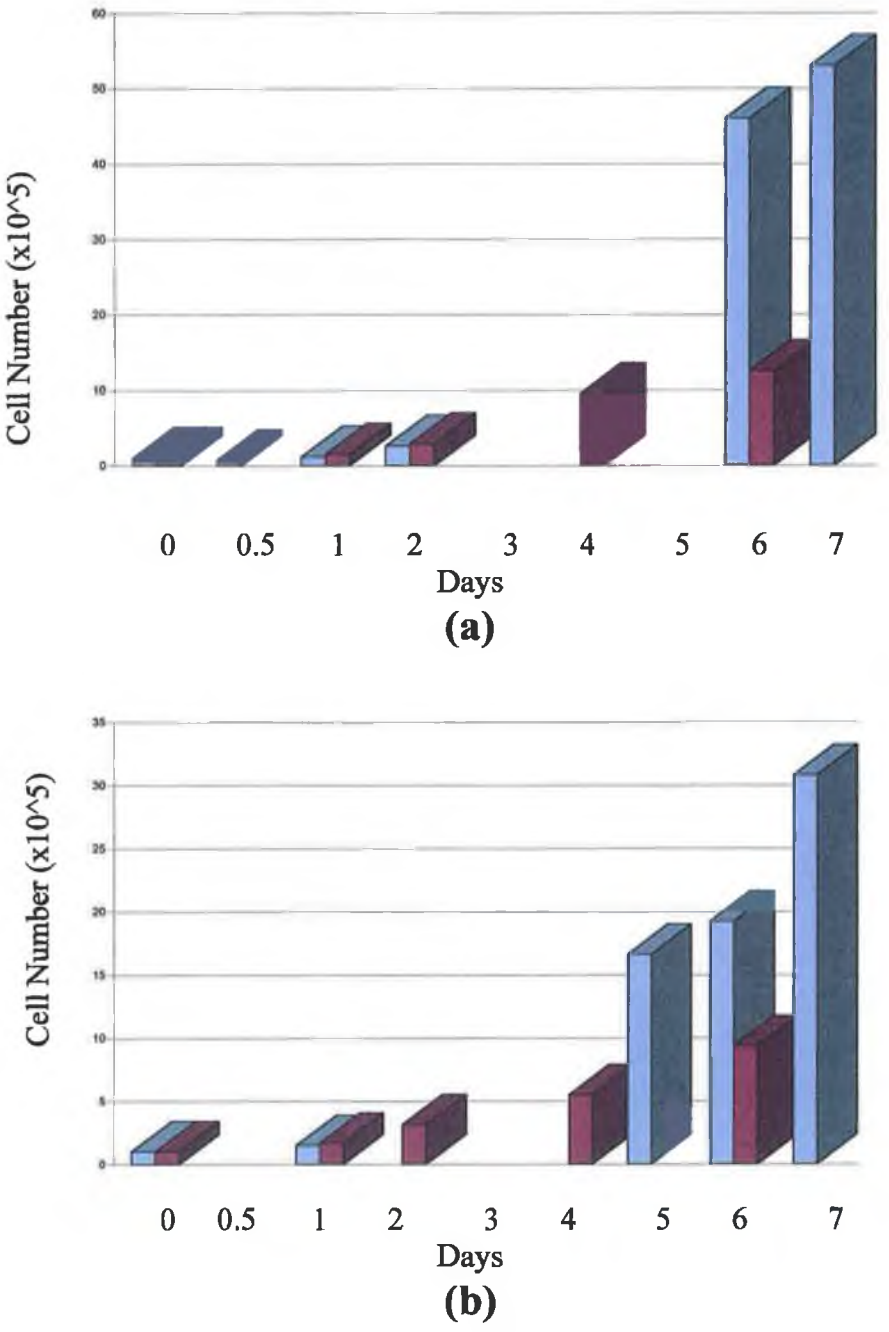



**Figure 3.1.2. Growth Inhibition observed in BrdU-treated (a) A549 and (b) DLKP.** Untreated control cells are in blue (left). BrdU-treated cells are in red (right).

### 3.1.3 Simple Keratin Expression in Differentiating Epithelia

Keratins are markers of epithelial differentiation (Section 1.2). BrdU-treated and untreated cells were stained with monoclonal antibodies to simple keratins, K8 and K18, as described in section 2.8, in order to investigate changes in keratin expression in differentiating epithelial lung cancer cells.

#### 3.1.3.1 Immunocytochemistry for Simple Keratin Expression in A549

Immunocytochemical analysis showed A549 expressed moderately high levels of keratin prior to treatment. However, the levels of expression of partner keratins K8 and K18 were found to increase upon exposure to 10μM BrdU. Keratin filaments are markers of epithelial differentiation and can be seen to radiate from the nucleus through the cytoplasm. The results are illustrated in figure 3.1.3.1. Again, the morphology of BrdU-treated cells can be seen to be quite distinct from that of the untreated control cells.

#### 3.1.3.2 Immunocytochemistry for Simple Keratin Expression in DLKP

Keratin expression was not detectable by immunocytochemistry in DLKP. DLKP is a very poorly differentiated NSCLC-NE/SCLC-V line (McBride et al, 1998), and as such do not express any keratin filaments as determined by immunocytochemistry. Treatment of these cells with 10μM BrdU results in the induction of K8 and K18 in about 10-15% of cells. Representative photographs are shown in figure 3.1.3.2. The induction in these cells is quite strong. Again, cytoplasmic filaments can be seen in these cells radiating through the cytoplasm from the nucleus.

#### 3.1.3.3 Keratin expression after prolonged exposure to BrdU (Immunocytochemistry)

Prolonged exposure to 10μM BrdU resulted in increased morphological changes in both A549 and DLKP, accompanied by increased staining in keratin positive cells. Representative photographs are shown in figures 3.1.3.3.1 and 3.1.3.2, in which cells

were exposed to BrdU for 21 days. Weak keratin filament formation was detectable in approximately 70% DLKP after exposure to BrdU for 3 months, while again a small percentage of cells exhibited very strong keratin filament staining (figure 3.1.3.3.3). Removal of BrdU and re-feeding with fresh medium in the absence of BrdU resulted in a "reversal" of the morphology of BrdU-treated DLKP (Section 3.1.1). However, keratin expression was maintained in these cells, as shown by immunocytochemistry (figure 3.1.3.3.4). These results suggest that BrdU is an irreversible maturational inducer of the epithelial lung cancer cell line, DLKP, in agreement with findings reported by Feyles et al (1991), using a small cell lung cancer cell line, NCI-H69.

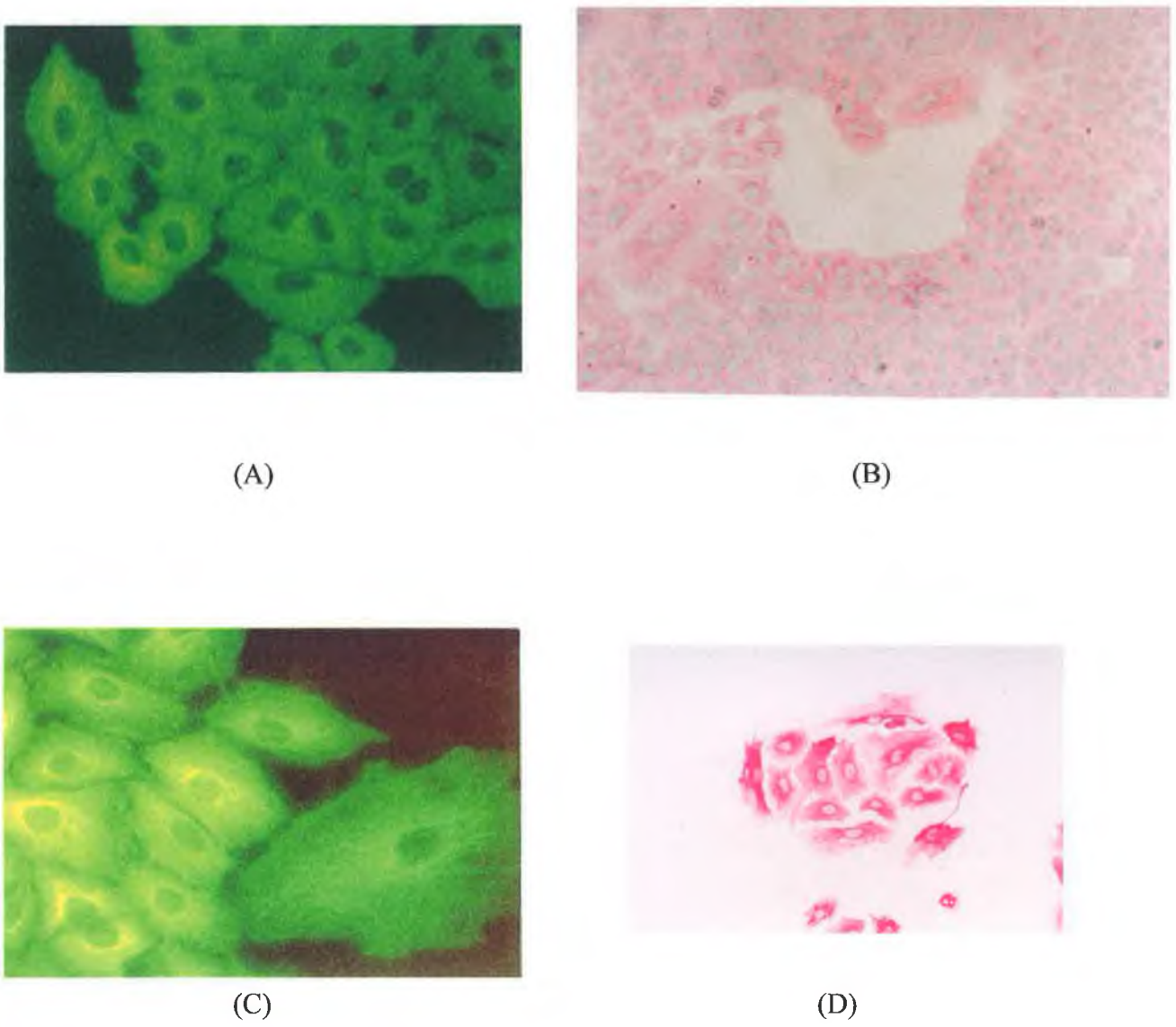



**Figure 3.1.3.1 Immunofluorescence/Immunocytochemistry for K8 and K18 expression in BrdU-treated A549.**
(A) 7-day Untreated A549 stained for cytokeratin 8. (B) Cytokeratin 18 stains in the same manner. (C) 7-day BrdU-treated A549 stained for cytokeratin 8. (D) 7-day BdU-treated A549 stained for cytokeratin 18. Immunofluorescence (A&C) (Section 2.8.3) was photographed at x40. Immunocytochemistry (B&D) (Section 2.8.2) was photographed at x10.

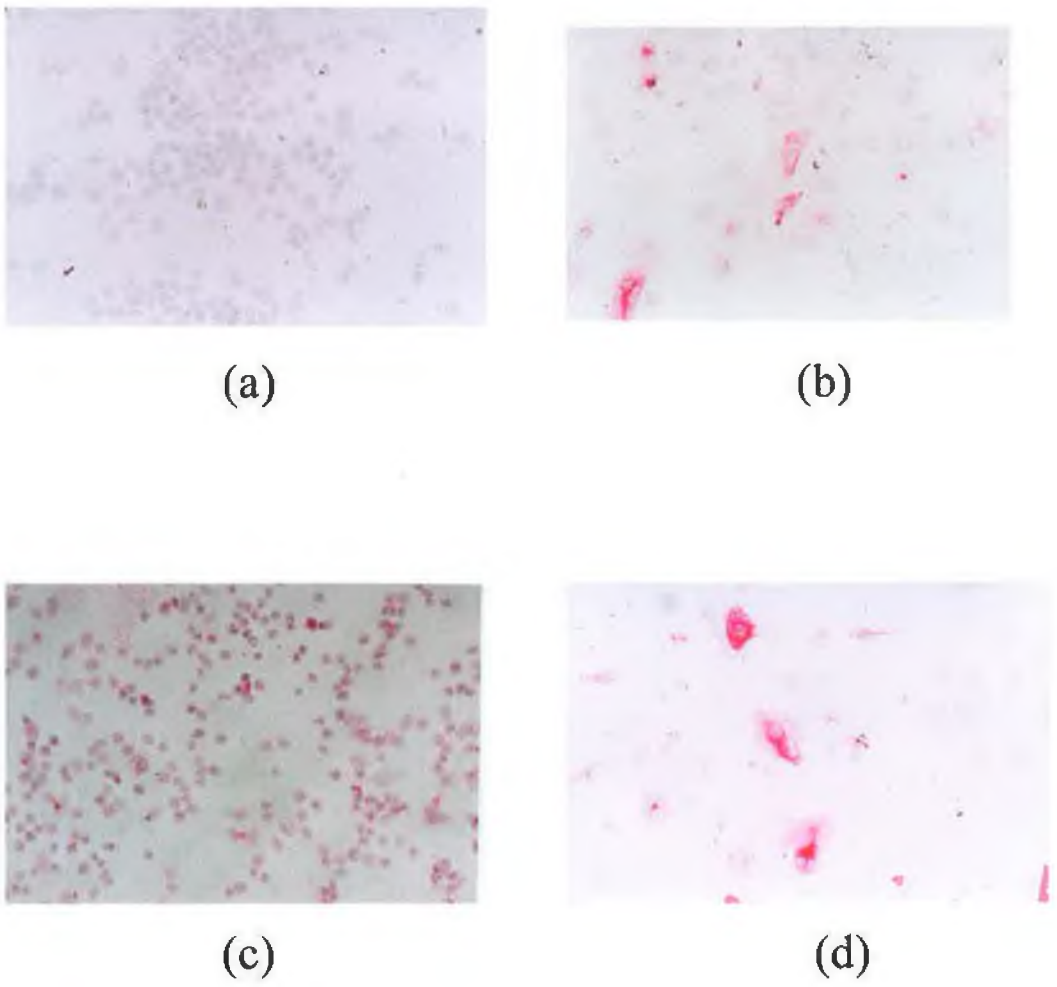
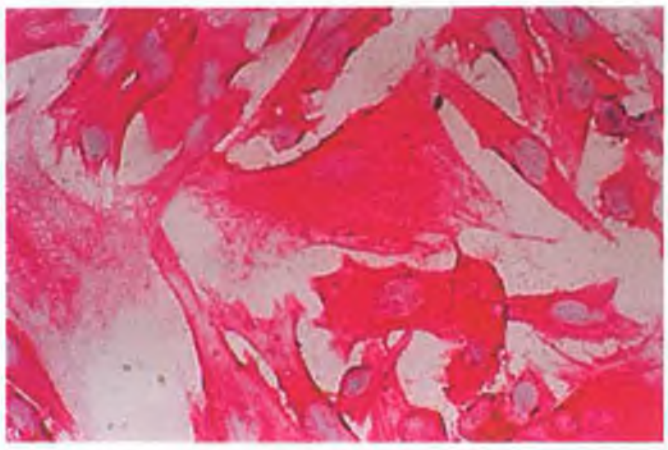
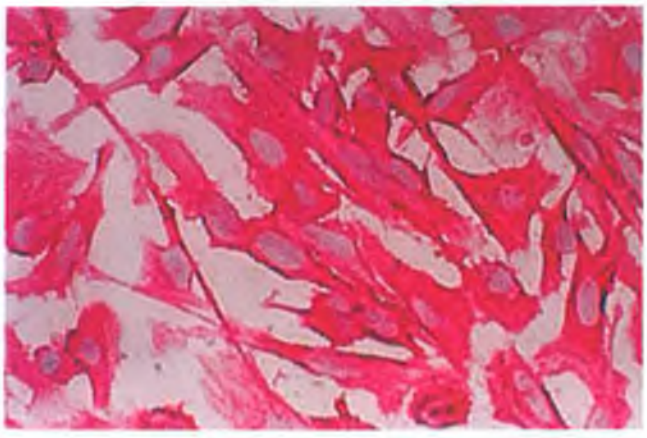



**Figure 3.1.3.2 Immunocytochemistry for K8 and K18 Expression in BrdU-treated DLKP.** (a) Untreated DLKP control cells stained with anti-K8 antibody. (b) 7-day BrdU-treated DLKP cells stained with the same K8 antibody as for (a). (c) Untreated DLKP control cells stained with anti-K18 antibody. (d) 7-day BrdU-treated DLKP cells stained with the same K18 antibody as for (c). Magnification x20.

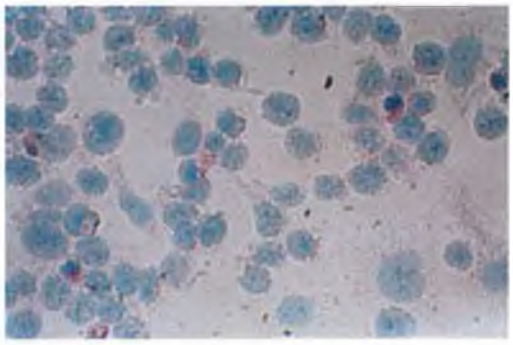


(a)

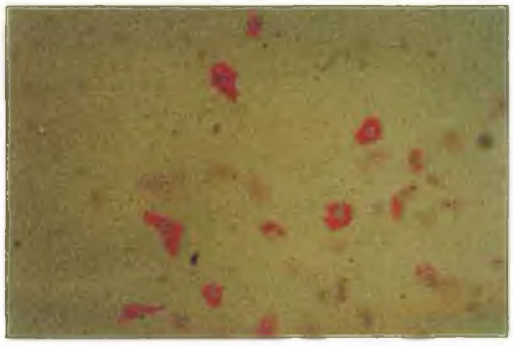


(b)

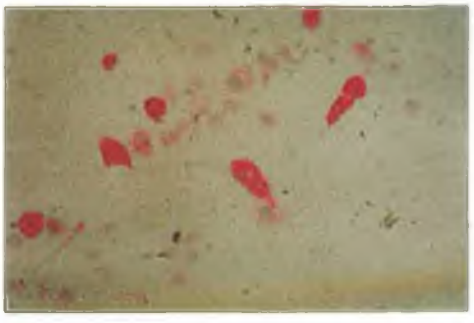
**Figure 3.1.3.3.1 The appearance of 21-day BrdU-treated A549 stained with anti-keratin antibodies.** (a) Keratin 8. (b) Keratin 18. Magnification x40.



(a)

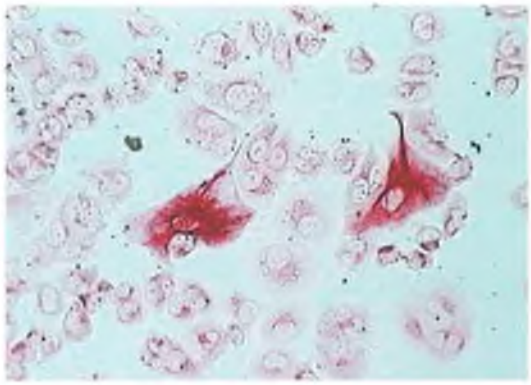


(b)

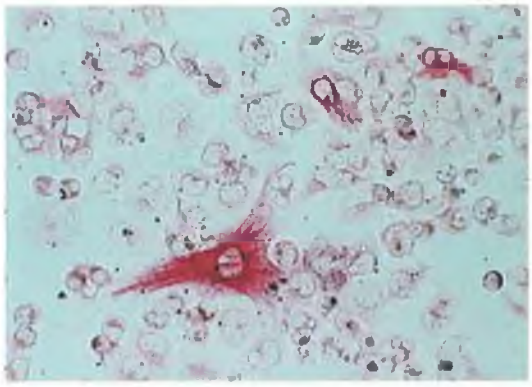


(c)

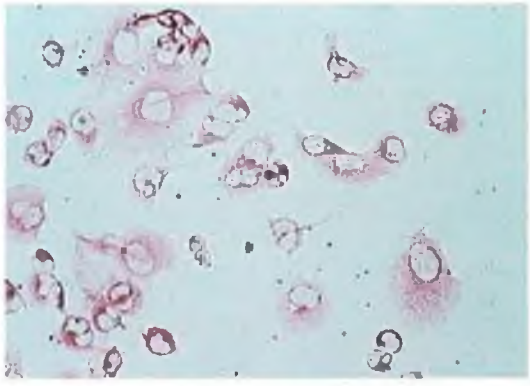
**Figure 3.1.3.3.2 The appearance of 21-day BrdU-treated DLKP stained with anti-keratin Antibodies.** (a) Untreated control cells are negative for Keratin 8 (and K18; not shown). Magnification x40. (b) 21-day BrdU-treated DLKP stained with anti-K8 antibody and (c) 21-day BrdU-treated DLKP stained with anti-K18 antibody. (b) and (c) Magnification x20.



(A)

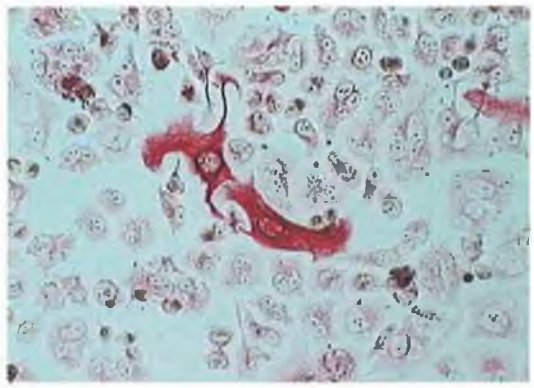


(B)

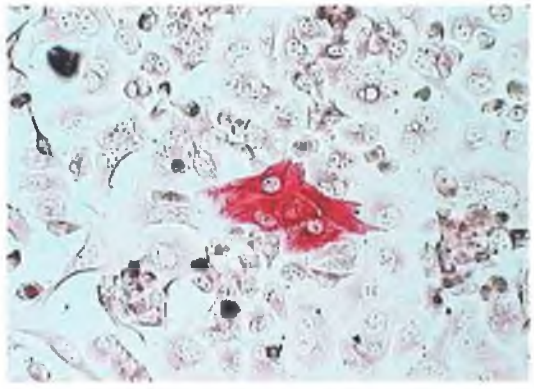


(C)

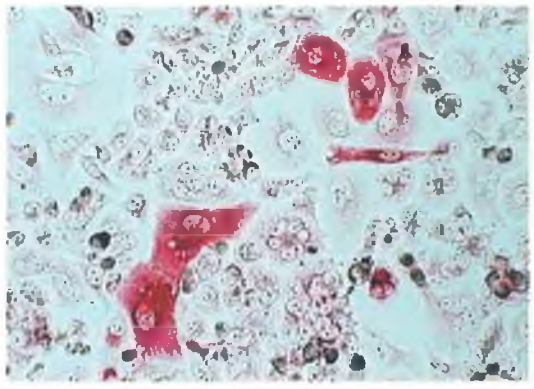
**Figure 3.1.3.3.3 Keratin Expression in BrdU-treated DLKP extended over 3 months.** (A) Keratin 8. (B) Keratin 18. (C) A large percentage of cells now stain weakly for keratin filaments (representative photograph is of K8 expression). Again, untreated parental DLKP were keratin negative (not shown). Magnification x20.



(A)



(B)



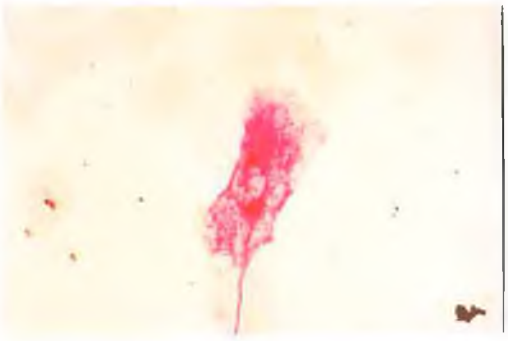
(C)

**Figure 3.1.3.3.4 Discontinued BrdU treatments of DLKP.** Cells were treated for 1 week with BrdU, washed in fresh medium and then cultured for 3 months in the absence of BrdU. These cells remain keratin positive. (A) Keratin 8. (B) Keratin 8. (C) Keratin 18. Again, untreated parental DLKP remain keratin negative (not shown). Magnification x40.

### 3.1.3.4 Keratin Expression in two DLKP clones

Two of the three clones identified in the clonal population that comprises DLKP were examined for keratin induction upon exposure to 10μM BrdU. This was done to ensure that a single clone from the parental population comprising DLKP was not responsible for the keratin induction observed upon exposure to BrdU. Keratin filaments from both K8 and K18 subtypes were shown to be inducible in both DLKP-SQ (Squamous) and DLKP-I (Intermediate) clones. Representative photographs are shown in figure 3.1.3.4.

(a)



(b)

**Figure 3.1.3.4 Induction of K8 expression in two of the clones identified in DLKP.** (a) BrdU-treated DLKP-SQ, stained with anti-K8 antibodies. (b) BrdU-treated DLKP-I, stained with anti-K8 antibodies. Untreated DLKP-SQ, DLKP-I and parental DLKP all remain keratin-negative (not shown). Magnification x20.

### 3.1.4. Western Blot Analysis for Keratin Expression

In order to confirm and quantify the changes in keratin expression observed by immunocytochemical analysis upon exposure to 10μM BrdU, western blot analysis was performed as described in section 2.9. All lanes are numbered in terms of the days of exposure to BrdU.

#### 3.1.4.1 Keratin expression in A549

Both K8 and K18 were shown to be upregulated in BrdU-treated A549. Moderately high levels of both keratins were detectable in untreated controls, with an increase in expression occurring within 24 hrs of exposure to BrdU. Elevated levels of both keratins were detectable up to 3 weeks after initial exposure to BrdU. This is illustrated in figure 3.1.4.1. K8 appears as a doublet due to major and minor forms of the protein detected by this antibody. Expression reaches a maximum after only 3-4 days, beyond which expression of both keratins remains elevated but constant.

#### 3.1.4.2 Keratin Expression in DLKP; Development of immunoprecipitation

Due to the fact that expression of keratins 8 and 18 is induced in only about 10-15% of cells (by immunocytochemistry) from a keratin negative cell line, initial attempts to detect K8 an K18 expression in these cells failed. It was decided to develop immunoprecipitation in the hope of detecting keratin expression in these cells. At first a simple RIPA-based precipitation procedure was used. However, persistent problems with this technique, primarily antibody decomposition to products of 30 and 50 kDa masking the 45-50 kDa keratin filaments of interest, led to the development of an alternative immunoprecipitation technique. Biotinylation of cellular proteins (Boehringer) allowed the specific immunoprecipitation of proteins of interest (in this case keratins) using monoclonal antibodies, followed by their detection by western blot analysis using an anti-biotin antibody (Section 2.10). Problems of antibody breakdown were overcome by the removal of reducing compounds such as DTT and mercapto-ethanol from all lysis, precipitation and loading buffers. Low levels of keratin expression were compensated for by increasing the incubation time for both

the precipitating antibody and protein-G beads from the recommended 1hr to an overnight incubation at $4^0$C.

Figure 3.1.4.2.1(a) shows the detection of keratin proteins in A549 using immunoprecipitation. Lanes 1 and 2 are controls containing antibody but no keratin protein, illustrating the elimination of the problem of antibody breakdown (IgG band is detectable only at 180 kDa). Keratin detection is shown in lanes 3, 6 and 8. Sonicated A549 cells were used to develop the technique to eliminate the possibility that any failure to detect keratin expression using this procedure might be due to the absence of keratin in samples, since A549 are already keratin positive. If BrdU-treated DLKP were used, it could not be 100% sure that any failure to detect keratin expression was not simply due to the failure of the cells to take up the drug or a problem with the drug itself, resulting in a failure to induce keratin expression in DLKP in the first place, i.e. no fault of the immunoprecipitation procedure at all !!!

Figure 3.1.4.2.1(b) shows an attempt made to use immunoprecipitation to quantify changes in keratin 8 expression in A549 cells upon exposure to Retinoic Acid. While the decrease in expression on day 1 is detected (Section 3.7.3.1), true increases at later time-points are not detectable. Immunoprecipitation is designed for accurate detection and quantification of low level expression, as in the case of BrdU-treated DLKP. However, A549 already express significant levels of keratin protein and as such, increases in keratin expression are unreliably detected using this technique due to saturation of the system with keratin protein.

It was then decided to test the immunoprecipitation procedure on a gradient of keratin protein established by diluting cell lysates from A549. This was used to confirm the ability of the system established to quantitatively detect changes in keratin protein levels within samples. The detection of a protein gradient is shown in figure 3.1.4.2.1(c).

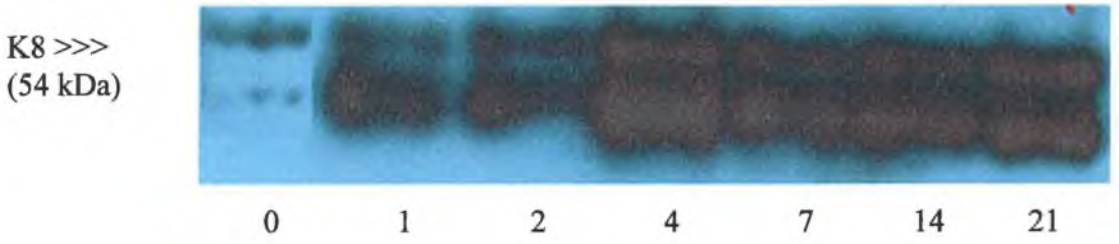
### 3.1.4.3 Keratin Expression in DLKP

Once the reliability of the immunoprecipitation procedure described in section 3.1.4.2. was established, keratin expression in BrdU-treated DLKP could be reliably quantified. Immunoprecipitation of keratin proteins from DLKP was optimised at between $10^6$ and $10^7$ cells, depending on the time period of exposure to BrdU under examination. Earlier time-points than 7-day treatments were not feasible due to prohibitive costs and time consumed in setting up enough flasks to obtain the required number of cells. Figure 3.1.4.3.1 shows the induction of keratin expression in DLKP exposed to 10μM BrdU over a seven-day time-period. $10^7$ cells were immunoprecipitated using K8 and K18 antibodies, followed by western blot analysis and detection using anti-biotin antibodies. Both K8 and K18 are strongly induced in DLKP upon exposure to BrdU. Of interest, a low level of keratin expression is detectable at such high cell numbers. This may represent extremely low level "leaky" expression or degradation products that do not form filaments detectable by immunocytochemistry.

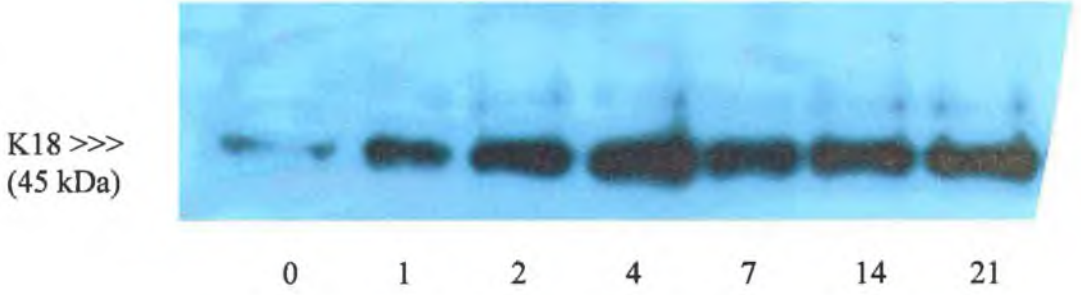
Figure 3.1.4.3.2. shows the increased expression of K8 in DLKP when exposure to 10μM BrdU is increased to 21 days. This is in agreement with the increased intensity with which the 21-day treated cells stain by immunocytochemistry. Due to the length of exposure to BrdU, only $10^6$ cells were immunoprecipitated per sample in this case. Therefore, the increase at day 7 is not visible. The use of $10^7$ cells in figure 3.1.4.3.1 could, therefore, be considered as a magnification or "zoom" on the earlier part of this treatment. Similar results were obtained for the partner keratin, K18 (data not shown).

### 3.1.4.4 Immunoprecipitation of Keratins in HL60 cells

The non-epithelial cell line, HL60, failed to show any keratin expression by immunoprecipitation in either BrdU-treated or untreated cells, suggesting an epithelial-specific induction of keratin expression by BrdU. This is shown in figure 3.1.4.4. $10^7$ cells were used per precipitation.




Keratin 8




Keratin 18

**Figure 3.1.4.1 Western Blot Analysis of Keratin Expression in BrdU-treated A549.** Numbers represent days of exposure to 10μM BrdU.

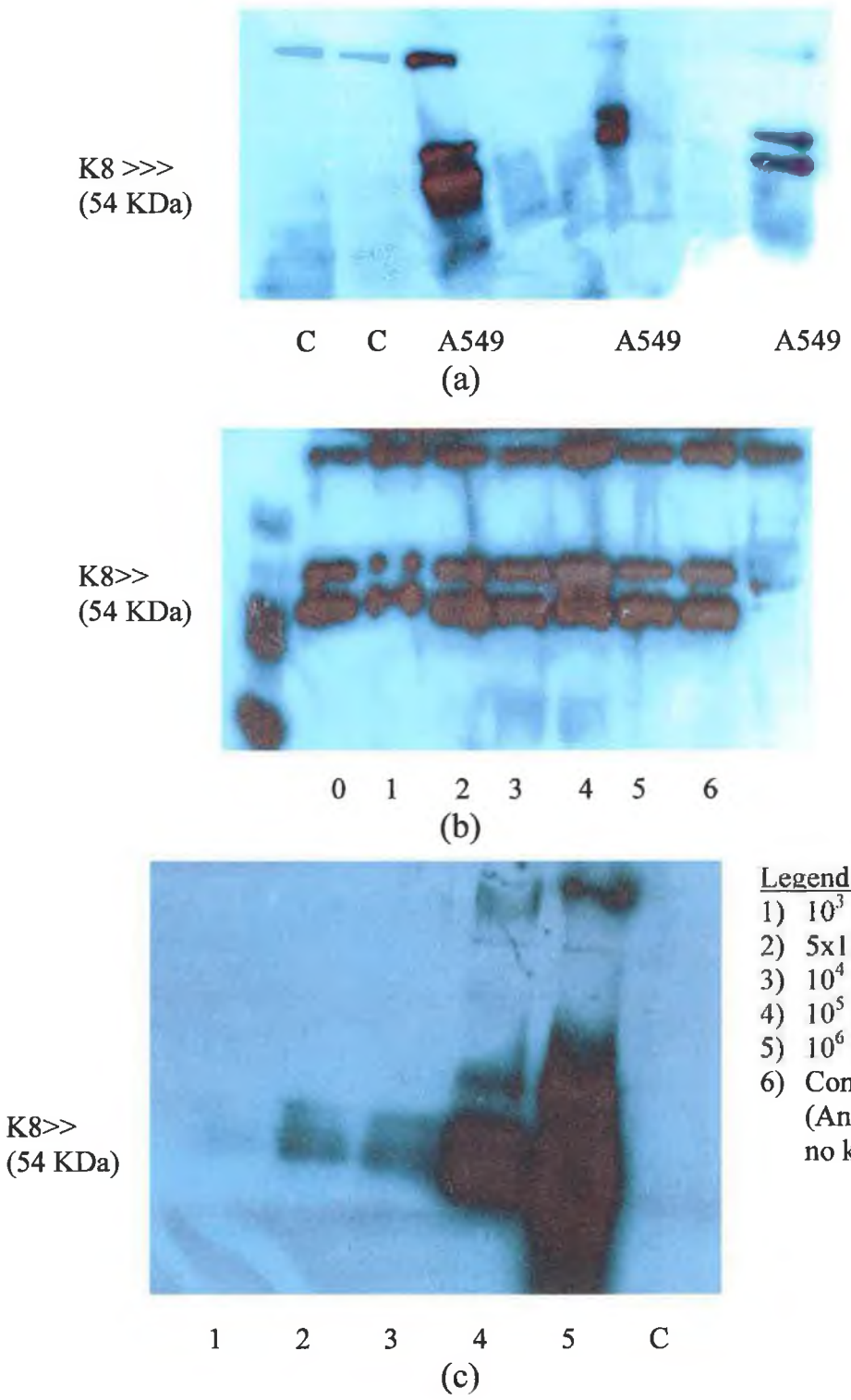
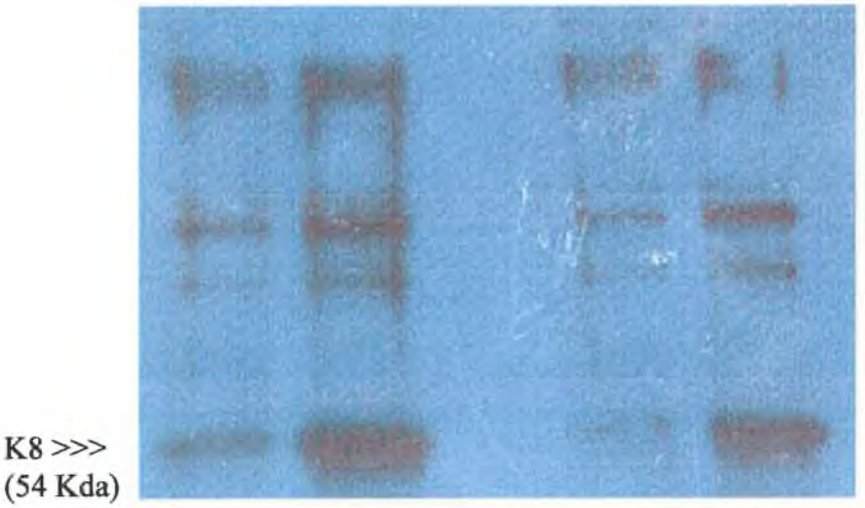
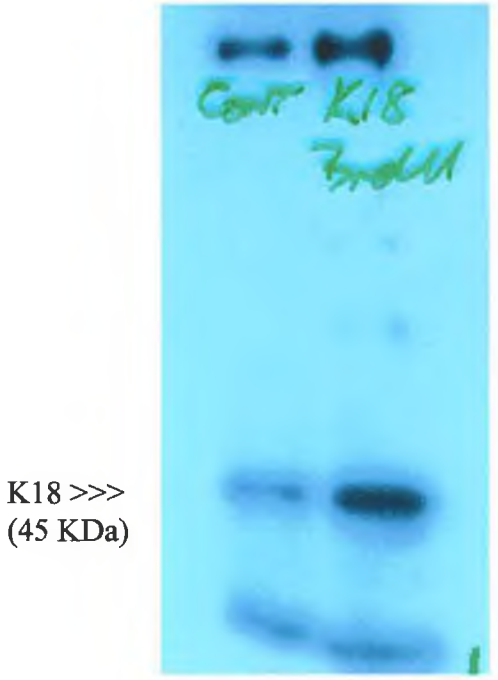



**Figure 3.1.4.2.1 Steps in the Development of a non-radioactive immunoprecipitation procedure to detect keratin protein.** (a) Immunoprecipitation of K8 from A549, a keratin positive cell line (gel leaked, so bands appear skewed). (b) Immunoprecipitation of Retinoic Acid-treated A549 cells. Immunoprecipitation systems become saturated at high antigen levels. Numbers represent days exposed to RA. (c) Immunoprecipitation of a Keratin gradient to test the quantitative capacity of the technique developed.




(a)




(b)

Note: U = Untreated Control Cells T = 7-Day BrdU-treated

**Figure 3.1.4.3.1 Immunoprecipitation of Keratins from BrdU-treated DLKP.** (a) K8 Immunoprecipitation in DLKP ($10^7$ cells used). (b) K18 Immunoprecipitaion in DLKP ($10^7$ cells used).

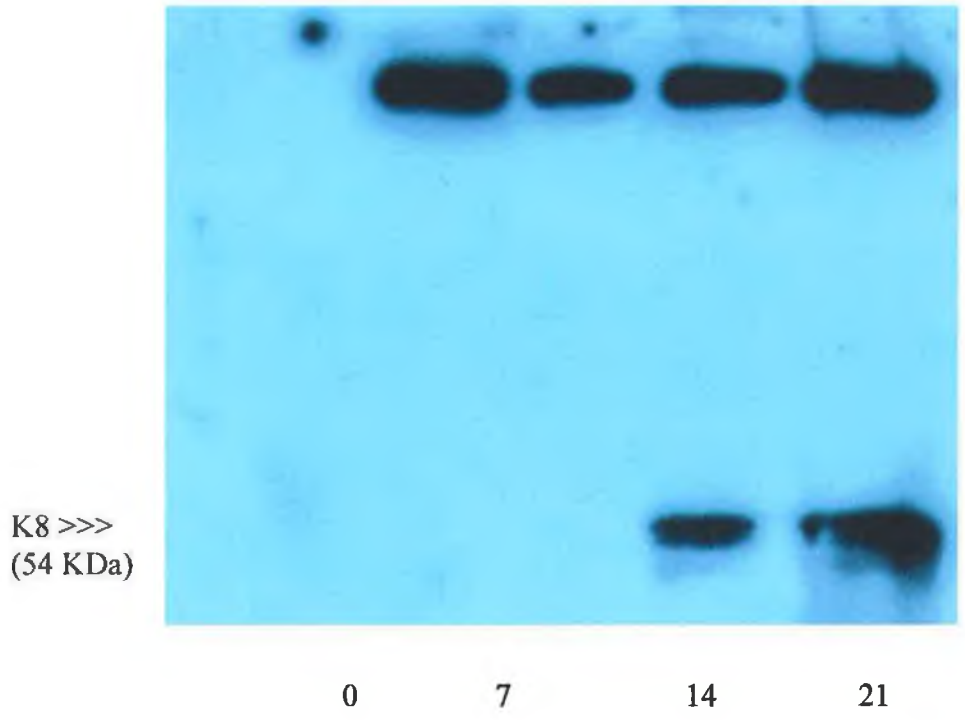



**Figure 3.1.4.3.2 Immunoprecipitation of K8 in BrdU-treated DLKP over 21 days.** Numbers represent days of exposure to 10μM BrdU. $10^6$ cells per precipitation.

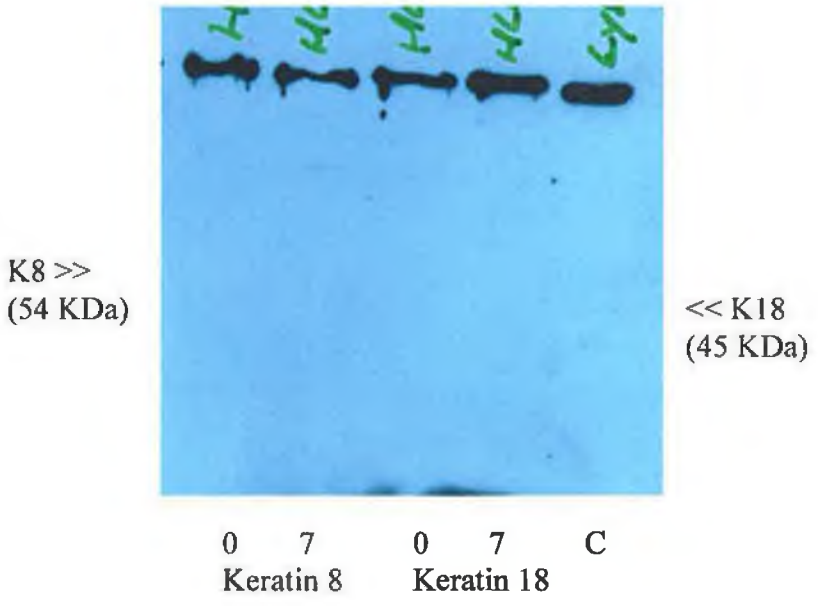



**Figure 3.1.4.4 Immunoprecipitation of K8 and K18 in 7-day BrdU-treated HL60s.** $10^7$ cells per precipitation. C = antibody control in lysis buffer. Numbers represent days of exposure to BrdU.

### 3.1.5 Northern Blot and PCR analysis for K8/18

Northern blot and PCR analysis for the expression of both K8 and K18 was performed by Dr. Shirley McBride and Dr. Noel Daly. Both K8 and K18 transcript levels were unaffected by exposure to 10μM BrdU, suggesting a post-transcriptional regulation of keratin expression in BrdU-treated A549 and DLKP cells. Representative Northern and PCR analysis is shown in figure 3.1.5. GAPDH and Ribosmal RNA levels were used as internal standards to confirm equal loading of RNA in each lane (not shown).

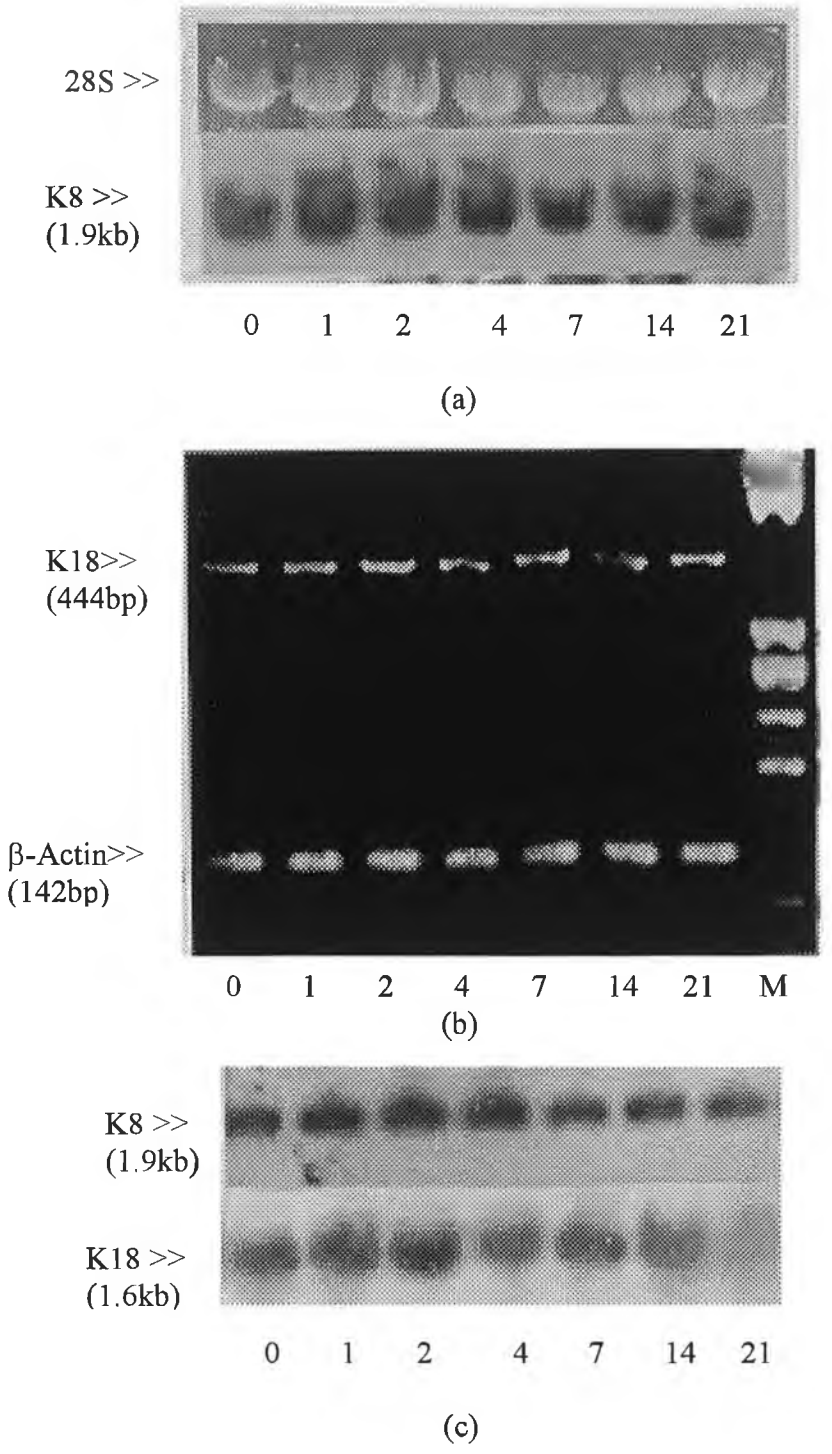



**Figure 3.1.5 Representative Northern Blot and PCR analysis of Keratin expression.** (a) Northern Blot Analysis for Keratin 8 (similar results for K18) (A549). (b) PCR Analysis for Keratin 8 (similar results for K18) (A549). (c) Northern blot analysis for K8 and K18 expression in differentiating DLKP. Analysis performed by Dr. Shirley McBride and Dr. Noel Daly. Numbers represent days of exposure to BrdU.

## Section 3.2 Bromodeoxyuridine and its effects on Translation

### 3.2.1 Immunocytochemistry for eIF-4E:

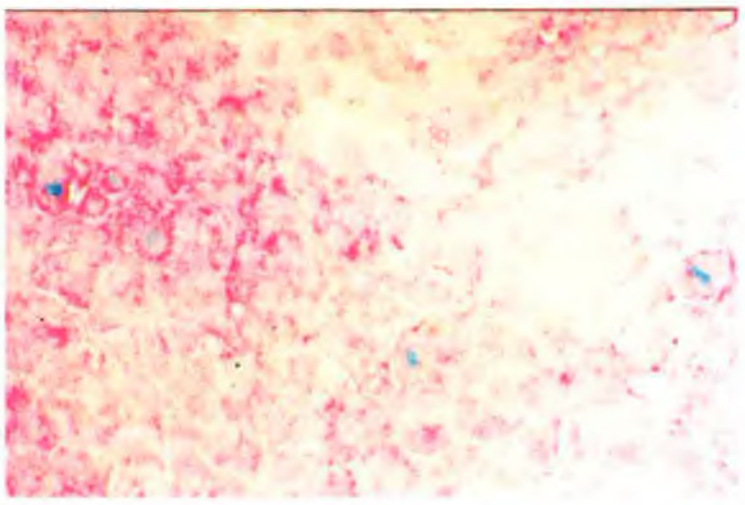
Immunocytochemical analysis (Section 2.8), using monoclonal anti-eIF-4E (Affiniti Research, UK) and polyclonal anti-eIF-4E (Dr. Simon Morley, Sussex, UK) antibodies, demonstrated the cytoplasmic localisation of eIF-4E. This small Cap-binding translation initiation factor is the limiting factor in the regulation of eukaryotic translation initiation (Section 1.6.3.2.4.3).

#### 3.2.1.1 eIF-4E Expression in A549

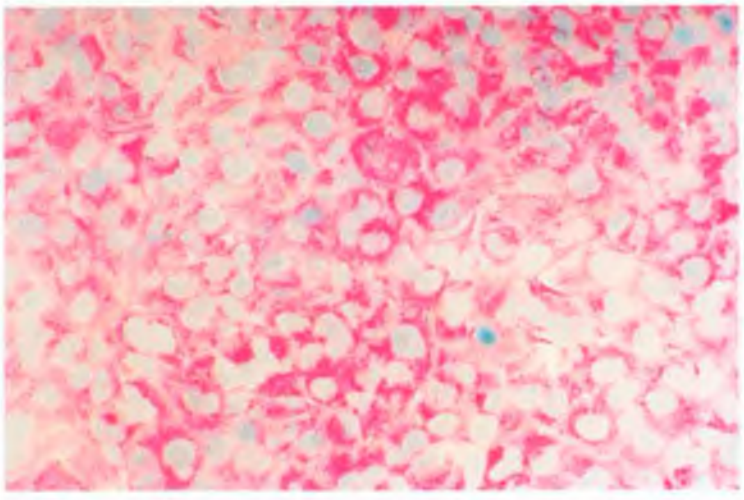
Treatment of A549 with 10μM BrdU results in an increase in eIF-4E expression. The pattern of overexpression was determined by immunocytochemistry, and was shown to be distributed throughout the population evenly. Representative photographs are shown in figure 3.2.1.1.

#### 3.2.1.2 eIF-4E Expression in DLKP

Immunocytochemical analysis of eIF-4E expression in DLKP shows the cytoplasmic expression of this factor in both treated and untreated cells. However, treatment with 10μM BrdU appears to induce a very strong upregulation in eIF-4E expression in a small percentage of cells (figure 3.2.1.2). Due to morphological changes and slight differences in coloration due to film development, it is difficult to determine if there is a universal upregulation in eIF-4E expression, as observed in A549 upon exposure to BrdU. However, it is clear that BrdU induces about 10-15% of DLKP to express very high levels of eIF-4E.

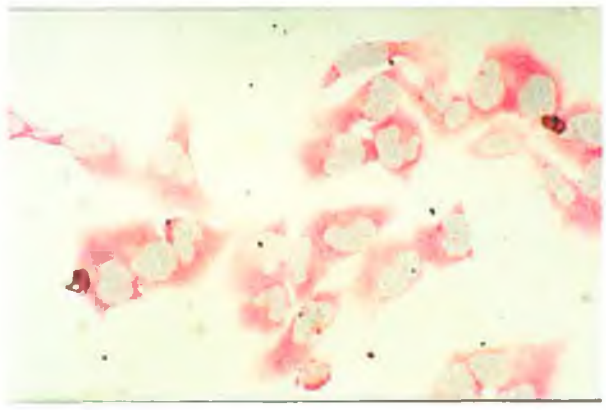


(a)

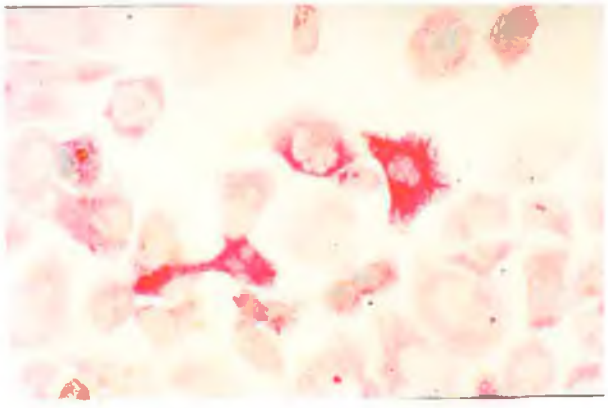


(b)

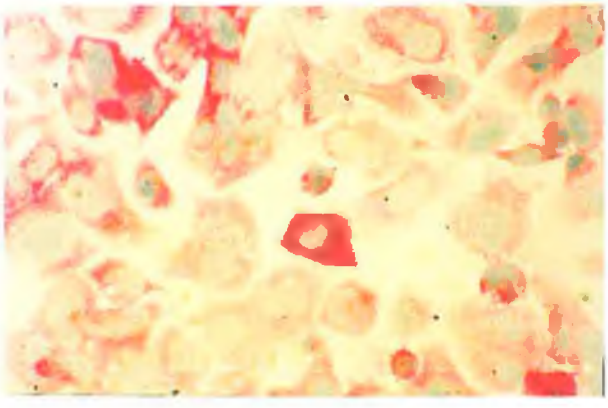
**Figure 3.2.1.1 Immunocytochemistry for eIF-4E expression in BrdU-treated A549.** (a) Untreated Control A549 and (b) BrdU-treated A549 on day 7, stained with monoclonal anti-eIF-4E.



(a)



(b)



(c)

**Figure 3.2.1.2 Immunocytochemistry for eIF-4E Expression in DLKP.**
(a) Untreated Control DLKP, (b) & (c) BrdU-treated DLKP show a percentage of cells that express very high levels of eIF-4E. Cells were stained with monoclonal eIF-4E. Magnification x20.

### 3.2.2 Western Blot Analysis of eIF-4E expression

Overexpression of eIF-4E in BrdU-treated cells was confirmed using western blot analysis. Antibodies used were mouse monoclonal anti-eIF-4E (Affiniti Research) and rabbit polyclonal anti-eIF-4E (kind gift of Dr. Simon Morley).

The BrdU-induced upregulation in eIF-4E expression is very clear in both epithelial lines examined, A549 (figure 3.2.2.(a)) and DLKP (figure 3.2.2.(b)). Of interest, western blot analysis suggested that the expression of eIF-4E appears to be higher in the more poorly differentiated and aggressive DLKP, which is in agreement with later findings by Northern blot analysis (section 3.2.5). The high level of expression of eIF-4E is illustrated in figure 3.2.2(b), while lower exposure illustrates the increased expression in these cells more clearly (figure 3.2.2(c)).

Figure 3.2.2(d) shows the effect of 10μM BrdU on eIF-4E expression in the Leukaemic cell line, HL60. eIF-4E was found to be downregulated upon exposure of HL60 cells to BrdU.

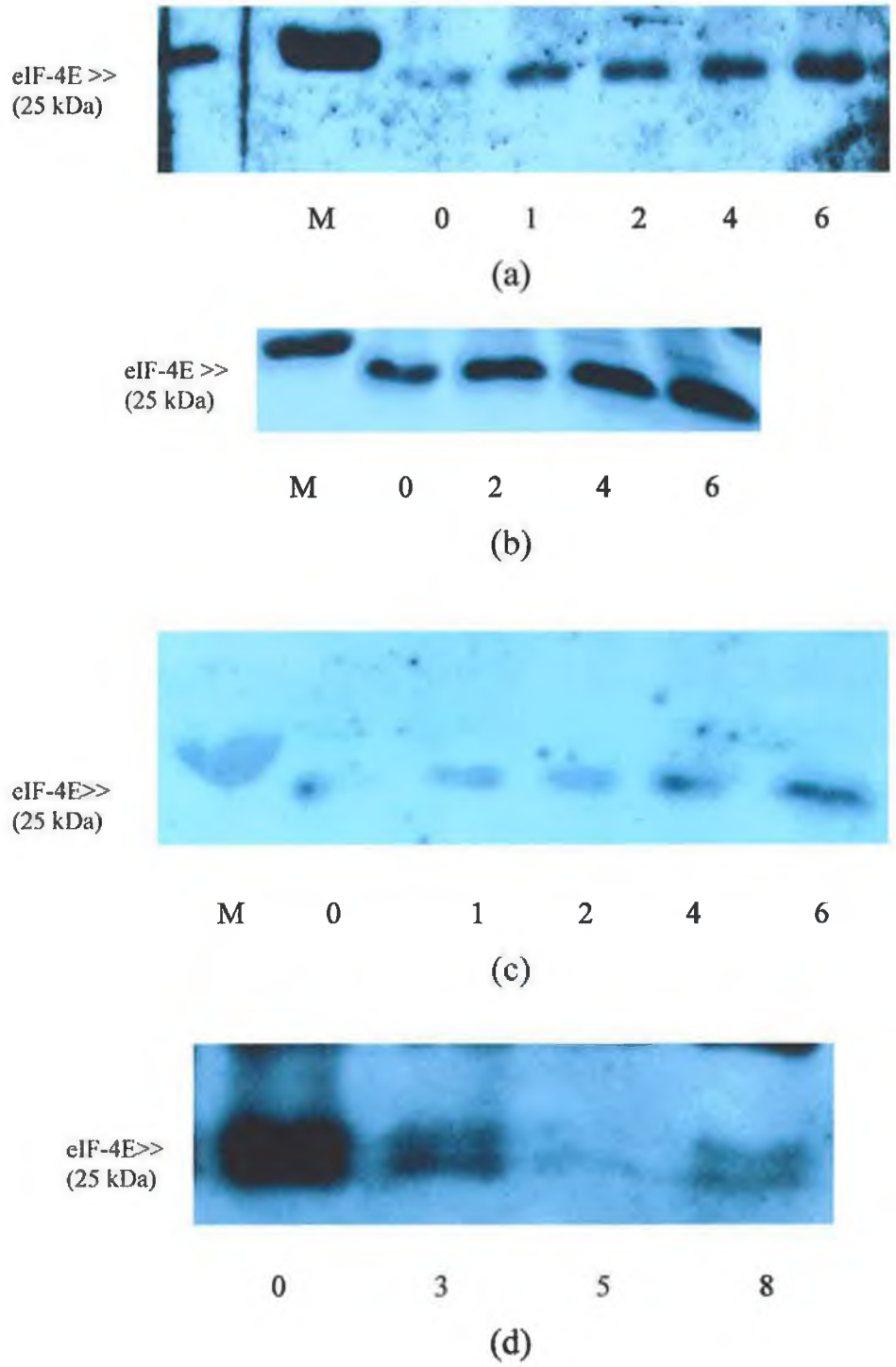



**Figure 3.2.2 Western Blot Analysis of eIF-4E expression in BrdU-treated cells.** (a) BrdU-treated A549, (b) BrdU-treated DLKP, (c) BrdU-treated DLKP (low exposure to reveal increase more clearly), detected with monoclonal eIF-4E. (d) BrdU-treated HL60, detected with polyclonal eIF-4E (a kind gift of Dr. Simon Morley). Numbers represent days of exposure to BrdU. M = 29 kDa marker.

### 3.2.3 PCR analysis of eIF-4E expression in differentiating lung cancer cells

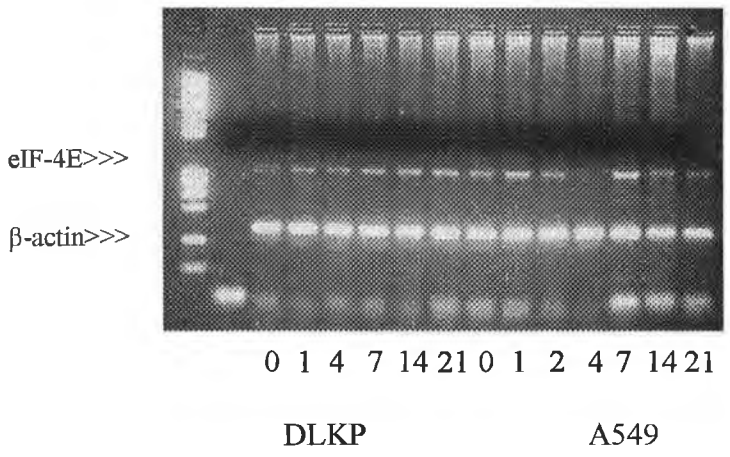
To investigate the level at which eIF-4E is induced in BrdU-treated cells, RT-PCR analysis was performed on total RNA isolated from A549 and DLKP. Initial RT-PCR reactions were performed using RNA that had been isolated almost 20 months previously. eIF-4E did not appear to be significantly upregulated in DLKP (figure 3.2.3(a)) upon treatment with BrdU. Figure 3.2.3(b) shows a repeat of this RT-PCR, using freshly isolated RNA, which suggests a small increase in eIF-4E transcript levels in BrdU-treated DLKP (days 7, 14, and 21 are upregulated when actin levels are accounted for).

Using the freshly isolated RNA, RT-PCR suggested an increase in eIF-4E transcript levels (day 2 appears to be degraded) in BrdU-treated A549 (figure 3.2.3(b)). The initial PCR reactions, using the older RNA, show clear increases on days 1 and 7, while increases on days 14 and 21 are evident when actin levels are accounted for (figure 3.2.3(a)). Results with A549 suggest that there may be minor increases in eIF-4E transcript levels, undetectable in DLKP.

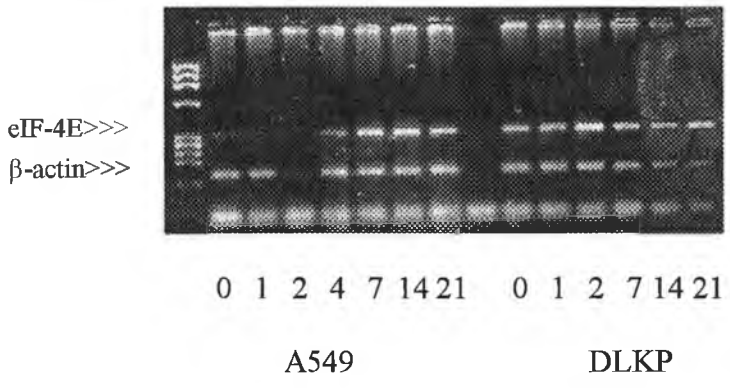
### 3.2.4 Northern Blot analysis of eIF-4E expression

Northern blot analysis for eIF-4E expression in BrdU-treated A549 and DLKP is shown in figure 3.2.4.1-3. Even loading of samples was visually assessed by the levels of ribosomal RNA in lanes. Overall, Northern blot analysis suggests that there is little or no increase in transcript levels for eIF-4E in BrdU-treated epithelial cell lines, DLKP and A549. Ribosmal RNA levels were used as internal standards to confirm equal loading of RNA in all lanes (not shown).

Taking all of the mRNA analysis into account, it appears that, if there is any increase in mRNA levels for eIF-4E following BrdU treatment, it is relatively small.

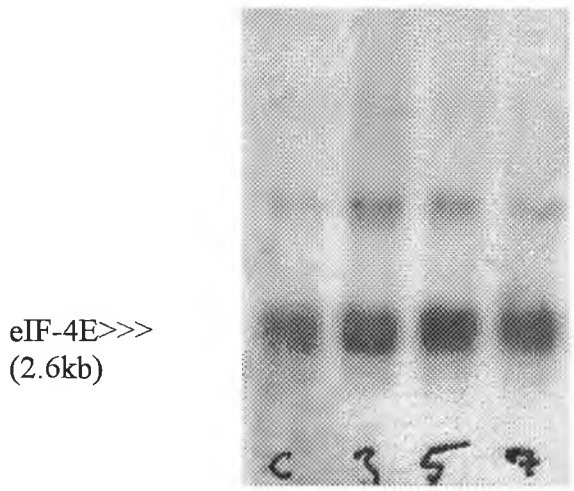



(a)

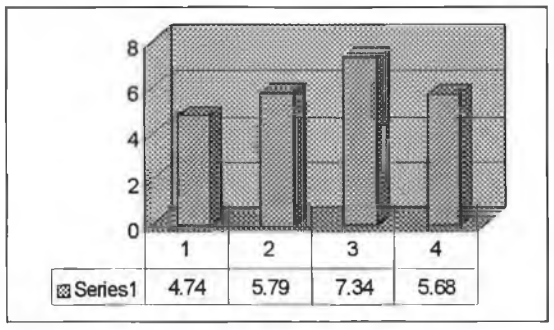



(b)

**Figure 3.2.3 PCR Analysis of eIF-4E expression inBrdU-treated cells.** Numbers represent days of exposure to BrdU. (a) PCR performed on 18-month-old RNA. (b) PCR performed on RNA isolated by Dr. Paula Meleady. Numbers represent days of exposure to BrdU. Primers were designed by Dr. Noel Daly. eIF-4E = 333bp. β-actin = 142bp.

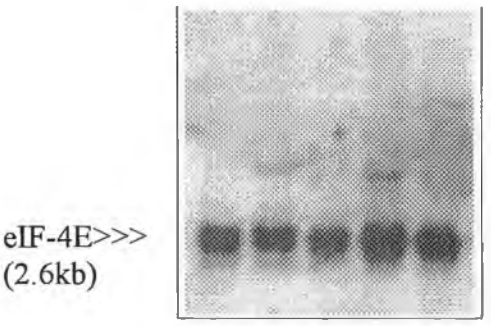



(a)




(b)

**Figure 3.2.4.1 Northern Blot Analysis and Densitometry of eIF-4E expression in BrdU-treated A549.** (a) Northern blot analysis. eIF-4E probes were made by Dr. Noel Daly. (b) Graphical representation of Densitometry readings. Numbers represent days of exposure to BrdU.




(a)

*= Poorly Loaded
(loss of sample)

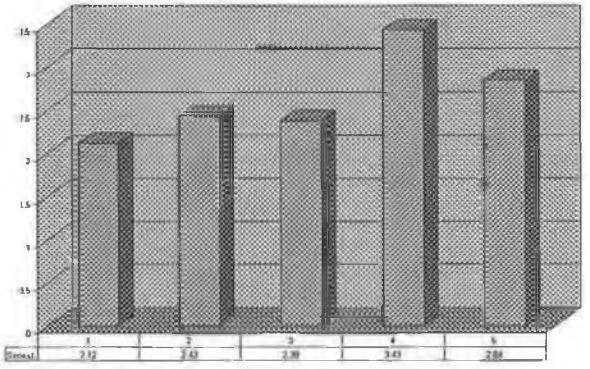
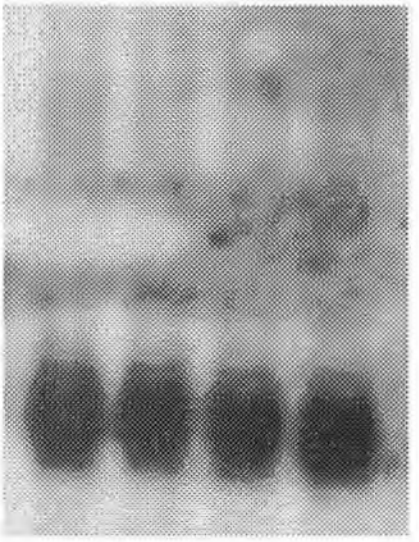
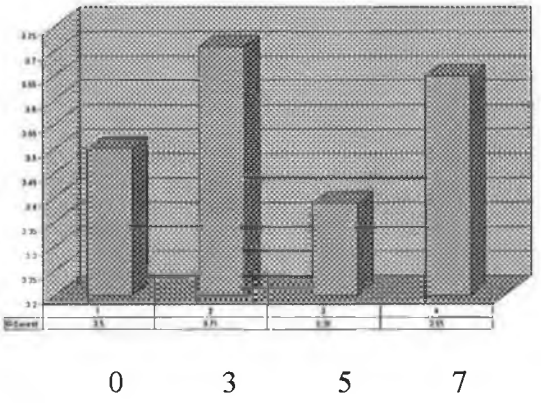



**Figure 3.2.4.2 Northern Blot and Densitometry of eIF-4E expression in BrdU-treated A549.** (a) Northern Blot analysis. eIF-4E probe was made by Dr. Noel Daly. (b) Graphical representation of Densitometry readings. It must be noted that the sample for day3 BrdU (lane 3) was poorly loaded due to loss of sample during loading.




(a)




(b)

**Figure 3.2.4.3 Northern Blot Analyis and Densitometry of eIF-4E in BrdU DLKP.** (a) Northern blot analysis. eIF-4E probe was made by Dr. Noel Daly. (b) Graphical representation of Densitometry readings.

### 3.2.5 Iso-electric Focusing (IEF) for eIF-4E

In order to assess the activity of eIF-4E in BrdU-treated and untreated cells vertical-slab Iso-Electric Focusing (IEF) was performed. IEF was developed for these cells with the kind help of Dr. Simon Morley (Sussex, UK). Vertical-slab IEF (Section 2.11) is an adaptation of standard isoelectric focusing techniques, in which samples are run through a low percentage polyacrylamide gel and pH gradient. This serves to separate proteins, not by size like conventional western blot analysis, but by changes in their pI (iso-electric point) due to phosphorylation. At the end of the run, proteins have reached equilibrium within the pH gradient of the gel. While multiple phosphorylation of eIF-4E is thought to occur, two forms predominate and are readily detected by this technique. They are the $Ser^{209}$ phosphorylated, more acidic form (upper band; pH 5.9) and the non-phosphorylated (lower band; pH 6.3) form of eIF-4E (Flynn and Proud, 1995; Sonenberg, 1996). Rabbit Reticulocyte lysate, due to the high content of translation factors and the manner in which it runs as a brown "smear" on IEF gels, is used as a positive control and also to orientate the gels for blotting.

IEF for eIF-4E in both DLKP and A549 is shown in figure 3.2.5. The increase in eIF-4E expression is again evident in both A549 (figure 3.2.5(a)) and DLKP (figure 3.2.5(b)). The upper band is the phosphorylated, more active form of eIF-4E (P-4E), while the lower band is the non-phosphorylated, less active form of the protein (4E). There appears to be a shift in the phosphorylation status of eIF-4E in both A549 and DLKP towards the more phosphorylated and active form of eIF-4E, in addition to an increase in eIF-4E levels. The high levels of eIF-4E in DLKP (mentioned in section 3.2.2) are reflected again in the IEF for eIF-4E (figure 3.2.5(a)).

Interestingly, when eIF-4E phosphorylation was examined in differentiating HL60s the majority of the observed reduction in eIF-4E detected by western blot analysis would appear to be in the non-phosphorylated form of the protein, as determined by IEF (figure 3.2.5(c)). There is a reduction in P-4E, however, illustrating that there is a reduction in active eIF-4E, but this is significantly less dramatic than the reduction in the non-phosphorylated, less active form of eIF-4E.

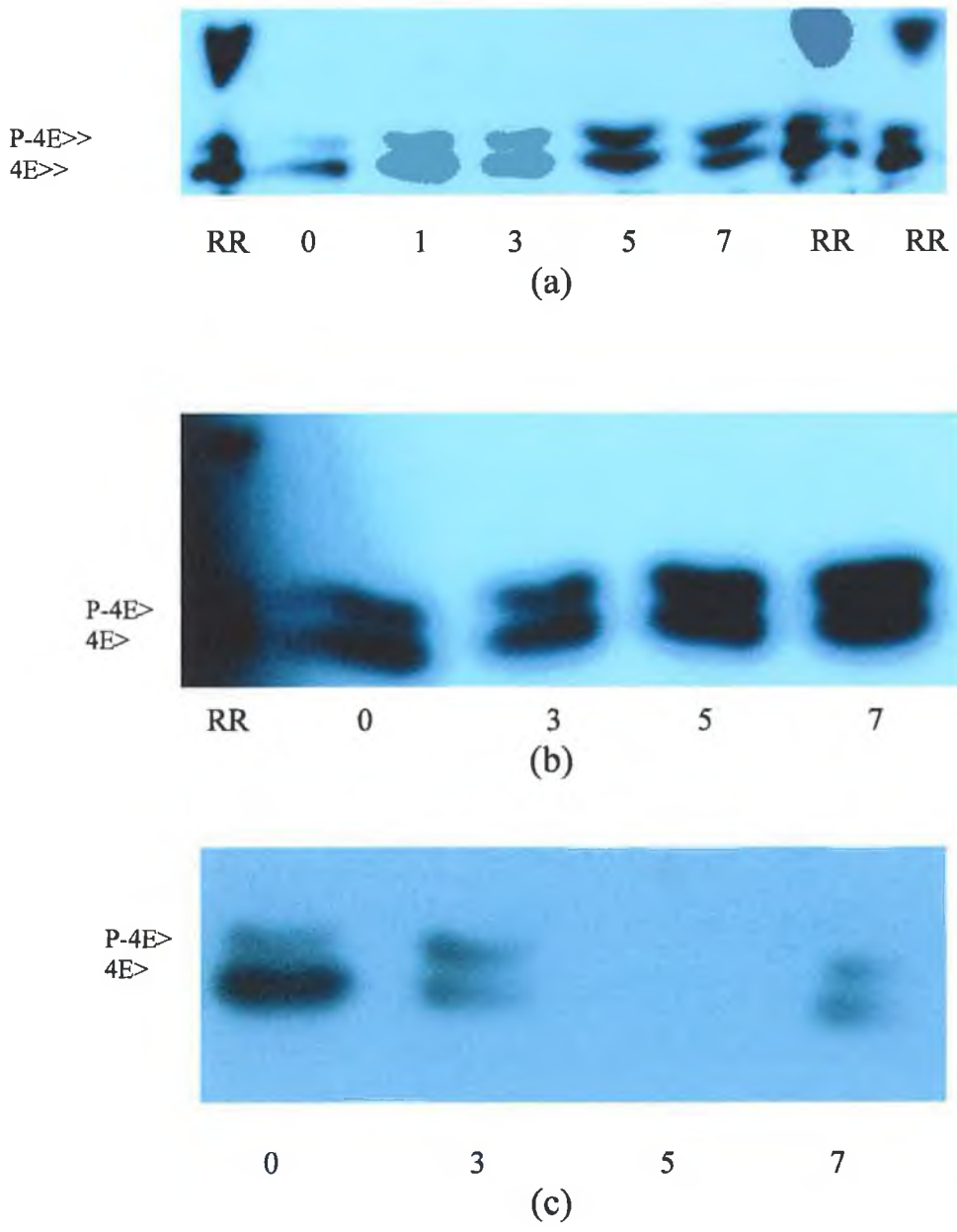



**Figure 3.2.5 Iso-Electric Focusing for eIF-4E in BrdU-treated cells.** (a) BrdU-treated A549, (b) BrdU-treated DLKP, (c) BrdU-treated HL60, detected using monoclonal eIF-4E. Numbers represent days of exposure to 10μM BrdU.
4E = eIF-4E (pI 5.9).
P-4E = Phosphorylated eIF-4E (pI 6.3)

### 3.2.6 ERK Activity in BrdU-treated cells

BrdU appears to affect the expression of numerous genes, including the upregulation of integrin expression (attachment and signalling molecules, important in metastasis and differentiation) (Meleady and Clynes, in preparation). It was decided, therefore, to investigate the possible contribution of ERK (Extra-cellular Signal Regulated Kinase) (Section 1.6.4 & 4.3) to the activity of eIF-4E in differentiating lung cancer epithelia. Figure 3.2.6 shows that there are slight increases in the expression of phosphorylated, active ERK in BrdU-treated A549, which may explain the shift in eIF-4E phosphorylation observed previously (Section 3.2.5). The levels of active ERK in DLKP do not appear to change significantly (figure 3.2.6).

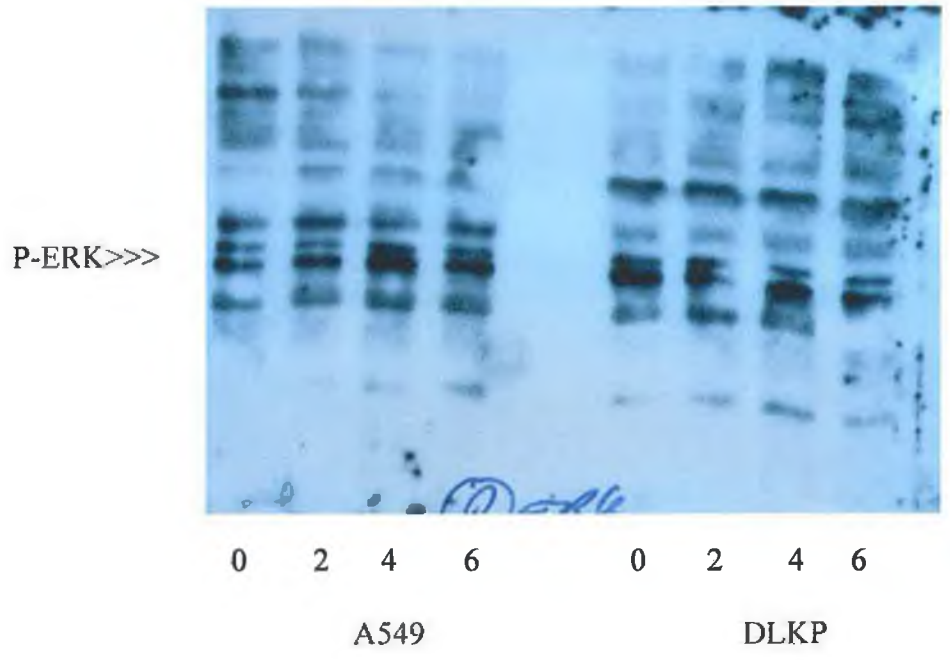



**Figure 3.2.6 Levels of Phosphorylated ERK in BrdU-treated A549 and DLKP.** Levels of phosphorylated (active) ERK were determined using phospho-specific antibodies (a kind gift of Dr. John Lyons). The doublet detected is ERK1 and ERK2, identified by this antibody. Numbers represent days of exposure to BrdU.

### 3.2.7 eIF-4F Complex Formation in Differentiating Lung Cancer cell lines

#### 3.2.7.1 Immunofluorescence for eIF-4G

Immunofluorescence (Section 2.8.3) was performed using a rabbit polyclonal antibody directed against amino acids 920-1396 that will detect both eIF-4GI and eIF-4GII (a kind gift of Dr. Simon Morley). Once optimum conditions for immunofluorescence using this antibody were determined (an overnight incubation at $4^0$C rather than 2 hours at room temp.) it was possible to assess eIF-4G I/II expression in both A549 and DLKP.

The cytoplasmic localisation of eIF-4G is illustrated in figure 3.2.7.1. The images for both treated and untreated A549 are a little unclear, due to a problem with the focus on the fluorescent microscope, but they illustrate the effect sufficiently. Immunocytochemical analysis suggests that no significant changes in eIF-4G expression occur during the differentiation of either cell line.

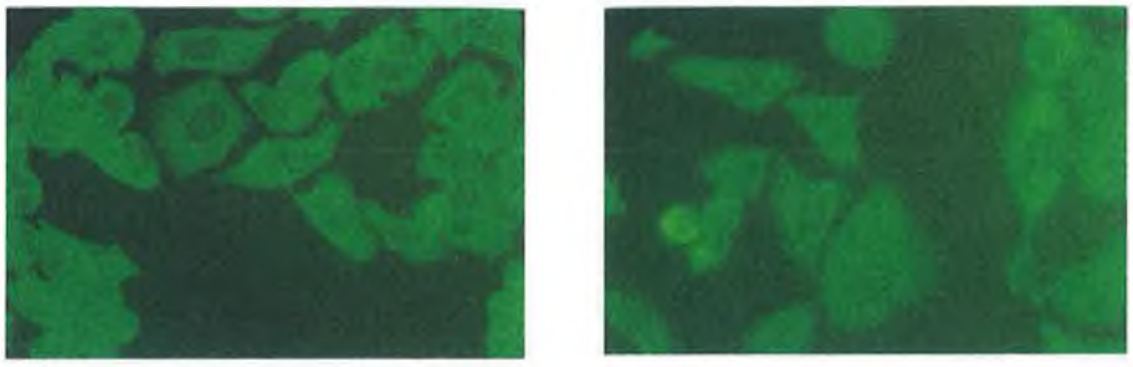
#### 3.2.7.2 Western blot analysis of eIF-4G expression

Western blot analysis on 7.5% gels run for long periods (3 hours at 325-350 V) separates and distinguishes both eIF-4GI and eIF-4GII. Lower bands are frequently detected and are C-terminal modifications of eIF-4G (Dr. Simon Morley, personal correspondence). eIF-4G expression appeared to remain unaltered during the differentiation of DLKP, while there appeared to be a low to moderate increase in A549 (figure 3.2.7.2). Low exposure blots for A549 are shown to illustrate the increase more clearly. Longer exposures show a significantly less dramatic increase in expression. This suggests that there are slight changes in eIF-4G levels beyond the detection capacity of immunofluorescence. These changes, however, are not as significant as those of eIF-4E or other proteins in this study.

### 3.2.7.3 Western blot analysis of 4E-BP1 expression

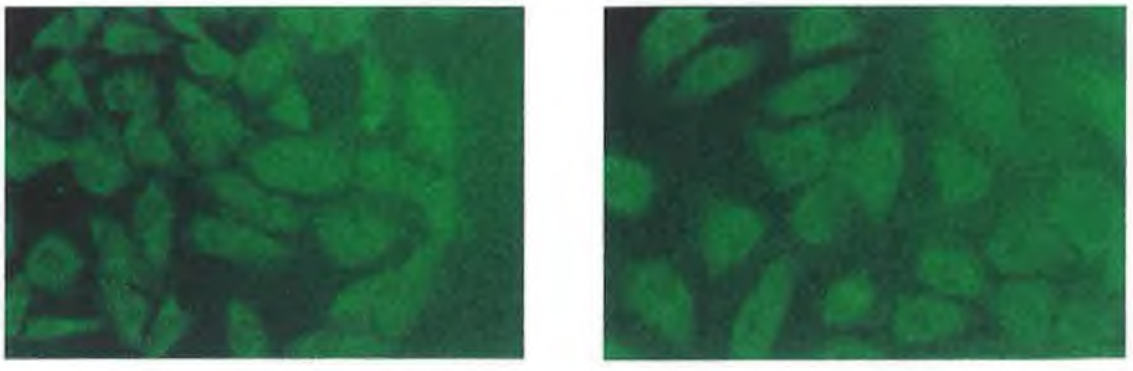
4E-BP1 expression was analysed on 15% polyacrylamide gels using a rabbit polyclonal anti-4E-BP1 antibody, a kind gift of Dr. Nahum Sonenberg and Dr. Anna-Claude Gingras. This antibody is capable of detecting three forms of 4E-BP1 when run on higher resolution gels (15%). These represent the α (hypo-phosphorylated), β (phosphorylated) and γ (hyper-phosphorylated) forms of 4E-BP1. Thus, the relative association of 4E-BPs with eIF-4E can be determined, since only the non-phosphorylated, α-form of the protein is capable of association.

When examined, the levels and ratios of the three forms of 4E-BP1 appeared to remain unchanged. To date, the resolution obtained has been sufficient for these studies, being a little unclear at times. A representative blot for A549 is shown in figure 3.2.7.3. Attempts will be made to improve resolution using larger gel systems that allow larger sample sizes and longer resolution times (Dr. Anna-Claude Gingras, personal correspondence).



(A) (B)

**DLKP**



(C) (D)

**A549**

**Figure 3.2.7.1 Immunofluorescence for eIF-4G expression in BrdU-treated DLKP and A549.** Immunofluorescence using anti-eIF-4G antibody (a kind gift of Dr. Simon Morley) shows no apparent change in eIF-4G levels. (A) DLKP Untreated, (B) DLKP 7-day BrdU. (C) A549 Untreated, (D) A549 7-day BrdU. Magnification x20.

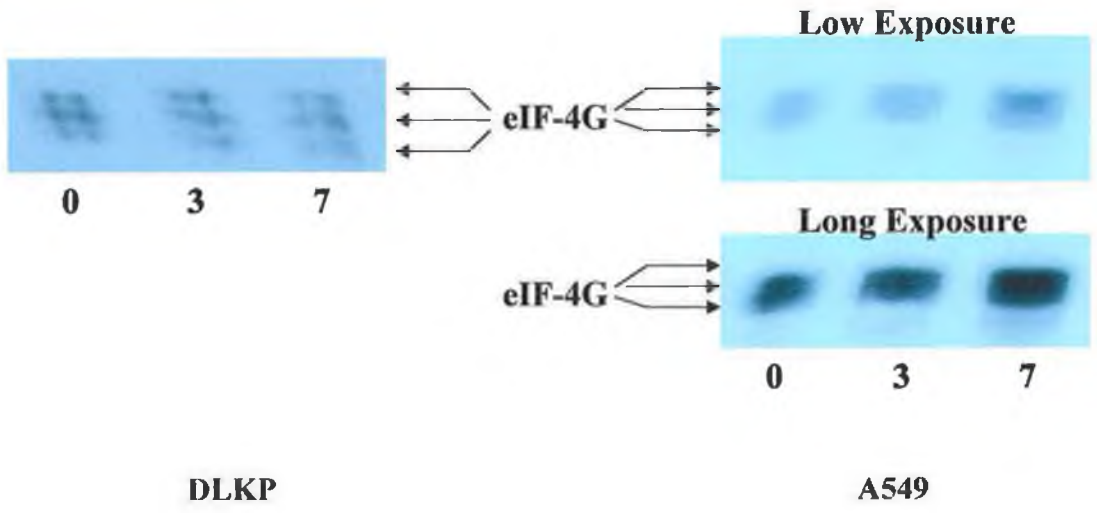



**Figure 3.2.7.2 Western Blot Analysis of eIF-4G expression.** Analysis shows a slight increase in eIF-4G expression in BrdU-treated A549, undetectable by immunofluorescence (Figure 3.2.7.1). Low exposures clearly illustrate slight changes in expression in A549, while longer exposure loses resolution of the isoforms of eIF-4G. No significant changes in eIF-4G expression were detected in DLKP during a 7-day treatment. Numbers represent days of exposure to BrdU.

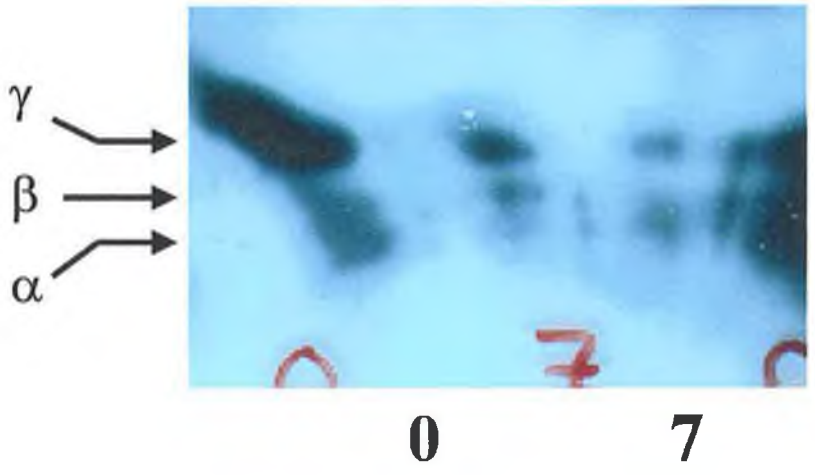



**Figure 3.2.7.3 4E-Binding Protein 1 (4E-BP1) expression in BrdU-treated A549.** 4E-BP1 levels and phosphorylation remain unchanged in untreated (0) and 7-day BrdU treated (7) A549. Techniques are currently being refined to improve resolution.

### 3.2.8 Crude Protein per Cell Readings

Crude calculations of total protein content per cell were made by lysing a known number of cells in a known volume of TG lysis buffer. Knowing the concentration of protein in each sample, from B.C.A. assays for protein content of samples, and the number of cells per sample, it was then possible to roughly estimate the levels of protein per cell. This involved dividing the sample concentration by the cell number (to reduce figures to "per cell") and multiplying by the sample volume (to determine "total protein") gave an estimate of total protein per cell. Averages of results are represented in figure 3.2.8.

There appears to be an increase in total protein in both epithelial lines, reflecting the morphological changes observed in these cells. Similarly, the morphologically smaller, non-adherent HL60 line proved to contain approximately half the level of protein of that of both DLKP and A549. Upon BrdU treatment these cells appear to decrease in size, reflected in the decreased protein content per cell.

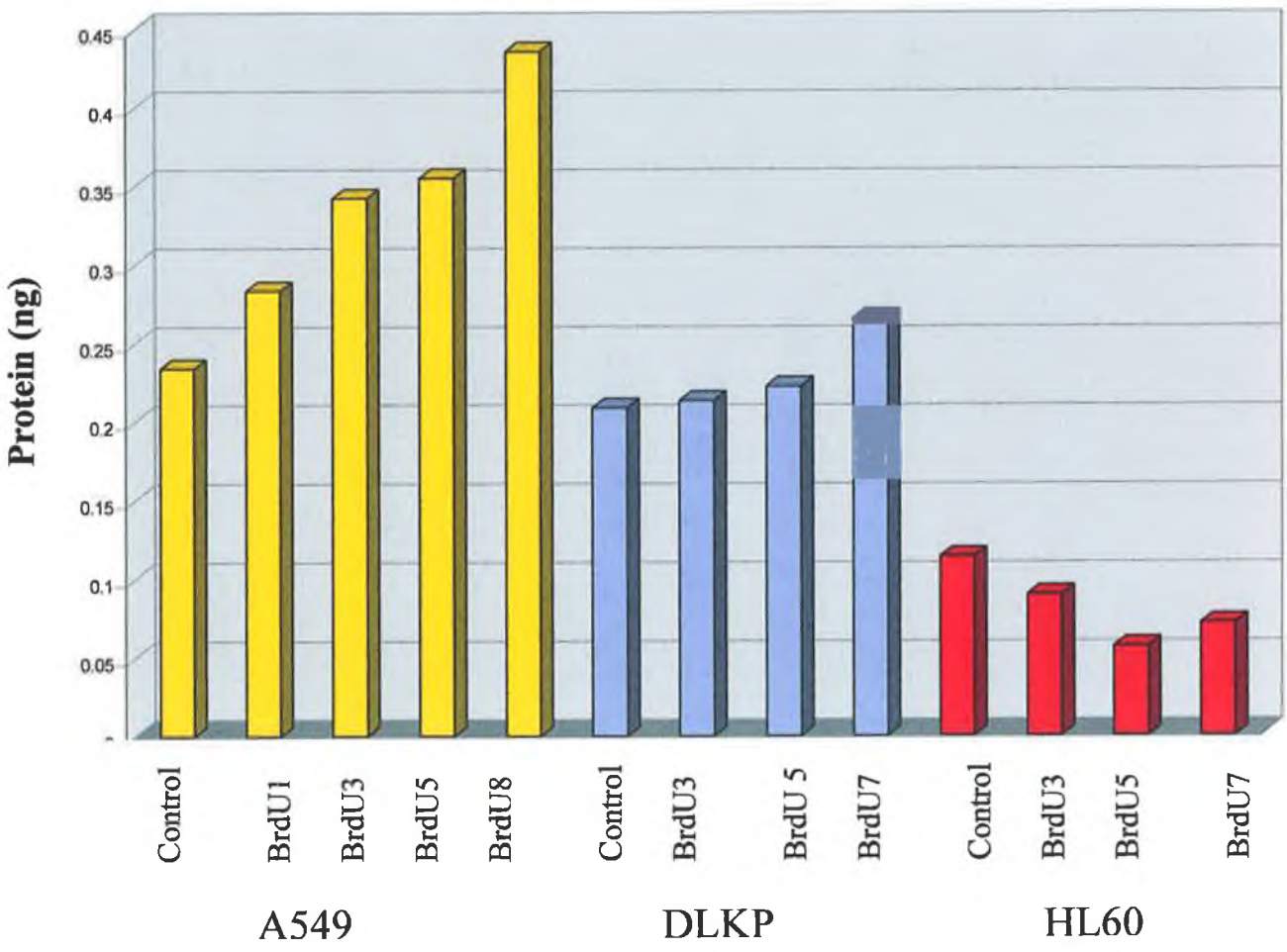



**Figure 3.2.8 Protein content per cell as determined by crude B.C.A. assay.** Increased protein-per-cell is observed in differentiating epithelial cells, A549 and DLKP, while decreased levels are observed in differentiating HL60s.

## Section 3.3 *In-Vitro* Translation

In light of the post-transcriptional induction of keratin expression in DLKP upon exposure to BrdU (Section 3.1.3), and the knowledge that translational control of gene expression is particularly important during differentiation and development, it was decided to investigate the possibility that there may exist a translational repressor of keratin expression in DLKP. If any cell line is to contain such a repressor of keratin translation it would be expected that DLKP would, due to its poorly differentiated state and epithelial origin.

### 3.3.1 Optimisation of In-Vitro Translation (IVT)

There are two main types of *in-vitro* translation system available; Rabbit Reticulocyte Lysate (RR) and Wheat Germ (WG) Extract Systems. Initially both systems were tested in order to assess which of them was best suited to our needs. A trial pack containing both systems was purchased from Promega for this purpose. Total RNA isolated from A549, as described in section 2.14, was used to develop the IVT procedure because high levels of transcript are present in these cells which are known to be translatable *in-vivo*.

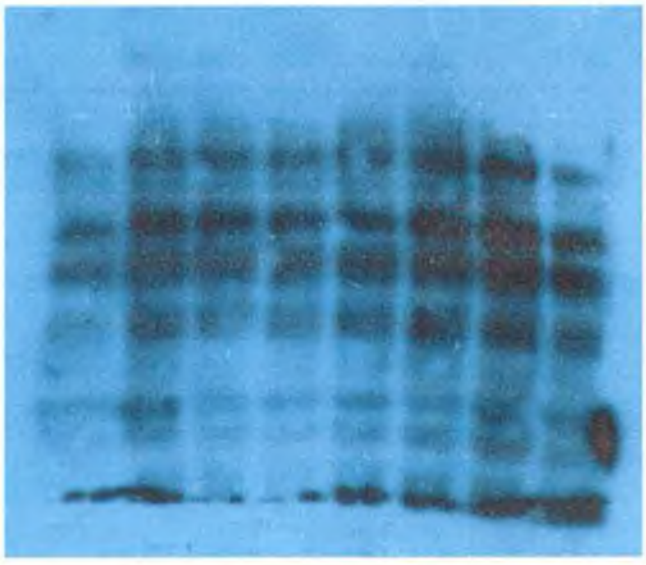
Figure 3.3.1(a) shows the problems encountered in trying to analyse the products of *in-vitro* translation of exogenous RNA. Due to the high protein content of the translation systems attempts to analyse the translation of keratin RNAs in these systems by western blot analysis produced so much background that it proved impossible to determine the band of interest. In parallel, attempts using the B.C.A. assay of protein concentration in systems with and without template mRNA to assess whether or not the translations had been successful failed (data not shown). The levels of protein in these systems proved so high that any increase due to the translation of added RNAs was not detectable.

It was decided to use the biotin-based immunoprecipitation technique modified for the detection of keratins in DLKP (section 3.1.4.2) as a "clean-up" to improve detection in translation mixtures. Completed IVT reactions were stopped on ice, diluted to 1ml

in chilled precipitation buffer, biotinylated and specifically immunoprecipitated using monoclonal antibodies. The resultant detection of keratin 8 expression in the Rabbit Reticulocyte (RR) translation system is illustrated in figure 3.3.1(b). Control lanes (C) containing no RNA showed no detectable keratin expression. Keratin protein was detectable in samples to which RNA was added, illustrating that the detection of keratins in these systems was due to translation of exogenously added RNAs.

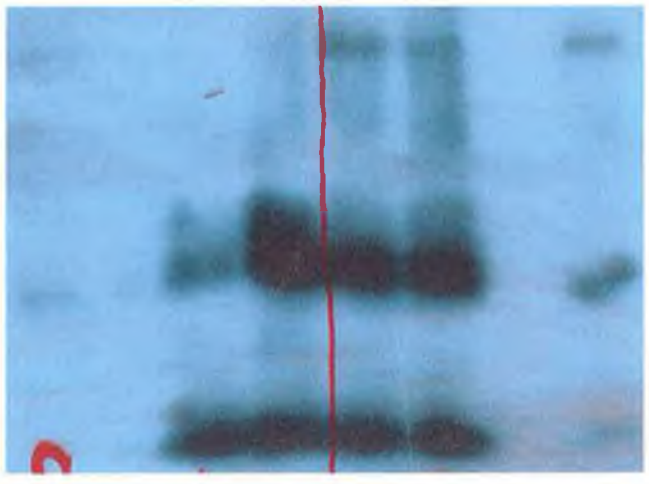
### 3.3.2 Optimisation of RNA concentrations for IVT

Once established that RRLs were the most efficient systems for our needs, a cheaper source of such systems was found due to the expensive nature of these products. The manufacturer (Boehringer) recommend a starting concentration of 1μg total RNA per reaction mixture, but that conditions should be optimised for individual sets of RNAs. Since no one set of RNA will be translated at the exact same efficiency a stock of RNA was isolated from A549 cells, diluted and aliquoted to avoid constant freeze-thaw. This RNA was then optimised for in-vitro translation by setting up a series of reactions at varying RNA concentrations. The results of these reactions are shown in figure 3.3.2. It is clear that the lower concentrations of RNA are translated more efficiently and consistently in terms of K8 detection. The higher concentrations begin to saturate the system and produce inefficient and inconsistent translation. From this, it was decided that the recommended concentration of 1μg per reaction would be used for future work using these IVT systems. All subsequent studies were performed using the same stock of RNA.



I----------WG---------I--------- RR---------- I

(a)



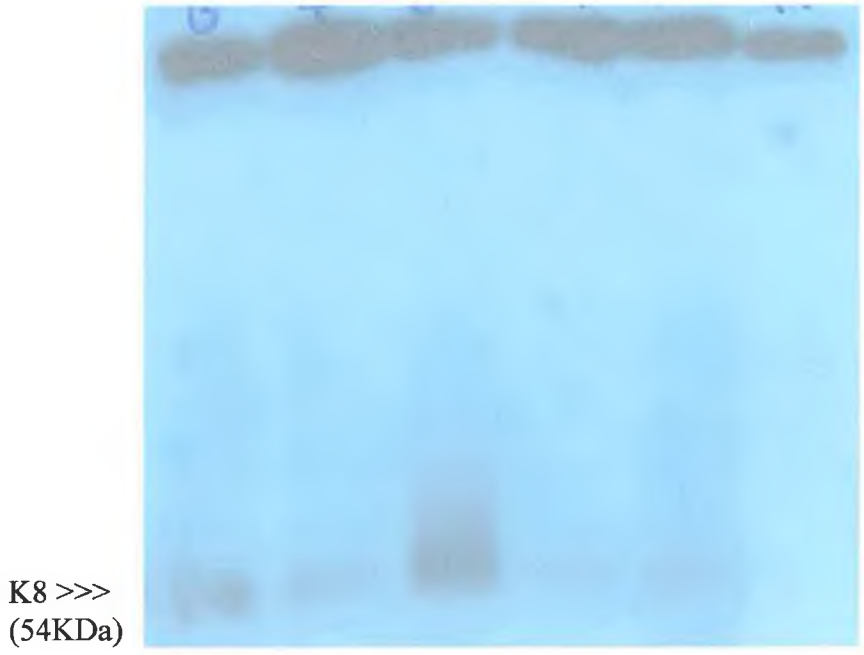
K8 >>>
(54 KDa)

C + + + + C +

(b)

(Note: C = Control with no RNA + = RNA present in reaction mixture)

**Figure 3.3.1 Development of *In-Vitro* Translation.** (a) Rabbit Reticulocyte Lysate and Wheat Germ Extract Translation Systems analysed for Keratin Protein by Western blot analysis (in the absence of Immunoprecipitation) after translation of A549 Total RNA. (b) Rabbit Reticulocyte Lysate System immunoprecipitated for Keratin expression after translation of total RNA isolated from A549.




**Legend:** Total RNA per reaction:

| | | | | |
|---|---|---|---|---|
| 1) 20µg | 2) 15µg | 3) 10µg | 4) 5µg | 5) 2µg |
| 6) 1µg | 7) 0.5µg | 8) 0.2µg | 9) 0.1µg | 10) 0.05µg |

11) Control (No RNA)

**Figure 3.3.2 *In-Vitro* Translation of a range of RNA concentrations.** (a) Lower concentrations of RNA are consistently translated. (b) Higher concentrations of RNA can become saturating and produce inconsistent translation.

### 3.3.3. Inhibition of Translation *in-vitro*:

To investigate the possibility that DLKP might express a novel translational repressor/regulator of simple keratin synthesis, experiments were designed to test this using *in-vitro* translation.

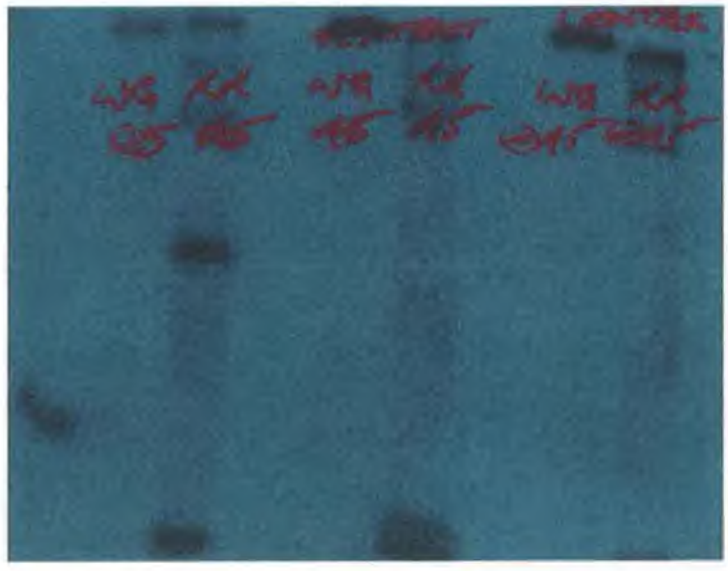
The principle behind the design of these experiments is simple; can a cytoplasmic preparation from DLKP inhibit the translation of keratin mRNAs in total RNA isolated from A549, known to be translatable both *in-vivo* and *in-vitro*? Cytoplasmic preparations from DLKP were isolated as outlined (Section 2.19.1.1). Prior to the addition of RNA these extracts were incubated with additional RNase inhibitor (RNasin) to eliminate any possible traces of RNase activity remaining in these extracts. The extracts were prepared by modifying a technique used to isolate cytoplasmic RNA for use in Northern Blot analysis, and as such RNase activity should be minimal. Total RNA from A549 was then pre-incubated with these extracts for 30 min, before *in-vitro* translation was performed. The cell extract was replaced with water as a positive control in these experiments.

Figure 3.3.3.1(a) shows that the Rabbit Reticulocyte Lysate (RRL) system worked well, while the Wheat Germ (WG) system showed no results. Inhibition of K8 translation was observed when RNA was pre-incubated with cytoplasmic extracts from DLKP. Figure 3.3.3.1(b) shows the results obtained using another source of RRL translation system (Boehringer). Again, significant inhibition of K8 translation appears to follow pre-incubation of RNA with DLKP extract. Notably, a small amount of keratin was detectable in lanes 3 and 4. This provides additional evidence that the RNA was not degraded in these samples. The cytoplasmic extracts from DLKP would appear to be translationally active (lane 3) when RNA from A549 is added. When exogenous RNA is not added (lane 2) no keratin protein is detectable. This suggests that keratin mRNAs are stable in these extracts and but that they are inefficiently translated in the presence of DLKP extract (with or without addition of RRL). We attribute this to repression of a large amount, but not all of the exogenously added RNA in these sytems.

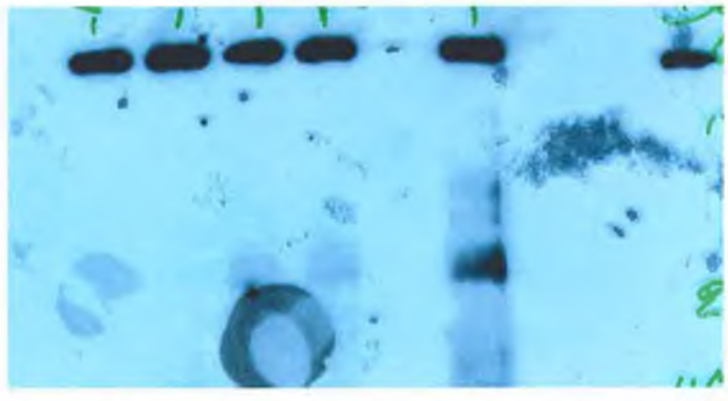
In contrast, incubation of RNA with DLKP extracts did not inhibit the translation of K18 mRNAs (figure 3.3.3.2), suggesting that RNases, as suspected, were not active in these cytoplasmic extracts. Scans are a little unclear due to darkness of blots, but illustrate the point sufficiently.

Unfortunately this approach is not suitable for use in A549 (figure 3.3.3.2). The high levels of Keratin mRNA and protein in cytoplasmic preparations from this cell line make it impossible to decipher any true results. We are currently designing modifications to the extract preparation potocol to eliminate these problems (antibody precipitation to remove protein, but the RNA may still pose a significant problem).

To demonstrate that the K8 mRNA transcripts in DLKP were functional and translatable, total RNA from DLKP was isolated, translated and specifically immunoprecipitated for K8 products of translation (figure 3.3.3.3).




**(a)**




**(b)**

**C1)** Control, RRL with no RNA or extract. **C2)** Control, Extract with no RRL or RNA. **C3)** Control, Extract and RNA, with no RRL.

+Extract Translation of RNA pre-incubated with DLKP Extract

-Extract Translation of RNA pre-incubated with Nuclease-free water.

**Figure 3.3.3.1 Inhibition of the translation keratin 8 mRNA from A549 total RNA by the addition of DLKP cytoplasmic extracts.** (a) In-Vitro Translation of A549 total RNA in both Rabbit Reticulocyte (RRL) and Wheatgerm (WG) Systems. Imunoprecipitation of Keratin 8 showed that the addition of crude cytoplasmic extracts from DLKP inhibited the in-vitro translation of Keratin 8. No results were obtained from the Wheatgerm system. (b) Repeat of the experiment described in (a) using the Rabbit Reticulocyte lysate system from Boehringer. Again, translation is severely inhibited, but not completely eliminated.

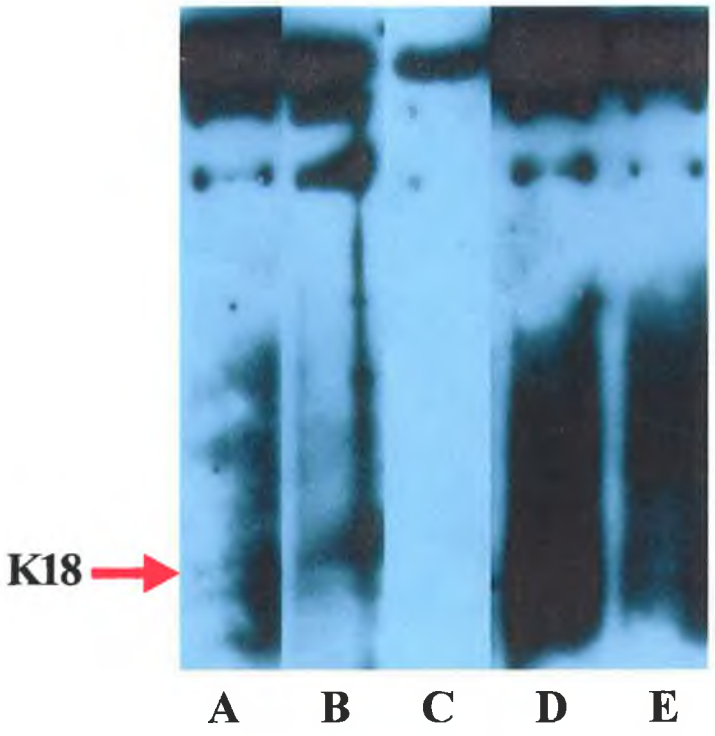



**Figure 3.3.3.2 Keratin 18 translation does not appear to be translationally repressed in DLKP.** A. A549 RNA. B. A549 RNA + DLKP Extract. C. Control (DLKP Extract, No RNA). D. A549 RNA + A549 Extract. E. Control (A549 Extract, No RNA). (D & E) High levels of both K18 protein and mRNA in A549 cytoplasmic extracts makes it difficult to determine whether or not repression is truly present in A549 or not.

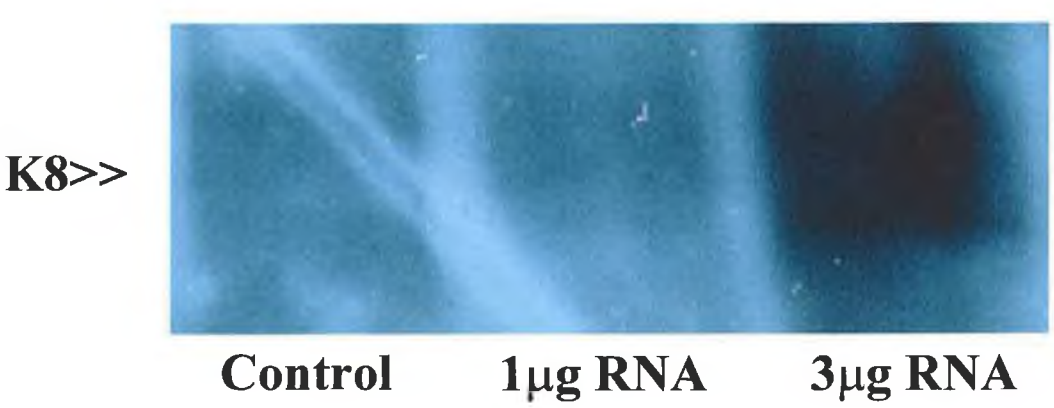



**Figure 3.3.3.3 Keratin 8 mRNAs in DLKP are translatable.** *In-vitro* translation of total RNA isolated from DLKP showed that the keratin 8 transcripts present in DLKP are translatable. Keratin 8 protein was detectable when 3µg RNA was used, probably due to the low level of expression of this mRNA as a percentage of total RNA in DLKP.

### 3.3.4 Identification of Unusual Sequences within the K8 mRNA that may play a role in translational regulation of K8 synthesis

**(A) The 5' UTR of K8 and K18:**

The 5' UTRs of both K8 and K18 show no significant homology when compared using blast sequence comparison programs. However, a very interesting repeat sequence was identified in the short 5' UTR of K8 (highlighted in red), situated only one nucleotide from the AUG start site. The repeat is separated by only a single nucleotide and is unique to K8 mRNAs in humans. This 10-nucleotide repeat (20-nucleotide in total) within the short, 60 nucleotide 5' UTR may be a prime candidate for a repressor binding site that regulates K8 translation during development.

**K8** (59 nucleotides)
CTGCTCCTTCTAGGATCTCCGCCTGGTTCGGCCCGCCTGCCTCCACTCCTG
CCTCCACC

**K18** (51 nucleotides)
CGGGGTCGTCCGCAAAGCCTGAGTCCTGTCCTTTCTCTCTCCCCGGACAGC

**(B) The 3' UTR of K8 and K18:**

Sequence comparisons revealed that the 3' UTR of K8 is significantly longer than that of K18. While the K18 3' UTR is encoded by nucleotides1344-1472 (128 nts), that of K8 spans nucleotides 1511-1752 (241 nts), approximately twice the length of the K18 3'UTR. Within this sequence we have identified an unusual almost identical 12-nucleotide triple-repeat, the core of which is a CCCACCTGGGGA sequence:

1606 CCCACCTGAGGC 1617
1635 CCCACCTGGGGA 1646
1651 ACTACCTGGGGA 1662

Complete matches in all three are marked in blue, while base mismatches to the core sequence are highlighted in red. A diagrammatic representation of these unusual sequences is presented in figure 3.3.4.

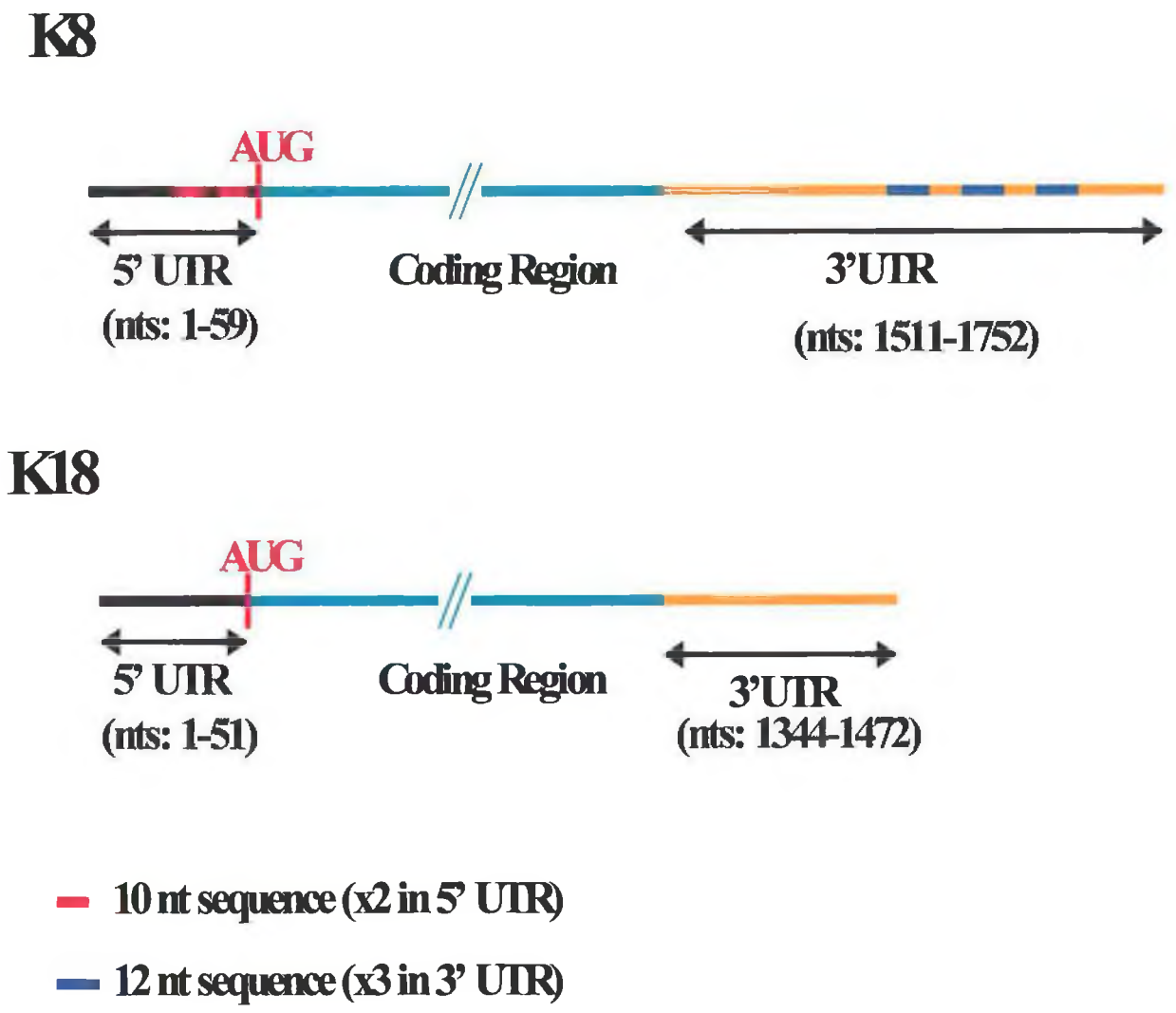



**Figure 3.3.4 Sequences identified in the keratin 8 mRNA that may be involved in translational control.** The keratin 8 5' UTR is only 60 nucleotides and yet it harbours a 10-nucleotide repeat placed a single nucleotide from the translation start site. Additionally, the 3' UTR of K8 is twice the length of its partner, K18, and harbours an interesting 11-nucloetide triple-repeat. The 3' UTR is thought to be involved in regulating the developmental translation of mRNAs.

## Section 3.4 BrdU and the regulatory factors possibly involved in the induction of eIF-4E

In order to investigate the possible mechanisms by which BrdU modulates the expression of eIF-4E and the possible role of transcription in differentiating lung cancer cells, factors thought to be involved in the regulation of eIF-4E expression were examined.

### 3.4.1 Expression of c-Myc

eIF-4E is regulated at the transcriptional level by c-*myc* (Rosenwald et al, 1993; Jones et al, 1996). There are two isoforms of c-Myc, of which c-Myc1 was examined in BrdU-treated epithelial cells. The significance of these isoforms, and particularly c-Myc1 is discussed in section 4.2.4.1.2. When levels of c-Myc1 expression were investigated using a p67-specific antibody (Santa Cruz), they were found to dramatically increase upon exposure to 10μM BrdU. Figure 3.4.1(a) and figure 3.4.1(b) show the increase in c-Myc1 levels in BrdU-treated A549 and DLKP, respectively. Due to the long film-exposure times required to detect the low level expression of c-Myc1 in untreated cells and the use of PVDF membranes for blotting, non-specific background bands are often detectable on autorads.

BrdU has been shown to cause down-regulation of *c-myc* gene expression in HL60 cells (Yen and Forbes, 1990). As such, to observe the possible downstream effects of BrdU-mediated changes in c-*myc* expression, conditions were reproduced from Yen and Forbes (1990). To date, c-Myc expression has proven undetectable in HL60s using two independent sources of c-Myc antibody (Santa Cruz and Biomol, UK). This may be attributable to the fact that c-Myc levels decrease in this cell line upon exposure to BrdU, making detection impossible when levels are not even detectable in untreated HL60s. The down-regulation of c-*myc* gene expression in these cells was confirmed by PCR on a single occasion, but photographs were lost due to poor film development.

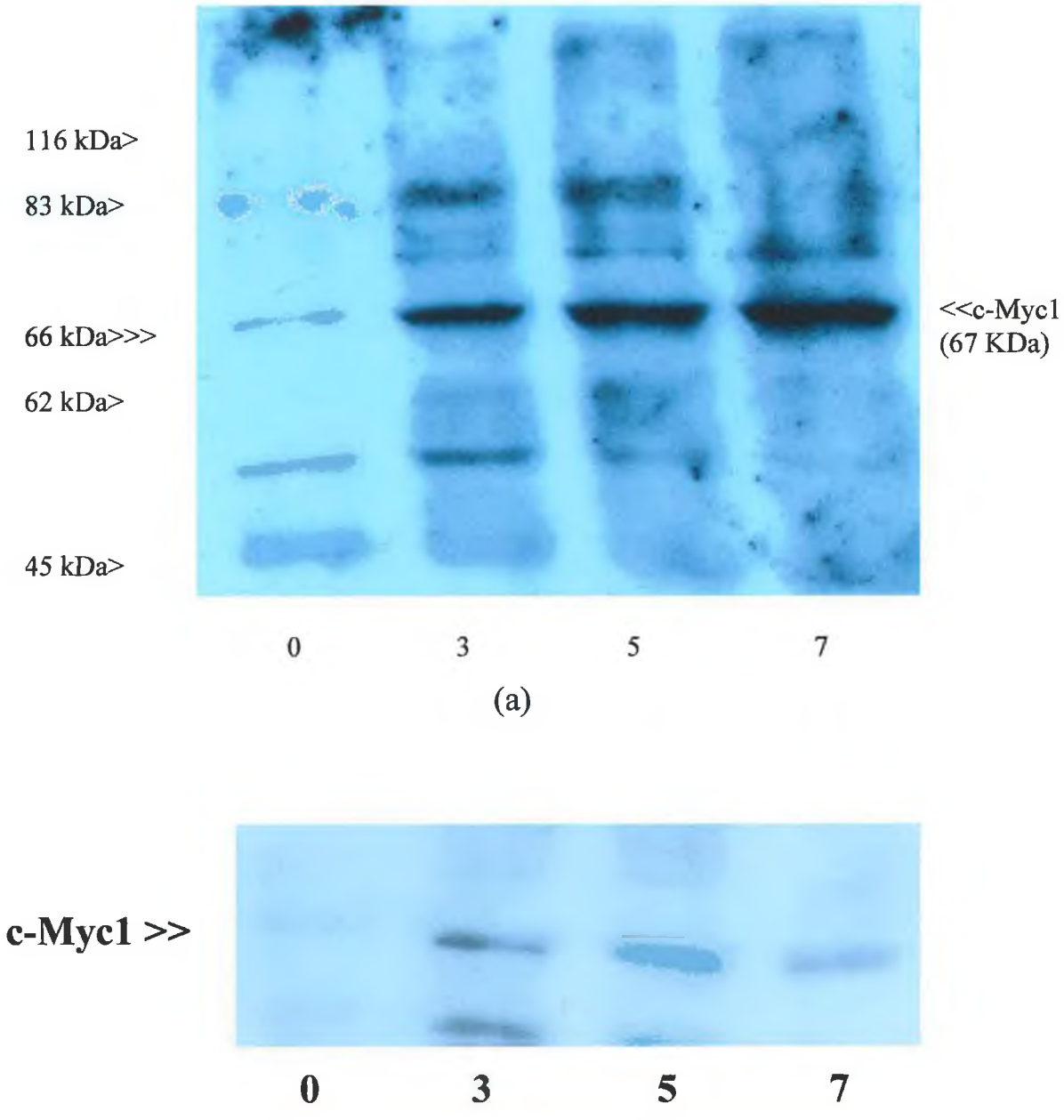
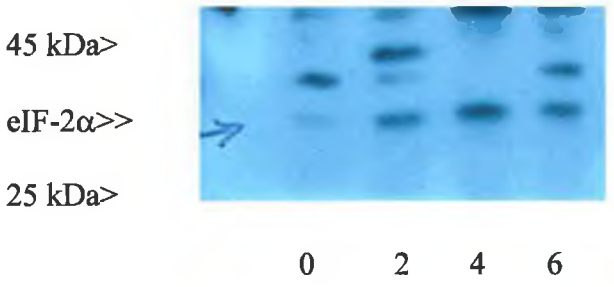



**Figure 3.4.1 Western Blot Analysis of c-Myc 1 Expression.** Numbers represent the days of exposure to BrdU. BrdU-treated (a) A549, (b) DLKP show a strong induction of c-Myc1. Long exposures are required to detect c-Myc 1 expression in untreated cells, which results in elevated background bands on blots.

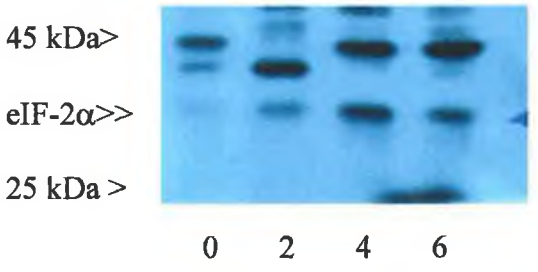
### 3.4.1.1 eIF-2α Expression in BrdU-treated Epithelial Cells

Probably the two most important of the translation initiation factors, eIF-4E (Section 1.6.3.2.4.3) and eIF-2α (Section 1.6.3.2.2) are among the few known c-*myc* regulated genes to date (Rosenwald et al, 1993). To indirectly test whether or not c-*myc* was actively involved in the BrdU-mediated upregulation in eIF-4E, the levels of eIF-2α, which should also increase, were investigated.

Western blot analysis revealed an increase in eIF-2α in both A549 and DLKP upon exposure to BrdU (Figure 3.4.1.1). eIF-2α is a 34-kDa protein and appears approximately mid-way between the 45kDA and 25kDa markers. Non-specific background binding is probably due to the use of PVDF membranes for blotting and the polyclonal nature of the antibody used. It must be stressed, however, that this western was not possible to repeat due to the scarcity of the antibody. The antibody used was a kind gift of Dr. Simon Morley, whose source no longer exists. Dr. Morley was kind enough to supply a small aliquot of this antibody from his limited supply.




(a)




(b)

**Figure 3.4.1.1 Western Blot Analysis for eIF-2α Expression.** Increased expression of eIF-2α was detected in BrdU-treated (a) A549, and (b) DLKP. These were one-off westerns using an antibody generously provided from a limited supply by Dr. Simon Morley. While background levels are high, the 34-kDa eIF-2α is relatively clear.

### 3.4.2 Expression of transciption factor, Yin-Yang 1 (YY1)

YY1 protein levels were found to be upregulated in BrdU-treated epithelial lung cancer cell lines, A549 and DLKP (figure 3.4.2a), while it is downregulated in the leukaemic line, HL60 (figure 3.4.2b). This is in agreement with the levels of c-Myc observed in both epithelial lines (section 3.4.1), and with the reported changes in c-*myc* levels in leukaemic lines (Yen and Forbes, 1990). YY1 has been shown to be upregulated in BrdU-treated embryonic myoblasts (Lee et al, 1992) in which case BrdU treatment was actually used as a substitute for transfection of the YY1 cDNA.

#### 3.4.2.1 Expression of cdc2

A recent report (Jun et al, 1998) sequenced the 5' promoter of the murine cdc2 gene, identifying a YY1 binding site and promoter characteristics that we suspect may make cdc2 another candidate for regulation by YY1. To test both the possible activity of YY1 in BrdU-treated cells, and at the same time confirm our suspicions that cdc2 may be another YY1 regulated gene, cdc2 levels were examined and shown to increase in BrdU-treated DLKP (figure 3.4.2.1).

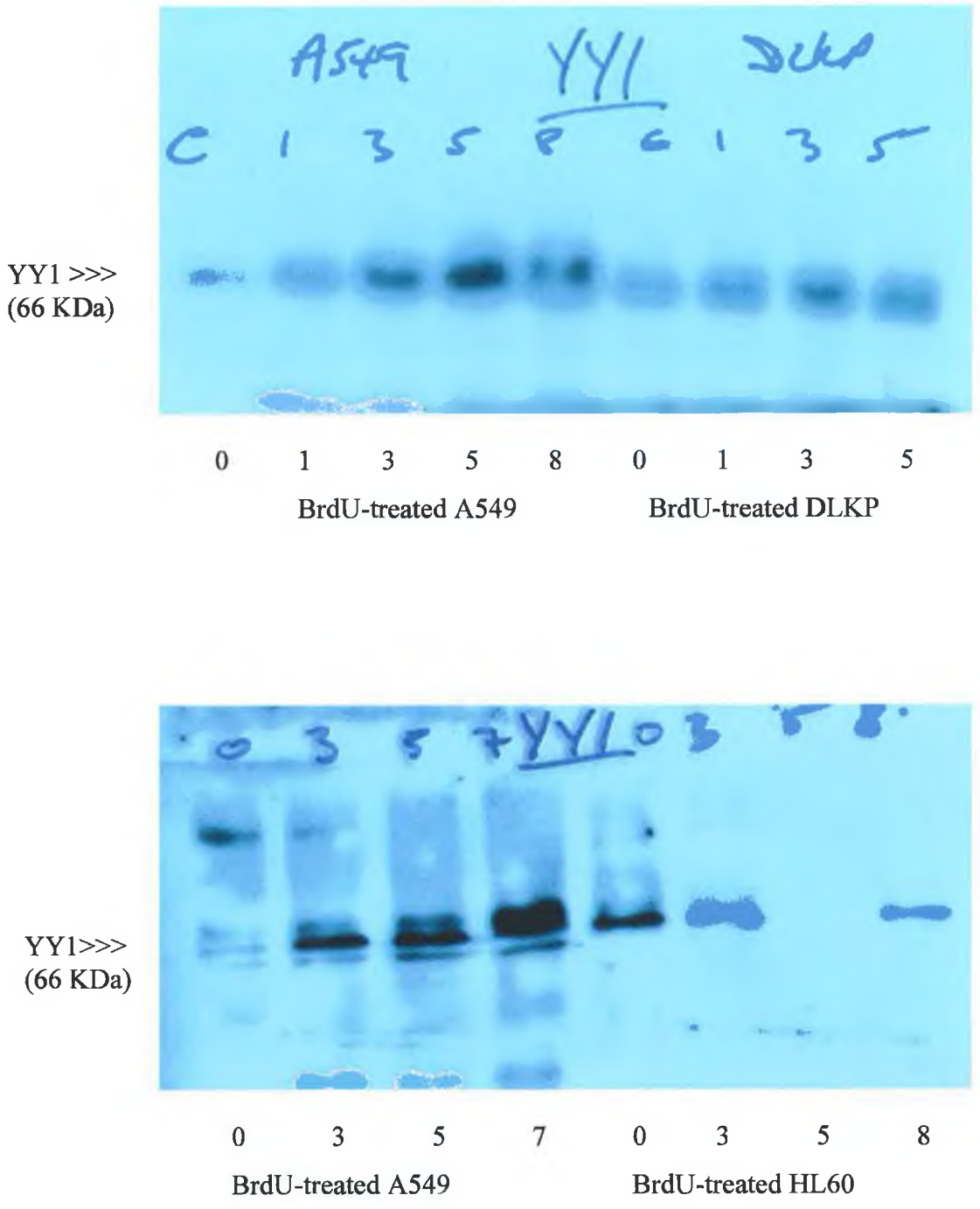



**Figure 3.4.2 Western Blot Analysis of YY1 expression in BrdU-treated A549, DLKP and HL60.** Exposure to BrdU increases the expression of YY1 in both epithelial cell lines, DLKP and A549, while it decreases levels of expression in the leukaemic line, HL60.

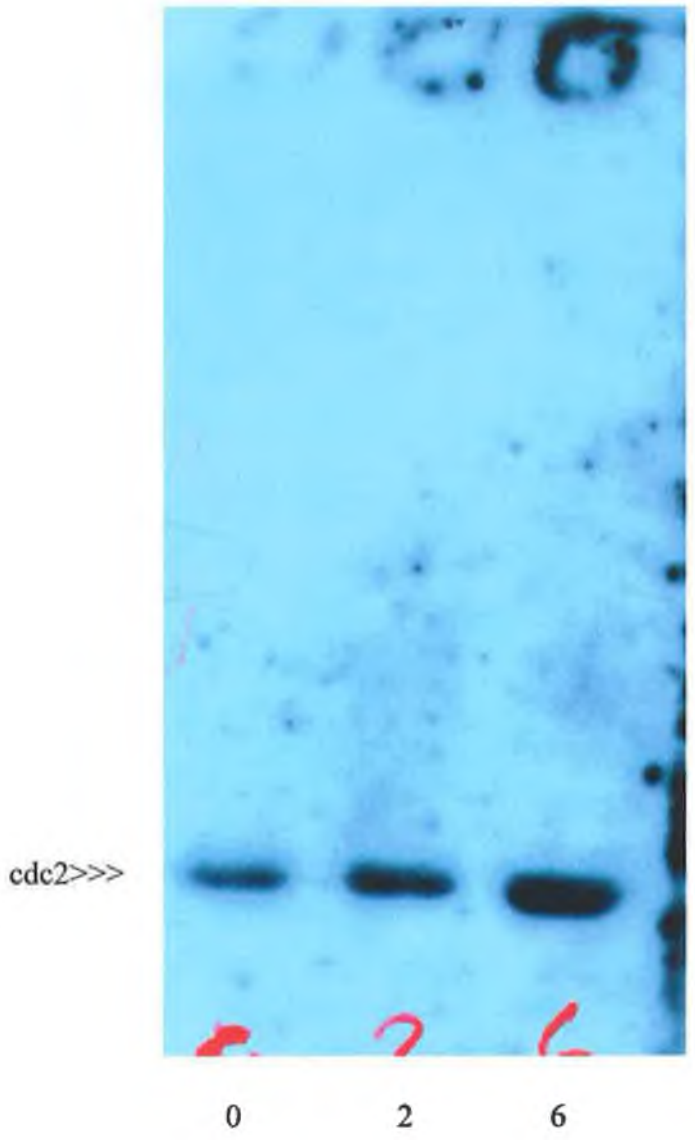
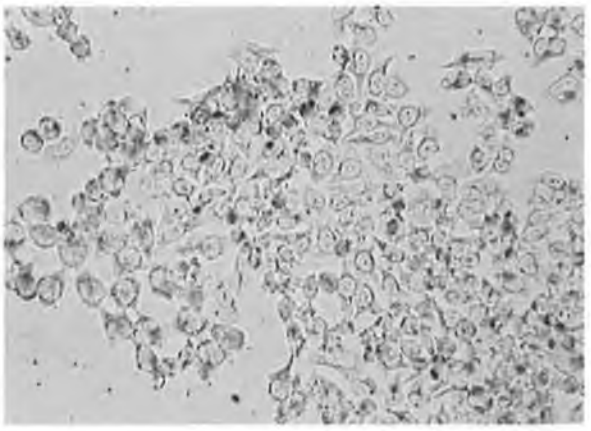



**Figure 3.4.2.1 Western Blot Analysis of cdc2 expression in BrdU-treated DLKP.** Levels of cdc2 protein expression in DLKP were found to be upregulated by BrdU. Numbers represent the days of exposure to BrdU.

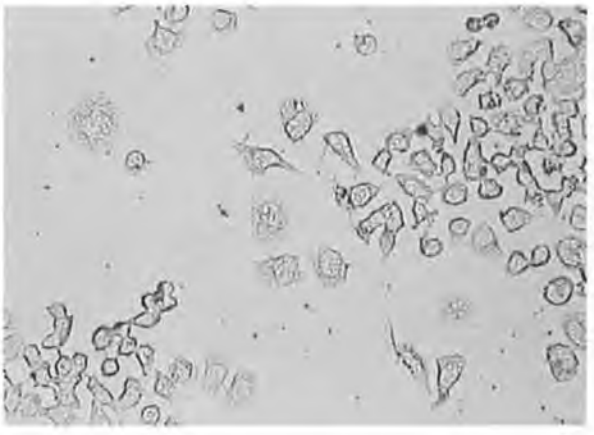
## Section 3.5 Overexpression studies

A number of important factors were identified over the course of these studies that we suspect play critical roles in the regulation of early lung cancer differentiation and development. A central role is proposed for the small cap-binding translation initiation factor, eIF-4E in the differentiation cascade induced by BrdU (Section 4.2.6). We suspect that the developmental transcription factor, YY1 is the initiating factor in this cascade. cDNA constructs for both eIF-4E and YY1 were obtained to study their roles in mediating the effects observed during BrdU-induced differentiation. Unfortunately, A549 proved extremely difficult to transfect. More importantly, however, the novel poorly differentiated cell line, DLKP proved to be transfectable.

It must be noted that these plasmids were obtained as gifts. Unfortunately control plasmids were not included, but in light of the interesting results they are currently being arranged. However, a temporary control, DLKP-SQ-pHβ (transfected with an empty vector encoding geneticin-resistance; NicAomhlaoibh, R., PhD Thesis, 1997) was used to ensure that exposure to geneticin or the general transfection protocol was not responsible for the effects observed in transfected cell lines. Immunocytochemical analysis of K8 expression showed no detectable induction (figure 3.5), suggesting that geneticin does not affect the pathway proposed to induce simple keratin expression in DLKP (Section 4.2.6).



(a)



(b)

**Figure 3.5 Both DLKP-SQ and geneticin-resistant DLKP-SQ-pHβ are negative for K8 protein by immunocytochemistry.** Slight background is detectable in both cell lines, but results show that geneticin does not alter the keratin status of resistant cells.

### 3.5.1 Overexpression of translation initiation factor, eIF-4E

A plasmid encoding the small cap-binding initiation factor, eIF-4E (Section 1.6.3.2.4.3) was obtained from Prof. Arrigo DeBenedetti (Louisiana, USA), a pioneer of eIF-4E function.

Several initial attempts to transfect DLKP with this plasmid failed, attributed to the size of the plasmid (13 Kb). This reduces its transfection efficiency to about 1% (Prof. DeBenedetti, personal correspondence). This is particularly relevant in A549, a cell line that appears to be particularly difficult to transfect in comparison with DLKP and its clones. A series of transfections using a range of plasmid concentrations and cell numbers finally yielded two sets of eIF-4E-transfected DLKP, named DLKP-4E1 and DLKP-4E2. Overexpression of eIF-4E was confirmed by Western blot analysis (Figure 3.5.1.3). Morphologically, eIF-4E overexpressing DLKP grow in looser colonies and are on average 1.5-fold larger than the parental line (Figure 3.5.1.1). Some cells exhibited distinct enlargement, suggesting that some cells may be expressing this plasmid significantly better than others. However, immunocytochemical analysis (Figure 3.5.1.2) showed that expression of eIF-4E was homogenous in the mixed population. We suspect that this is simply due to the fact that this is an episomally replicating vector, eliminating variability normally associated with integrating vectors (their expression is dependent upon the site of integration and its relative transcriptional activity). Unlike standard transfection protocols, these transfections were not cloned, for two reasons. Firstly, the very poor efficiency of transfection yields very few transfected cells after selection using geneticin. Attempting to transfer them to 96-well plates would result in further loss of transfected cells. It takes a considerably long time for selected cells to "recover" and grow to workable numbers, due to very low density within the flask after selection. Secondly, DLKP is a mixed population. Cloning of transfected parental DLKP results in a situation where there is no parental line for comparison, since there is no way of distinguishing the cellular origin of the cloned transfectants (i.e. was the cell from the DLKP-SQ, DLKP-I, DLKP-M or an as yet unidentified sub-clone). Both eIF-4E- and YY1-transfected DLKP (Figures 3.5.1.1 and 3.5.2.1, respectively), retain their "mixed population" appearance, suggesting that the geneticin-resistant clones that replenished

these populations consist of a representative mix of the parental populations and not one particular sub-population.

Both DLKP-4E1 and DLKP-4E2 exhibited c-Myc1 expression (Figure 3.5.1.3). This is in agreement with predictions and findings using eIF-4E overexpressing CHO-cells in Prof. DeBenedetti's laboratory (Carter et al, 1999). In addition, induction of simple keratins, K8 and K18, was observed in these lines (Figure 3.5.1.2 & 3.5.1.3). eIF-4E overexpressing DLKP also appears to express higher levels of a larger form of the YY1 protein (Figure 3.5.1.3.1), suggesting a similar mechanism of isoform regulation to that proposed for c-Myc (Carter et al, 1999) (Section 4.2.4.1.2).

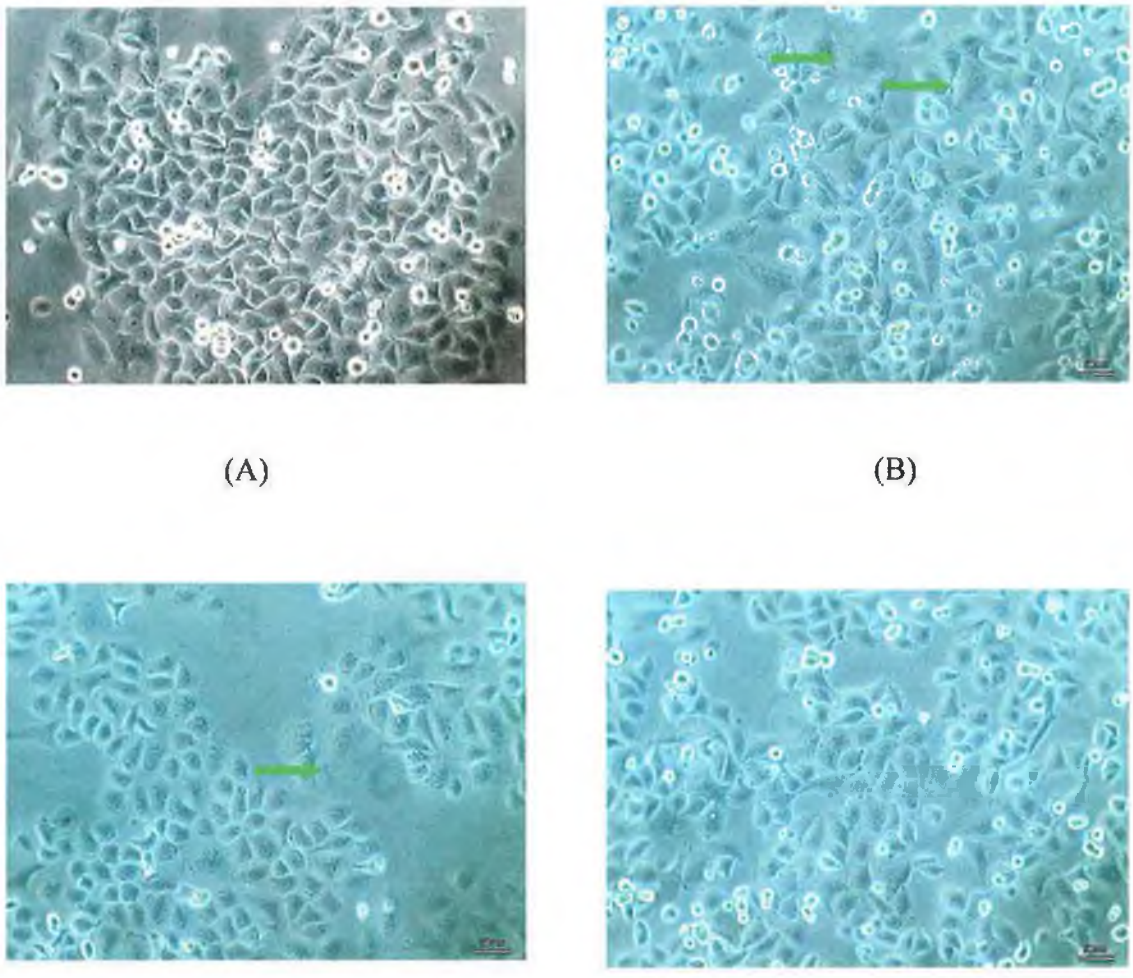
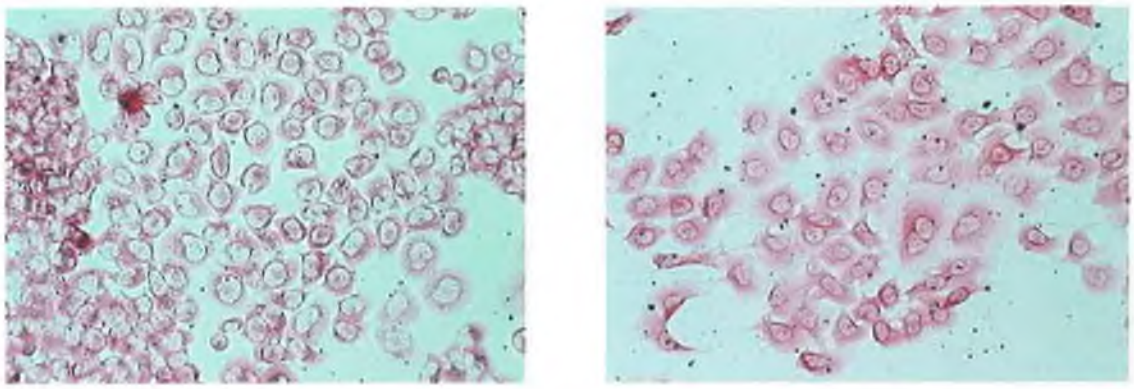
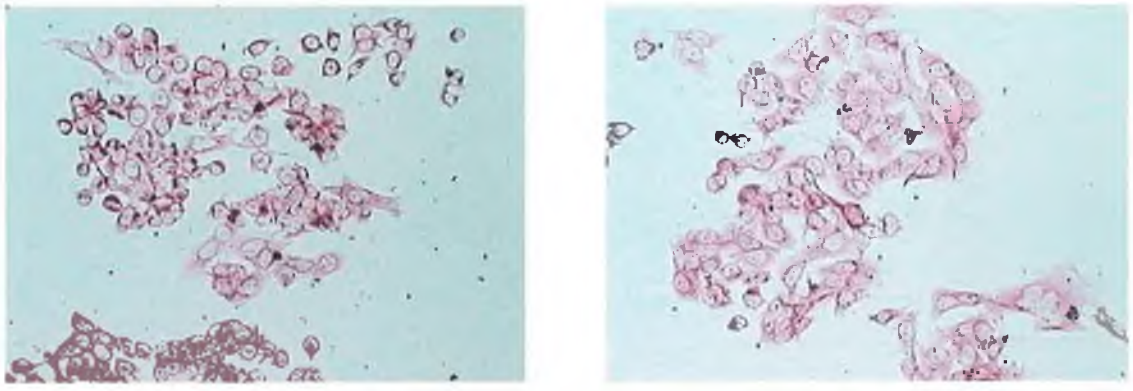



**Figure 3.5.1.1 Morphological appearance of DLKP and DLKP-4E cells.** (A) DLKP grow in clustered colonies. (B) DLKP-4E1 are morphologically larger and grow in looser colonies. Some significantly enlarged cells are illustrated by the green arrows. (C) DLKP-4E2 exhibits a similar morphological appearance to DLKP-4E1. (D) DLKP-4E2. Image analysis revealed an average 1.5-2-fold increase in cell size.



**(1) Parental DLKP** **(2) DLKP-4E1**

**(A)**



K8 K18

**(B)**

**Figure 3.5.1.2 Immunocytochemistry in eIF-4E overexpressing DLKP, DLKP-4E1.** (A) (1) Untransfected parental DLKP and (2) DLKP-4E1, stained with polyclonal anti-eIF-4E antibody (a kind gift of Dr. Simon Morley). (B) Induction of both K8 and K18 expression in DLKP-4E1. Again, untransfected parental DLKP were keratin negative (as in figure 3.1.3.2) (not shown).

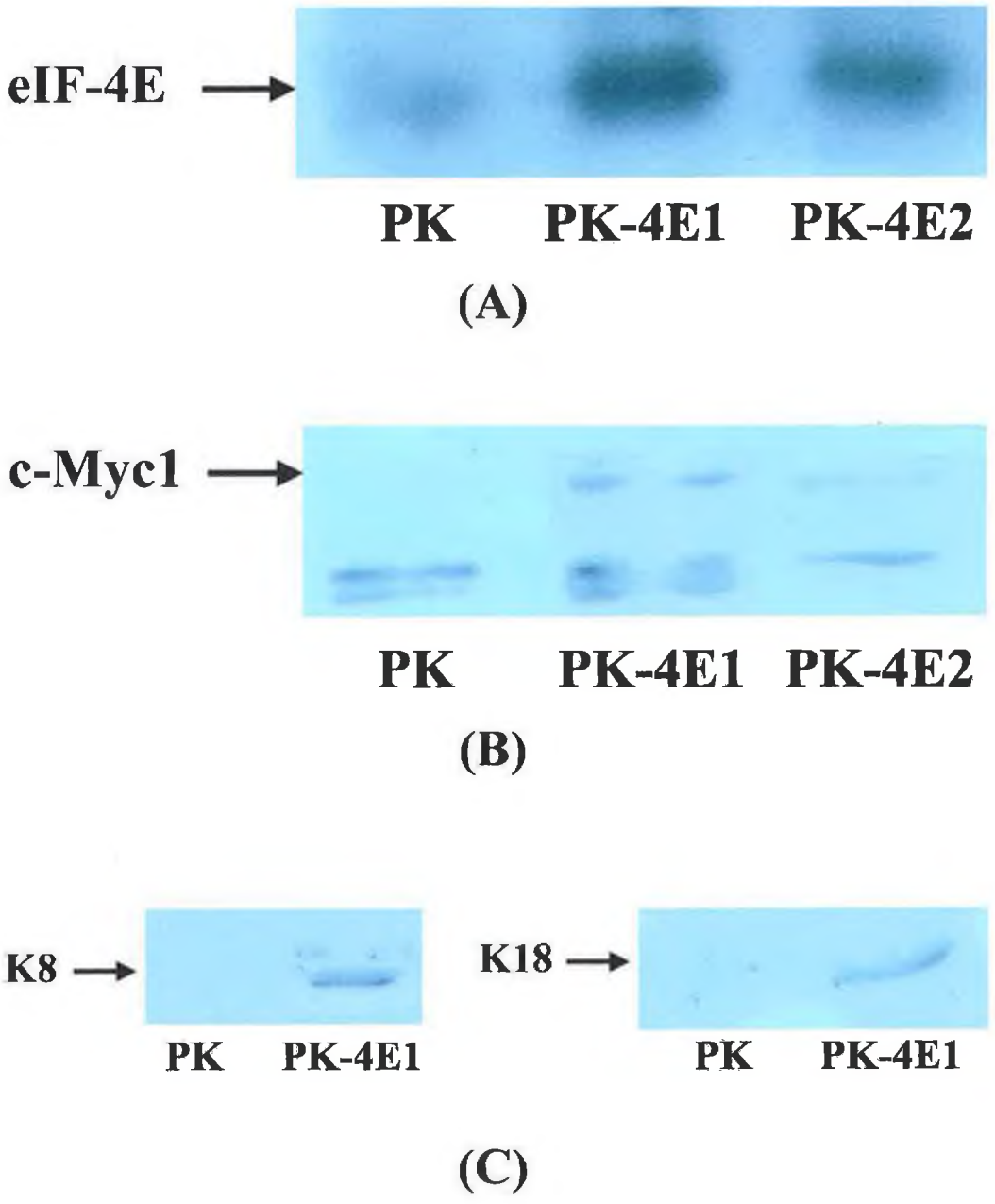



**Figure 3.5.1.3 Western blot analysis of eIF-4E-transfected DLKP.** (A) Levels of eIF-4E in transfected DLKP (PK-4E1 & PK-4E2) are higher than in parental DLKP (PK). (B) Overexpression of eIF-4E induces c-Myc1 expression in DLKP. (C) Over-expression of eIF-4E induces the expression in simple keratins, K8 and K18 in DLKP.

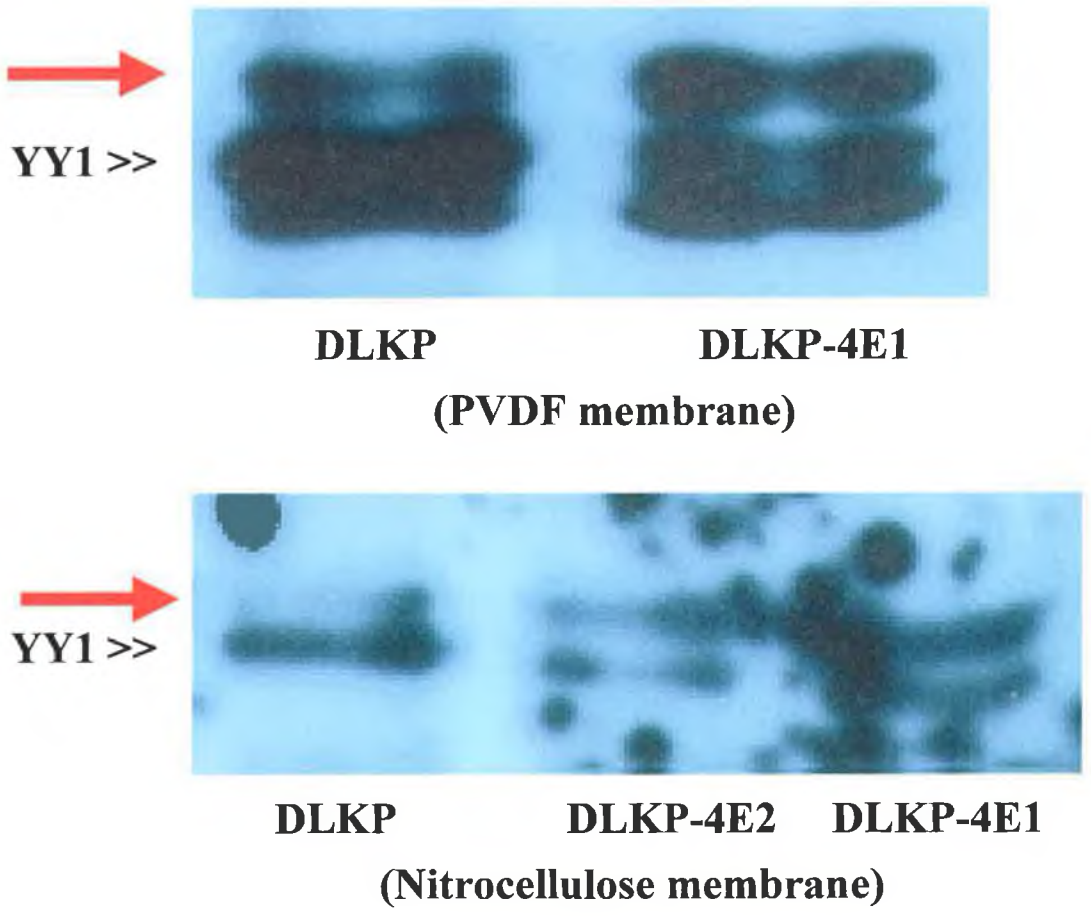



**Figure 3.5.1.3.1 YY1 expression in eIF-4E Overexpressing DLKP.** There appears to be a shift in YY1 expression towards a larger form of the protein in eIF-4E-transfected DLKP. Levels of YY1 do not appear to change significantly overall, but expression of this larger isoform may have significant effects on YY1 activity, similar to those described for c-Myc (Section 4.2.4.1.2).

### 3.5.2 Overexpression of Yin-Yang 1, YY-1

A plasmid encoding the transcription factor, YY1 (Section 1.5.2.3) was obtained from Dr. Finian Martin (Dublin, Ireland). This plasmid proved reasonably efficient to transfect. However, to retain the mixed population that comprises DLKP for comparison with the parental line (as discussed in section 3.5.1), three flasks of the parent population were transfected, selected with geneticin but not cloned. Again, the morphology of resistant cells suggested that a representative mixture of cells comprising the parent population was present in transfected populations (Figure 3.5.2.1). Some cells exhibited significantly enlarged morphologies, suggesting that these cells expressed the plasmid more efficiently than others. The YY1 plasmid is an integrating vector and, unlike the eIF-4E plasmid, its expression is influenced by the site at which it integrates, explaining the variable expression suspected in these cells. A clone of DLKP, DLKP-SQ was also transfected and compared to its parental clone (Figure 3.5.2.1). DLKP transfectants were termed DLKP-Y1 to DLKP-Y3, while the DLKP-SQ transfectant was termed SQ-Y.

Overexpression of YY1 was confirmed using western blot analysis (Figure 3.5.2.2). It is suspected that the low level of YY1 overexpression in some of these transfectants is attributable to the overall heterogeneity in expression of the plasmid in this mixed population, since this plasmid is an integrating vector. Unfortunately the YY1 antibody available was not suited to immunocytochemistry. However, in agreement with this speculation, both Keratin and eIF-4E staining in transfected cells was found to be heterogenous (Figure 3.5.2.2).

Figures 3.5.2.2 and 3.5.2.3 show that transfection of YY1 was capable of inducing simple keratin, K8 and eIF-4E expression. Additionally, two out of three YY1-transfected DLKP populations showed elevated c-Myc1 expression (Figure 3.5.2.3b).

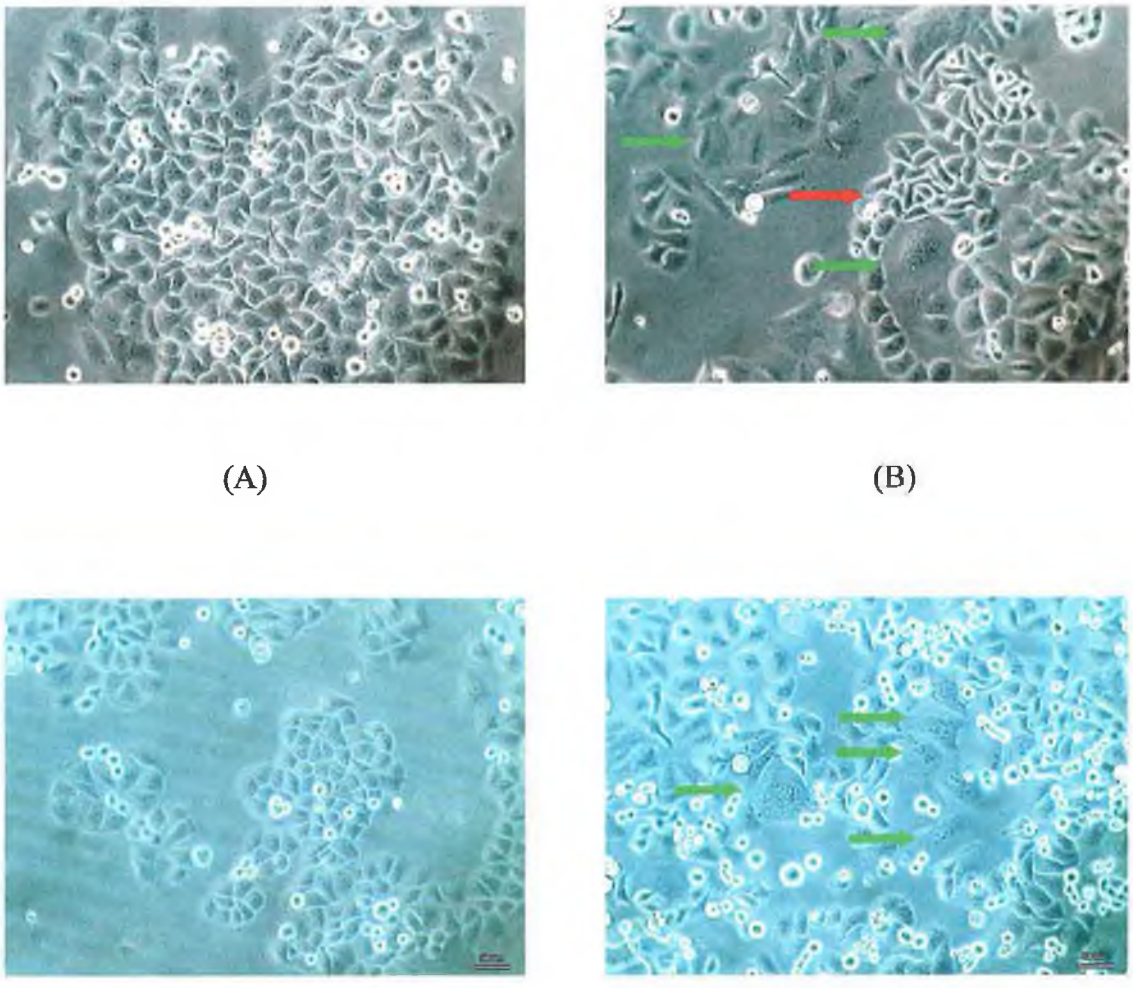



**Figure 3.5.2.1 Morphological changes in YY1-transfected DLKP and DLKP-SQ.** (A) Morphology of DLKP. (B) DLKP-YY1 exhibits distinct enlargement of cells (green arrows), while sub-populations remain relatively unchanged and retain the clonal appearance of parental DLKP (red arrow). (C) Morphology of DLKP-SQ. (D) SQ-YY1 are larger and grow in more dispersed colonies than parental DLKP-SQ. Image analysis showed that both DLKP-YY1 and SQ-YY1 were morphologically larger than their respective parental lines.

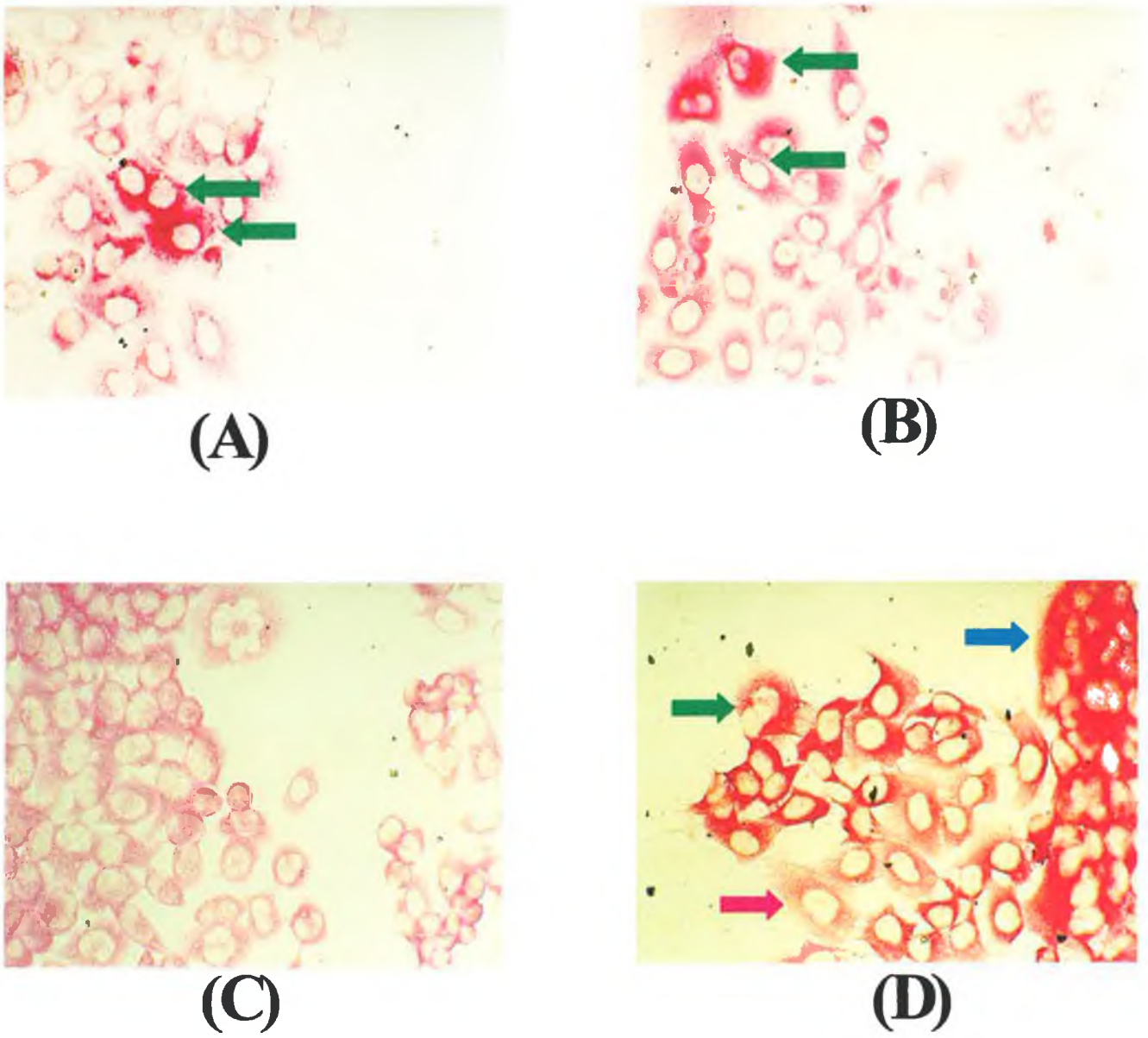
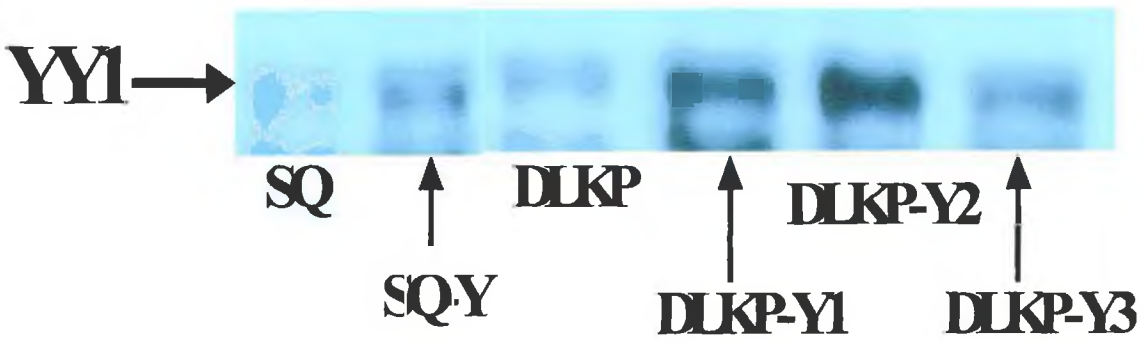
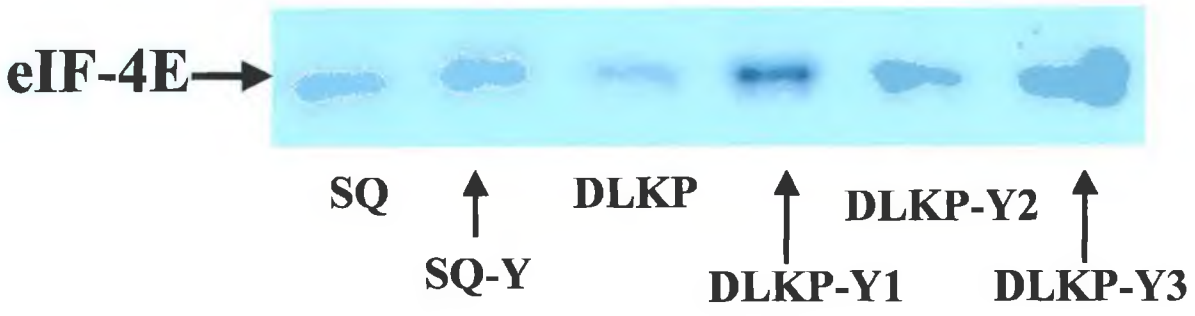



**Figure 3.5.2.2 Keratin 8 and eIF-4E expression in YY1-transfected DLKP.** (A) and (B) YY1 induces Keratin 8 expression in the keratin-negative cell line, DLKP. K8 induction is not uniform (green arrows), suggesting heterogenous expression of transfected YY1 plasmid in this mixed, uncloned population. (C) eIF-4E expression in parental DLKP. (D) eIF-4E induction in YY1-transfected DLKP appears heterogenous (coloured arrows), in agreement with the mixed nature of these transfections and the pattern of keratin induction.

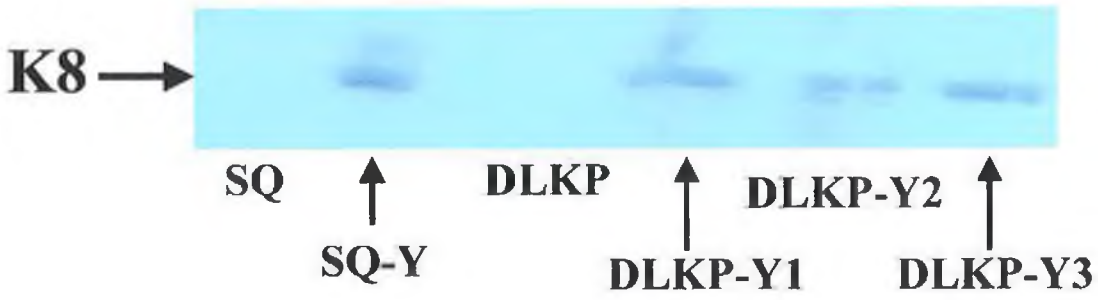



(A)




(B)

**Figure 3.5.2.3A Western blot analysis of YY1-transfected cells.** (A) Levels of YY1 are higher in transfected lines than those in parental DLKP or DLKP-SQ. (B) eIF-4E expression in YY1-transfected lines.




(A)


(B)

**Figure 3.5.2.3B Western blot analysis of YY1-transfected cells.** (A) Keratin 8 is induced in YY1 overexpressing lines. (B) Conclusive elevations in Myc1 expression were only detectable in DLKP-Y1 and DLKP-Y3. DLKP-Y2 samples may have been degraded.

## Section 3.6 The control of eF-4E activity in A549

Serum starvation studies were used to further examine the regulation of eIF-4E activity in the epithelial cell lines used in these differentiation studies. By removing the mitogenic stimulation of serum, cells shut down their signal transduction cascades via dephosphorylation. Subsequent re-stimulation with serum then allows the roles of various kinases to be examined as signal pathways are reactivated, by measuring the relation between reactivation of kinases versus their target proteins, and through the use of specific kinase inhibitors. Attempts to serum starve DLKP failed (data not shown), probably due to the suspected high level expression of autocrine growth factors. In agreement, DLKP has been shown to grow very well in serum-free medium (Meleady and Clynes, 1995). However, after 48 hrs serum starvation, A549 exhibited sufficient dephosphorylation of ERK and eIF-4E for use in such studies. Ras protein turnover in the absence of any mitogenic signals was probably sufficient to overcome the K-12 Ras "GTP-loading" of these cells (Mitsudomi et al, 1991).

### 3.6.1 ERK and eIF-4E Phosphorylation in serum-stimulated A549

Inactivation after 48 hrs serum starvation and re-activation of both ERK and eIF-4E upon re-stimulation with 10% serum is shown in figure 3.6.1. ERK levels within the cell do not change, as illustrated using anti-ERK antibodies (3.6.1a). Use of Phospho-specific antibodies to ERK reveal, however, that the levels of phosphorylation of ERK protein were hugely increased upon re-stimulation with serum after only 5 min (figure 3.6.1b). The phosphorylation of eIF-4E exhibited the same sort of behaviour (figure 3.6.1c). The level of eIF-4E phosphorylation was determined by Iso-Electric Focusing, and as such is interpreted differently. The phosphorylation of eIF-4E is assessed by the relative ratio of phosphorylated, active eIF-4E (upper band) compared to the levels of non-phosphorylated, less active eIF-4E (lower band). It is not the intensity of bands in different lanes that is compared in the case of IEF, but changes in the relative intensity of the upper and lower bands in the same lane, which reveals the activity of the eIF-4E present in these lanes. Serum starved cells show only the lower, non-phosphorylated form of the protein. Upon serum stimulation of these cells a shift

occurs, with the appearance phosphorylated eIF-4E (upper band) (see also; Section 3.2.5).

### 3.6.2 ERK and eIF-4E Phosphorylation profiles in A549

An extended profile of rephosphorylation for both ERK and eIF-4E (figure 3.6.2) showed that both profiles exhibited "normal" characteristics; a peak in ERK phosphorylation around 10-15 min after re-stimulation with serum due to sudden re-exposure of signal cascades to mitogenic stimulation, causing an initial "hyperactivation" of the kinases in these pathways. eIF-4E phosphorylation follows a similar profile. The phosphorylation of these factors then subsides and returns to normal, suggesting that ERK autoregulation is active in these cells, despite the reported presence of Ras mutations in this cell line (Mitsudomi et al, 1991).

### 3.6.3 The effects of Specific Kinase inhibitors on eIF-4E Phosphorylation in A549

Prior incubation of cells with specific kinase inhibitors allows the role of individual kinases to be evaluated in cells after re-stimulation with serum. To date Mnk is the only known direct kinase for eIF-4E, and its activity is regulated mainly by ERK, as well as by the stress response p38 kinase. The use of specific kinase inhibitors in such studies can be used to confirm current understandings in relation to the regulation of eIF-4E activity. Anomalies/unexplained inhibition of eIF-4E rephosphorylation by kinase inhibitors can be the earliest indication of possible novel kinases or pathways involved in this control, perhaps as yet undiscovered. The results are shown in figure 3.6.3.1 and 3.6.3.2. The inhibitors used were (see also; Figure 4.10, Section 4.3.2):

| Inhibitor | Point of Inhibition | Concentration used |
|---|---|---|
| PD98059 | MEK-ERK Phosphorylation | 50 μM |
| SB203580 | p38-Mnk phosphorylation | 30 nM |
| Wortmannin | PI-3K Phosphorylation | 100 nM |
| Rapamycin | FRAP/mTOR Phosphorylation | 20 nM |

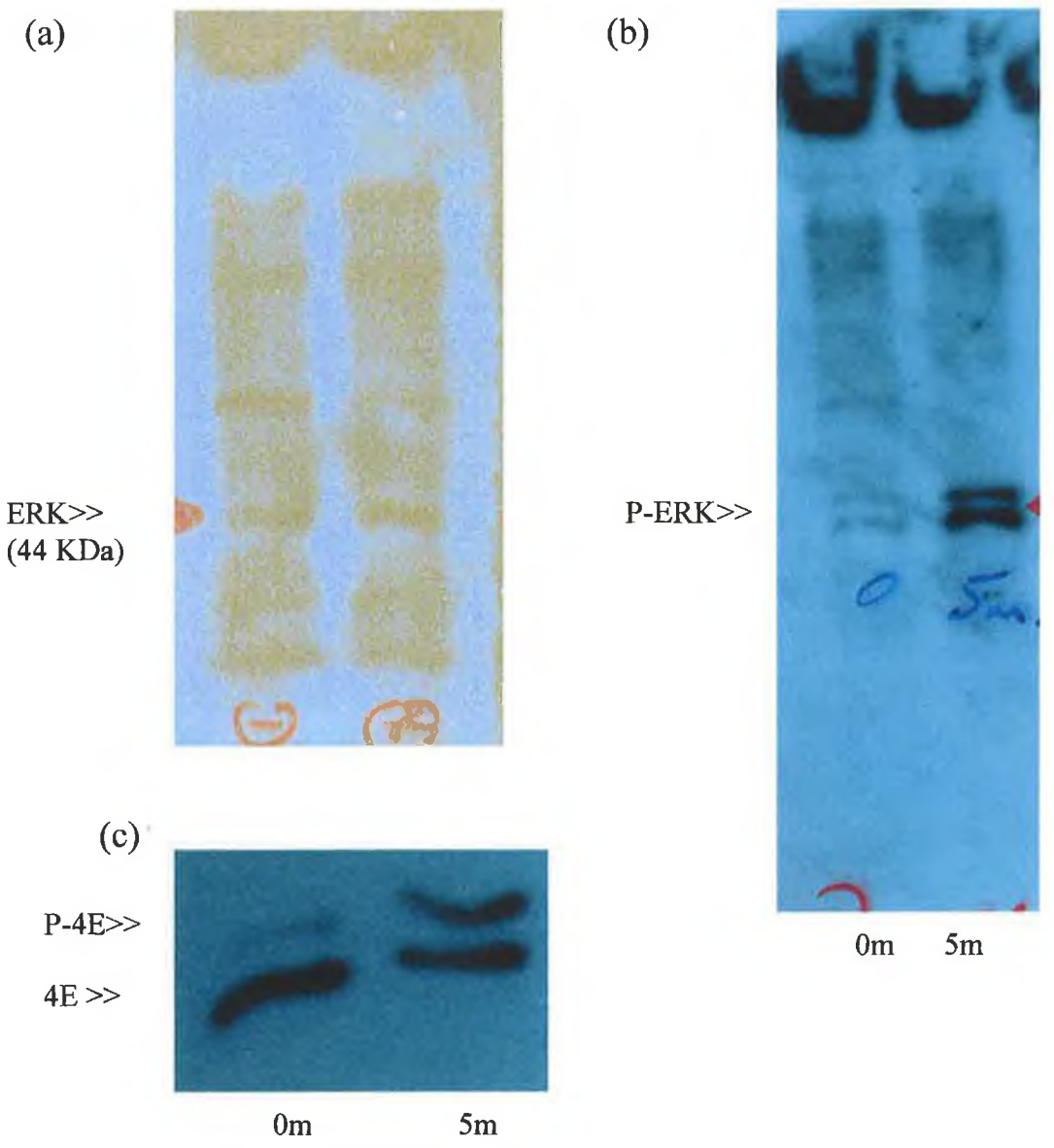
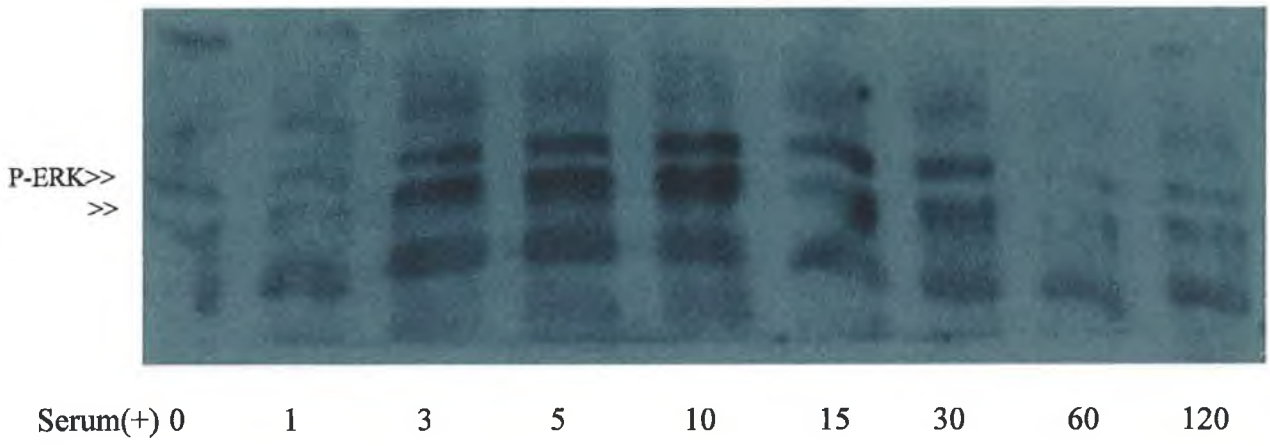
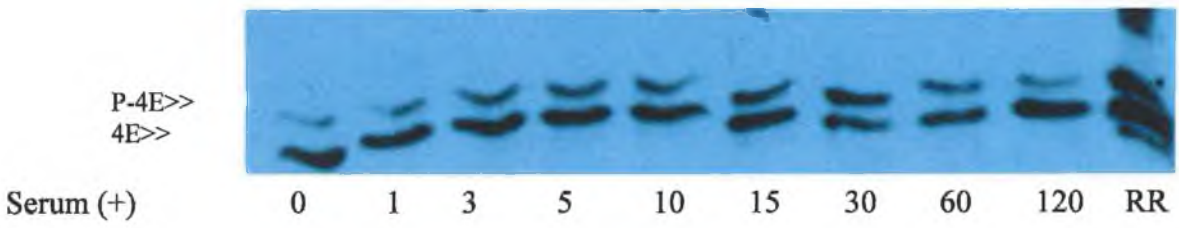



**Figure 3.6.1.** Re-stimulation of serum starved A549. After only 5 min (a) ERK levels in both stimulated and unstimulated samples are the same while (b) Phosphorylation levels of ERK increase as detected using phospho-specific antibodies (c) Re-phosphorylation of eIF-4E as assessed by IEF.

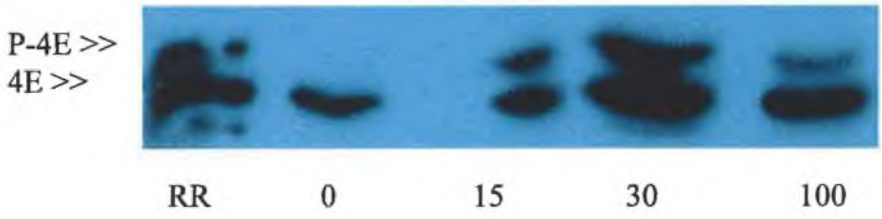



(a)

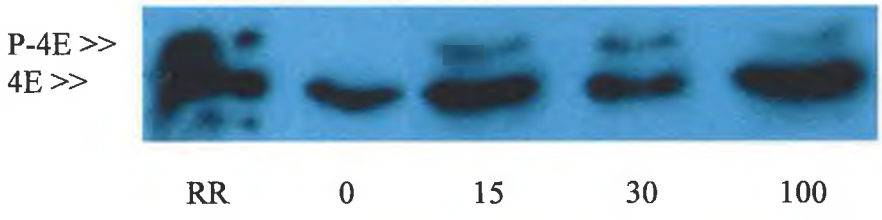



(b)

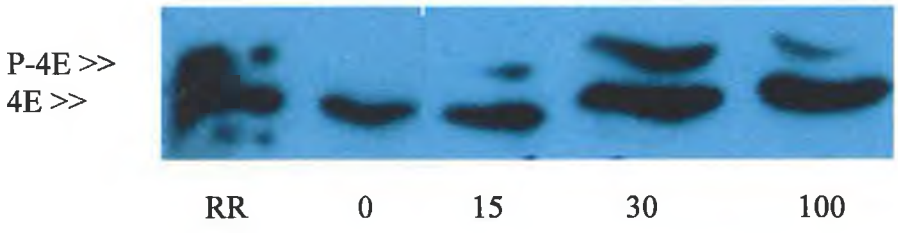
**Figure 3.6.2 Rephosphorylation Profiles for ERK and eIF-4E in serum stimulated A549.** (a) ERK phosphorylation exhibits a "classic" profile due to autoregulation. Doublet represents ERK1 (42 KDa) and ERK2 (44 KDa). (b) eIF-4E rephosphorylation peaks between 15-30 min and returns to normal by 120 min. Rabbit Reticulocyte lysate is used as a control and means of orientating gels.




(a) Serum Stimulation

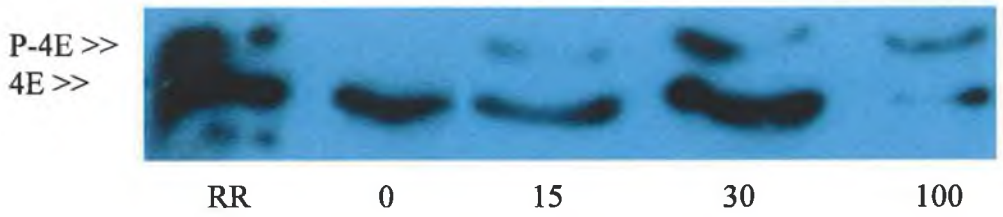



(b) PD98059

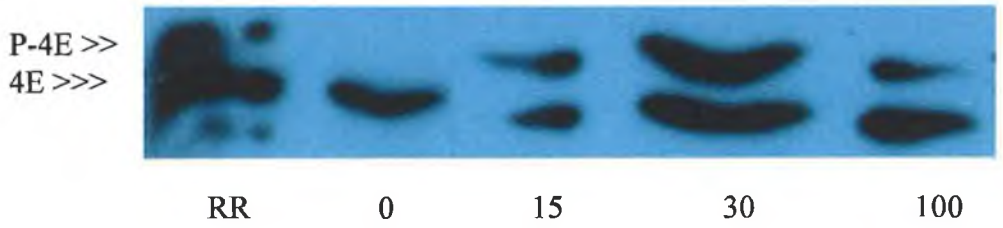



(c) SB203580

**Figure 3.6.3.1. The Effects of various specific kinase inhibitors on the Re-phosphorylation of eIF-4E in A549 cells.** (a) Serum stimulation induces rephosphorylation of eIF-4E. (b) PD980589, an ERK inhibitor, significantly reduces the rephosphorylation of eIF-4E. (c) SB203580, a p38 kinase inhibitor, slightly affects initial eIF-4E rephosphorylation, but has no significant or lasting effects.




(a) Wortmannin




(b) Rapamycin

**Figure 3.6.3.2 The effects of various specific kinase inhibitors on the rephosphorylation of eIF-4E.** (a) Wortmannin, an inhibitor of PI-3K, produces a mild inhibition of eIF-4E rephosphorylation. (b) Rapamycin appears to have no effect on the rephosphorylation of eIF-4E.

## Section 3.7 Retinoic Acid and Keratin Expression

Retinoic Acid (RA) was used to investigate the effects of physiological differentiating agents on keratin expression in the epithelial lines, A549 and DLKP. Initial attempts to treat cells with RA, using DMSO as a solvent, proved unsuccessful due to the fact that DMSO is a differentiating agent itself. Keratin expression was found in control treatments exposed only to DMSO (data not shown). All cells were exposed to a final concentration of 20μM RA, using 95% Ethanol as a solvent.

### 3.7.1 Growth profiles of cells grown in 20μM RA

Both DLKP and A549 exhibited significant growth inhibition when grown in the presence of 20μM RA. Results are presented in figure 3.7.1.

### 3.7.2 Immunocytochemistry for Keratin Expression

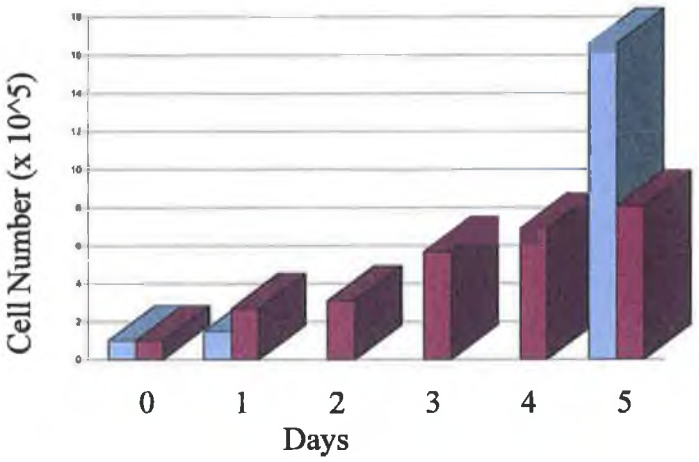
Immunocytochemistry (Section 2.8) on 6-day RA-treated epithelial cells was performed using monoclonal antibodies to K8 and K18 in order to investigate changes in keratin expression in epithelial lines exposed to RA.

#### 3.7.2.1 Keratin Expression in A549

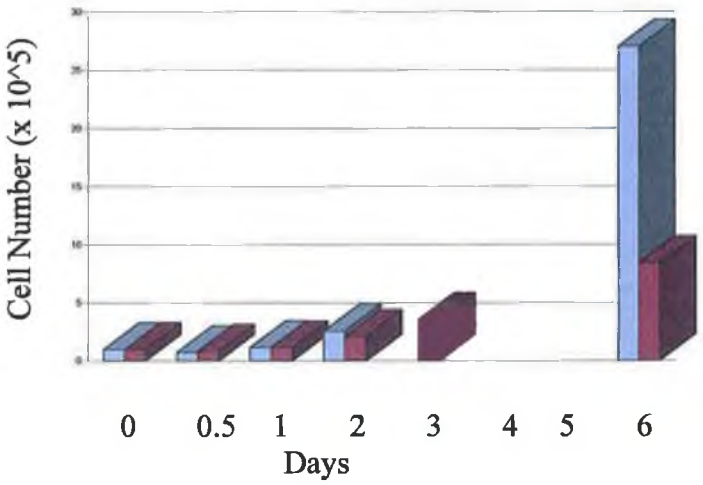
Retinoic Acid-treated A549 cells stained strongly for both K8 and K18 expression (figure 3.7.2.1a). On occasions a decrease in K18 expression was detectable (figure 3.7.2.1b). Keratin filaments were seen to radiate from the nucleus throughout the cytoplasm. Cells appeared to grow in loosely contacted colonies, similar to those described in other cell lines exposed to RA (Kopan et al, 1987).

#### 3.7.2.2 Keratin Expression in DLKP

Retinoic Acid-treated DLKP did not stain for either K8 or K18 expression. Unlike BrdU-treatment, RA did not appear to be capable of altering keratin expression in DLKP.

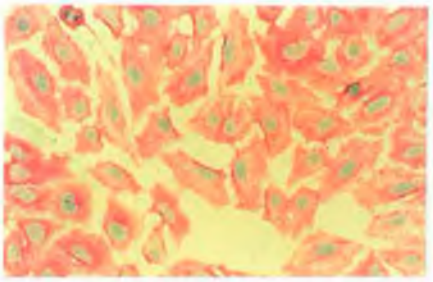



(a)

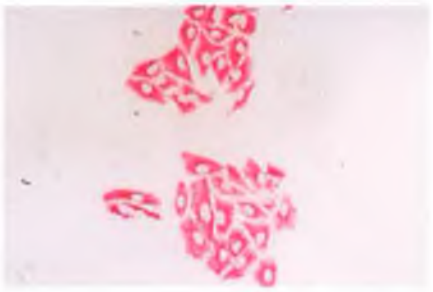



(b)

**Figure 3.7.1 Growth inhibition in Retinoic Acid-treated (a) DLKP and (b) A549.**
Untreated Control Cells are in blue (left)
RA-treated cells are in red (right)



(a)



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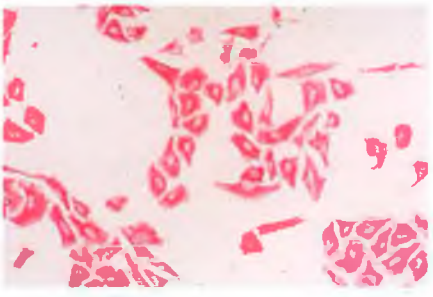
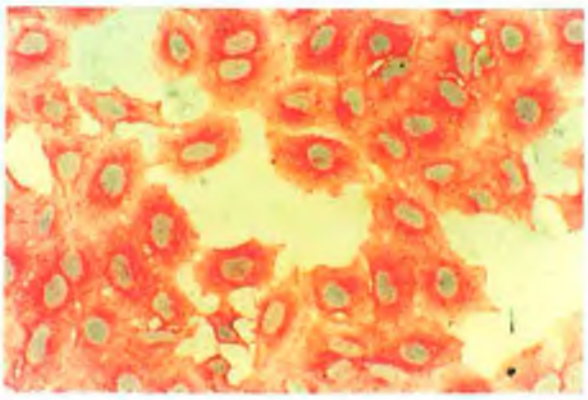
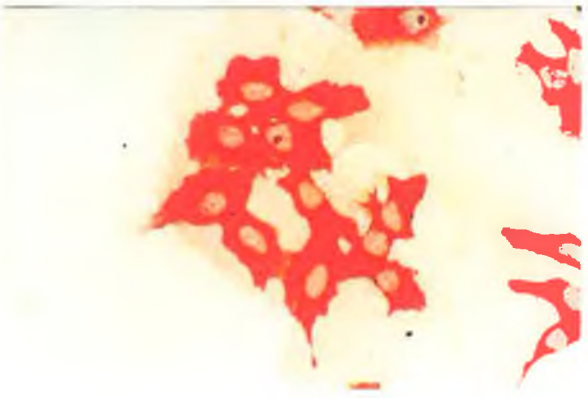


(c)

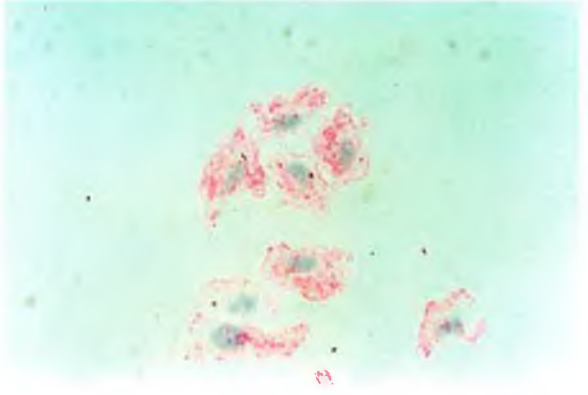
**Figure 3.7.2.1a Immunocytochemistry for Keratin expression in RA-treated A549.** (a) Untreated A549 cells stained with anti-K18 antibody. Cytokeratin 8 stains in the same manner (data not shown). (b) 6-day RA-treated A549 cells stained with anti-K18 antibody (c) 6-day RA-treated A549 cells stained with anti-K8 antibody.



(a) K8 Control

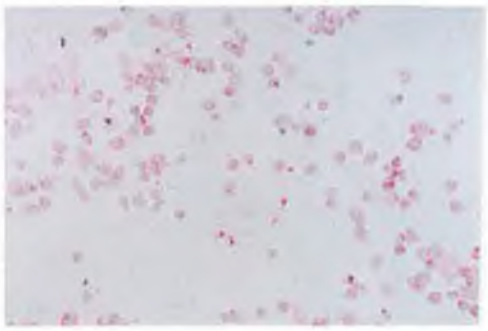


(b) K8 7-day RA



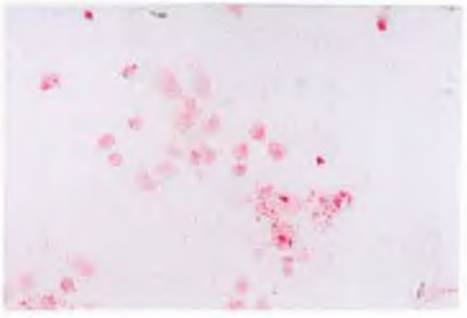
(c) K18 7-day RA

**Figure 3.7.2.1b Immunocytochemistry for Keratin expression in RA-treated A549.** (a) Untreated A549 cells stained with anti-K8 antibody. Cytokeratin 18 stains in the same manner (data not shown). (b) 7-day RA-treated A549 cells stained with anti-K8 antibody (c) 7-day RA-treated A549 cells stained with anti-K18 antibody.



(a)

(b)



(c)

**Figure 3.7.2.2. Immunocytochemistry for Keratin Expression in RA-treated DLKP.** (a) Untreated Control DLKP stained with anti-K18 antibody (Cytokeratin 8 stains in the same manner (data not shown). (b) 7-day RA-treated DLKP stained with anti-K8 antibody. (c) 7-day RA-treated DLKP stained with anti-K18 antibody. Slight background staining is evident in some cells.

### 3.7.3 Western Blot Analysis for Keratin Expression

In order to quantify the changes in keratin expression observed in cells upon treatment with 20μM RA western blot analysis was performed using the monoclonal antibodies, K8 and K18 (Sigma). All lanes are marked in terms of the number of days cells were exposed to RA.

#### 3.7.3.1 Keratin Expression in A549

Western blot analysis showed that K18 expression was decreased upon exposure to RA (figure 3.7.3.1b), while K8 expression consistently showed a decrease on day one, which was restored by day two and was either maintained or actually increased with continued exposure to RA (figure 3.7.3.1a). The levels of K18 expression are quite strong and blots exposed for relatively short periods show that K18 expression, while decreased, remain high in RA-treated A549 (figure 3.7.3.1c). This may explain the apparently conflicting immunocytochemical staining of K18 in RA-treated A549 compared to control cells (figures 3.7.2.1a & 3.7.2.1b). Morphological changes in cells can be misleading when protein levels are assessed by immunocytochemistry. As such, immunocytochemistry is never taken as completely quantitative in the absence of western blot analysis.

#### 3.7.3.2 Keratin Expression in DLKP

DLKP is a very poorly differentiated carcinoma, and as such expresses virtually no keratin proteins. Figure 3.7.3.2(a) shows that there is no induction of K8 expression upon exposure to 20μM RA as detected by Immunoprecipitation and western blot analysis. Increasing the cell number to $5x10^7$ cells per precipitation, the point where low level "leaky" keratin expression is detectable, shows that Keratin expression does not change in DLKP exposed to RA (figure 3.7.3.2(b)).

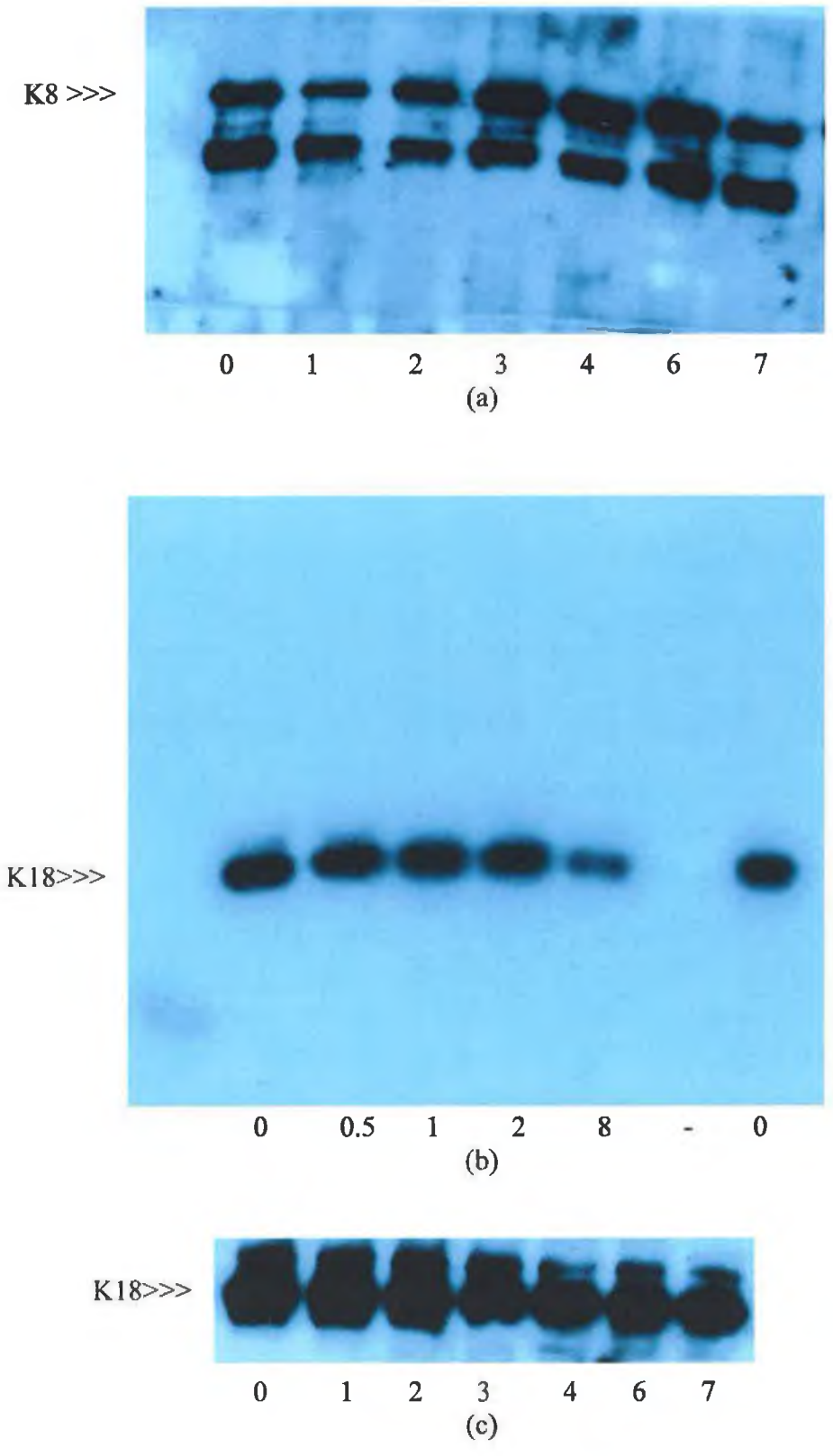
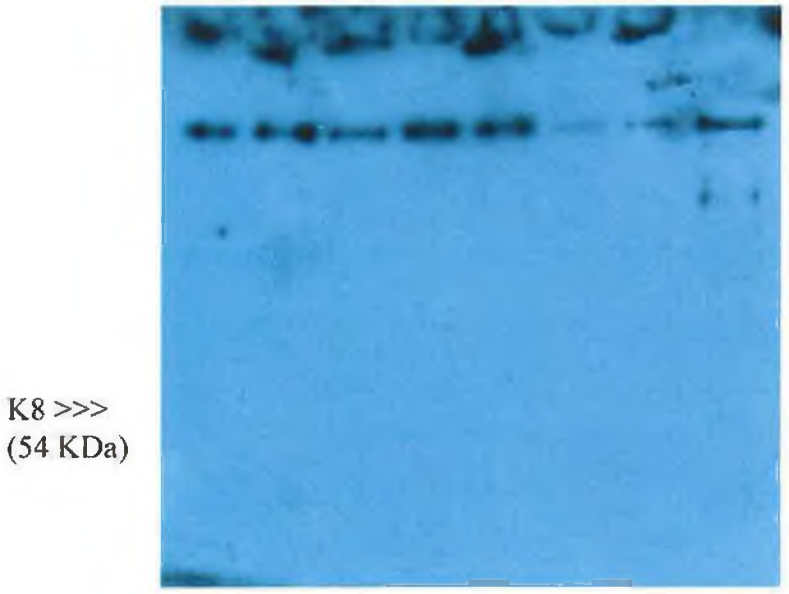
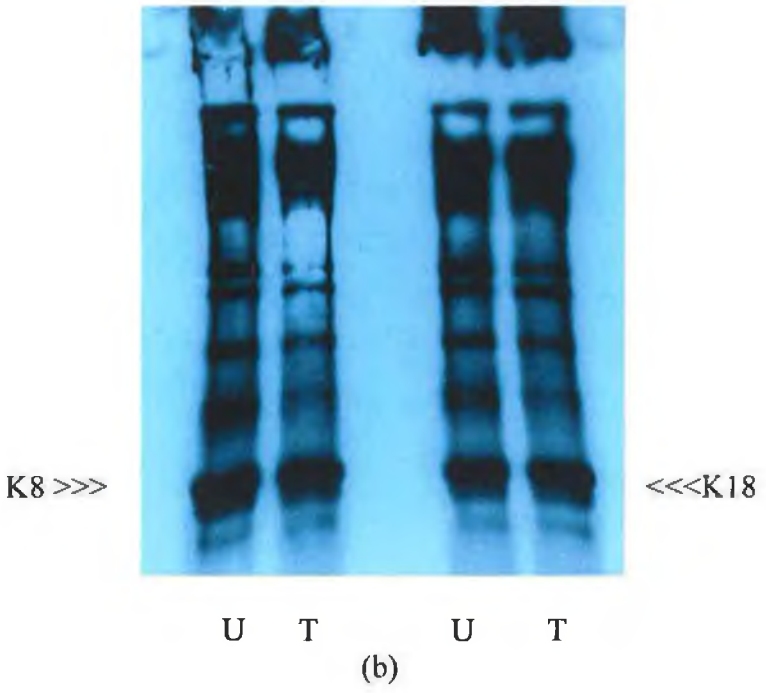



**Figure 3.7.3.1.** Keratin Expression in RA-treated A549. (a) Keratin 8 expression in RA-treated A549, (b) Keratin 18 Expression in RA-treated A549, (c) Keratin 18 expression in RA-treated A549. Numbers represent days of exposure to RA.




Note: C1=EGF antibody control C2=Lysis buffer control

**Figure 3.7.3.2 Immunoprecipitation of Keratins from RA-treated DLKP.**
(a) Immunoprecipitation of K8 from RA-treated and untreated DLKP. $10^6$ cells per precipitation. Numbers represent days of exposure to RA. (b) Immunoprecipitation of Keratins from 7-day RA-treated (T) and untreated (U) DLKP. $5x10^7$ cells per precipitation.

### 3.7.4 Northern Blot analysis of Keratin Expression

Northern blot analysis revealed that there was no change in keratin 8 mRNA levels in RA-treated A549 and DLKP (figure 3.7.4). Analysis was performed by Dr. Noel Daly. Even loading was confirmed by ribosomal RNA levels (not shown). K18 mRNA levels have been previously shown to be downregulated by RA in A549 (Ledinko and Costantino, 1990), in agreement with the findings reported here in relation to K18 protein expression.

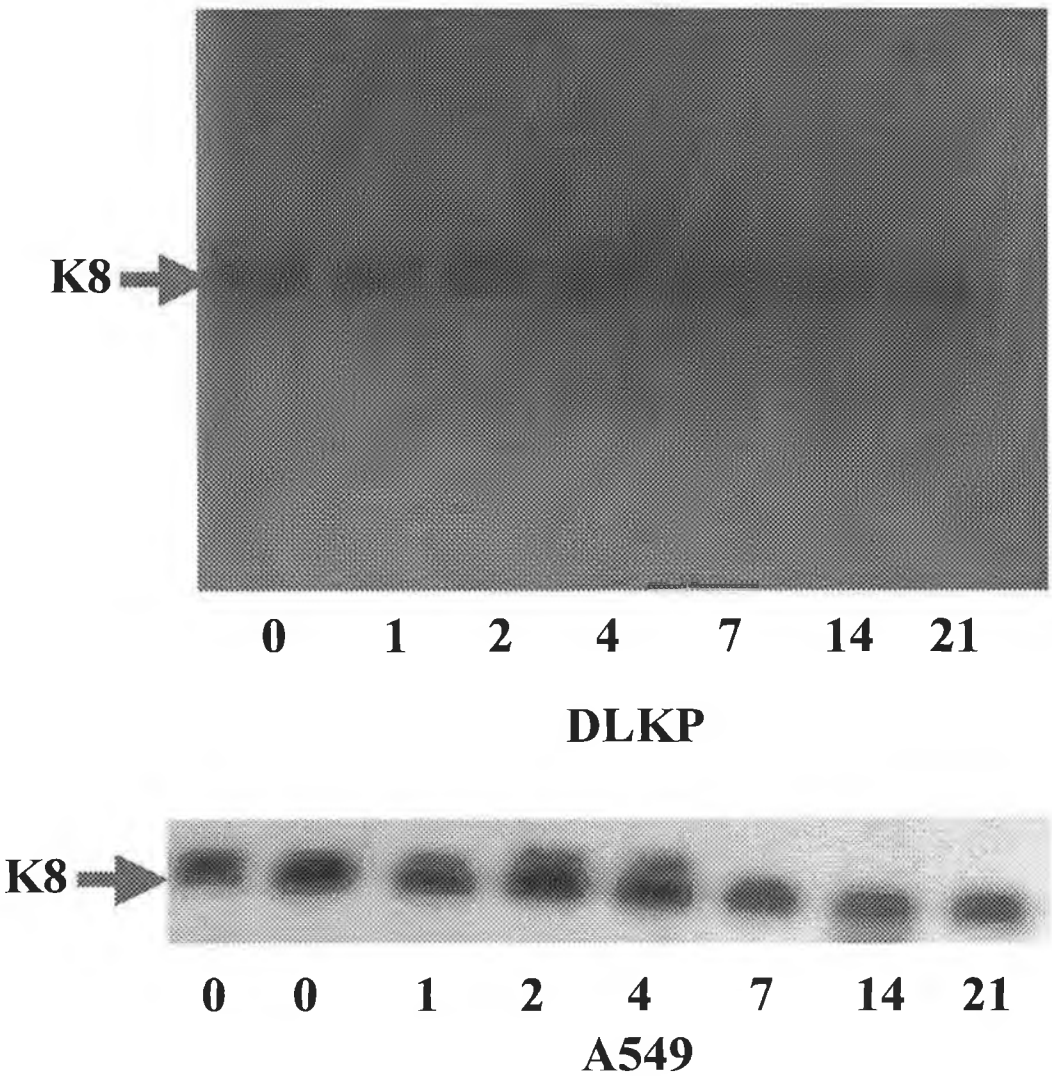



**Figure 3.7.4 Northern blot analysis of K8 expression in DLKP and A549 upon exposure to RA.** Northern blot analysis was performed by Dr. Noel Daly. Numbers represent days of exposure to RA.

### 3.7.5 Retinoic Acid Receptor (RAR) Analysis

In order to investigate possible mechanisms whereby these cells differ in their response to RA, the expression of two important Retinoic Acid Receptors (RARs) (Section 1.3.1), RAR-α and RAR-β, were examined in both cell lines.

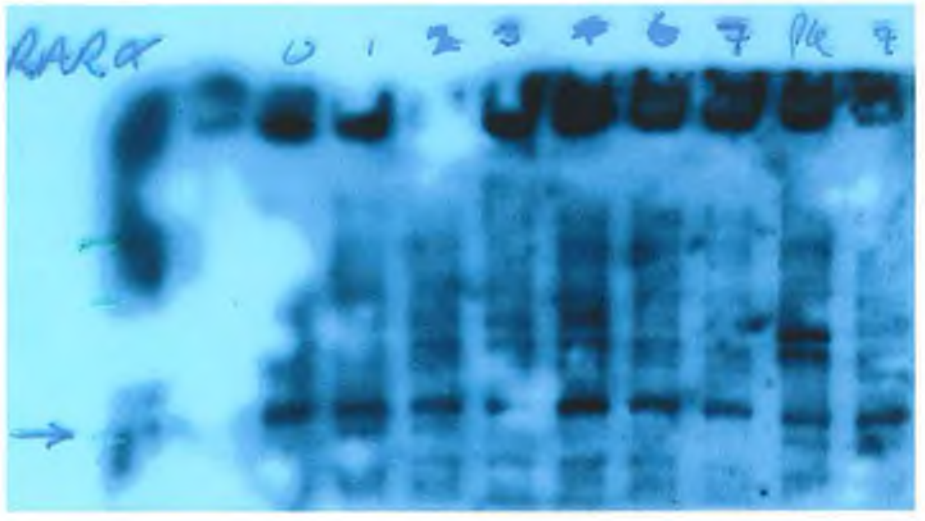
#### 3.6.5.1 RAR-α Expression in A549 and DLKP

Western blot analysis of RA-treated and untreated A549, as well as DLKP, showed that both cell lines expressed this receptor at relatively similar levels (Figure 3.7.5.1). RAR-α has been implicated in the mediation of growth arrest and at times induction into apoptotic pathways in RA-treated cells, and may explain the growth inhibition observed in both cell lines.

#### 3.7.5.2 RAR-β Expression in A549 and DLKP

Western blot analysis of RAR-β expression in RA-treated and untreated A549, as well as DLKP revealed that A549 expresses this receptor, which appears to be modestly upregulated upon exposure to RA (figure 3.7.5.2). RAR-β contains a RARE (Section 1.3.1) and is autoregulated, thereby increasing the response to RA in RA-responsive cells.

On the other hand, it would appear that DLKP lacks or expresses a truncated form of the RAR-β receptor (figure 3.7.5.2). A second sample of day-7 RA-treated A549 was loaded in the lane beside the DLKP sample to ensure that the absence of a band in DLKP was not due to defects in transfer during blotting or skewing of the gel. Longer exposure of blots revealed a lower band present in DLKP, at about 40-42 kDa. This may simply represent a background band, or alternatively, this may be a truncated form of the RAR-β receptor. In this case, it may be that the truncated form of the protein is inactive. The importance of RAR-β expression is discussed in section 4.4.2.

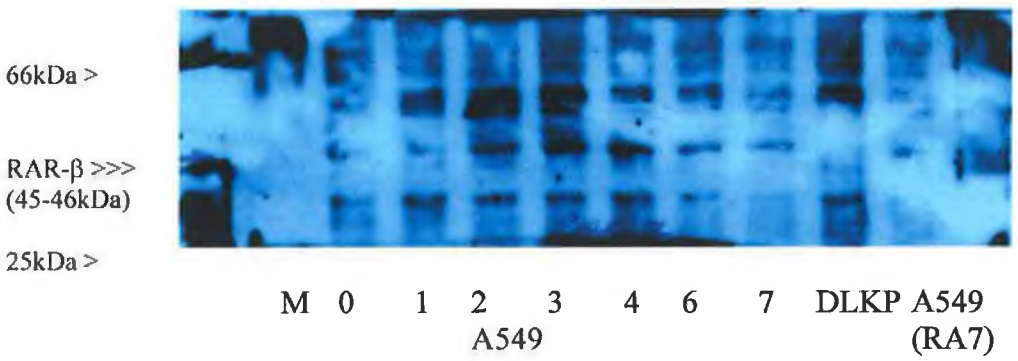



66kDa >>

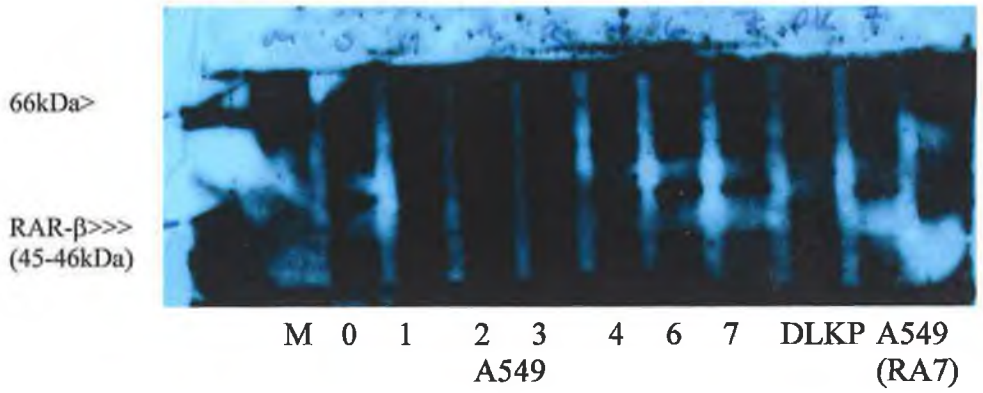
RAR-α>>
(46KDa)

M 0 1 2 3 4 6 7 DLKP A549 (RA7)
A549

**Figure 3.7.5.1 RAR-α Expression in RA-treated A549 and DLKP.** RAR-α is expressed in RA-treated and untreated A549, as well as in a sample of DLKP (Lane 9).




(a)




(b)

**Figure 3.7.5.2 RAR-β Expression in RA-treated A549 and DLKP**. (a) RAR-β is detectable in A549 and appears to be upregulated slightly upon exposure to RA. The receptor is absent in DLKP (lane 9). (b) Longer exposure reveals a band at about 40-42kDa. This may simply be background appearing due to longer exposure of film, or may represent a truncated form of this receptor in DLKP, which may be inactive. Numbers represent days of exposure to RA.

Section 4.0

# *Discussion*

## 4.1 General Introduction:

Developmental genetics itself is undoubtedly in its infancy, and the failure to identify a stem cell(s) of the lung (Emura, 1997) only acts as a further obstacle to progress in understanding the mechanisms regulating early development of the lung. We have been afforded a unique opportunity to study an *in-vitro* model for early lung development using a very poorly differentiated lung cancer cell line, DLKP, isolated here at the NCTCC (Law *et al,* 1992).

Recent advances have suggested that the role of translation in the regulation of gene expression may be very significant, particularly during development. To date, models are based largely on discoveries in Drosophila, *Xenopous* and *C.elegans* (Klein and Melton, 1994; Curtis *et al,* 1995; Vassalli and Stutz, 1995). Translation is now suspected to play critical roles in regulating precisely timed stages of development. While now accepted as an oncogene (DeBenedetti and Rhoads, 1990), the possible role of translation initiation factor, eIF-4E in regulating the differentiation process has gone largely unstudied. In addition, it has been suggested that a major function of transcription factors, such as c-*myc*, is to indirectly regulate the translational efficiency of the cell (Grandori *et al,* 1996). This could be a partial explanation for the lack of transcriptional targets identified for the enigmatic c-*myc* to date, which include two rate-limiting translation initiation factors (Rosenwald *et al,* 1993) and an RNA helicase (Grandori *et al,* 1996).

The work detailed in this thesis, using the poorly differentiated lung cancer cell line, DLKP highlights the complex inter-relationship between transcription and translation factors during differentiation. Deregulation of such intricate mechanisms is thought to result in the onset of cancer, a "disease of abnormal differentiation" (Sporn and Roberts, 1983). We propose a model for the regulation of simple keratin filament formation in early lung development under certain circumstances. Elucidation of such models will be critical to improving the overall understanding of lung development and the design of therapies to treat the greatest cancer killer of our time (Parker *et al,* 1996).

## 4.2 BrdU-induced Differentiation in Epithelial Cell Lines:

The speculation that the poorly differentiated NSCLC-NE/SCLC-V (Non-small cell lung carcinoma-neuroendocrine differentiation/Small cell lung cancer-variant) DLKP may represent a stem cell-like population of the lung provided a unique opportunity to study some of the possible mechanisms regulating early lung development *in-vitro*. Studies were carried out on DLKP, in addition to a more differentiated adenocarcinoma cell line, A549, exposed to the differentiating agent BrdU. Key findings were also investigated in the non-epithelial, leukaemic cell line, HL60, to identify possible cell-type specific effects observed during the differentiation process. Additional studies using the physiological agent, Retinoic Acid, are detailed in Section 4.4.

### 4.2.1 Morphological Changes during BrdU-induced differentiation:

When both the adenocarcinoma cell line, A549, and the very poorly differentiated cell line, DLKP were exposed to 10μM Bromo-deoxyuridine, induction of a differentiated phenotype was evidenced by changes in morphology, including cell flattening and enlargement (Figures 3.1.1.1. and 3.1.1.2.), accompanied by increased protein content per cell (Figure 3.2.8) and decreased growth rate (Figure 3.1.2). In order to assess whether or not a terminal differentiation was induced, these cells were initially treated for 7 days to induce differentiation, followed by removal of BrdU and continued feeding of these cells in fresh medium (Figure 3.1.1.3). Removal of BrdU results in what appears to be a gradual reversion towards the parental phenotype. Reversion occurs in the absence of passaging, reducing the possibility of a simple dilution of terminally differentiated cells upon passaging by outgrowth of less differentiated or unresponsive cells.

Over the first 10-14 days the reversion is rather slow and growth rates (assessed by consumption of medium and passaging requirements) are relatively low over this period. This is presumably due to the need for the cells to "dilute" out the BrdU incorporated into their DNA through cell division. Growth rates are slow at first, but as cells divide in the absence of BrdU the effect is gradually depleted. By day 30 of removal of BrdU, cells

have all but reverted to "normal" morphology and growth rate. This, combined with the fact that continued exposure to BrdU appears to maintain morphological characteristics of differentiation in DLKP would suggest that BrdU does not induce a terminal differentiation but probably a pre-commitment to differentiate. BrdU has been suggested to induce pre-commitment to differentiation in a leukaemic cell line (Yen and Forbes, 1987) and can replace replace Retinoic Acid (RA) over the first 24 hrs. (i.e. 48 hour treatment with RA is the same as 24 hour with BrdU followed by 24 hr with RA). Therefore, in HL60 cell differentiation BrdU appears to be capable of inducing some of the early effects induced by RA, and as such may act to "prime" cells for differentiation.

### 4.2.2 BrdU induces Simple Keratin Filament formation:

Immunocytochemical analysis of DLKP with anti-keratin antibodies showed that it lacks the expression of many of the major keratins, including keratins 8 and 18 (Section 3.1.3.2), which would be indicative of simple epithelial differentiation (Daly *et al,* 1998). This is in agreement with its characterisation as a very poorly differentiated, stem cell-like NSCLC-NE/SCLC-V cell line. K8 and its partner K18 are the first intermediate filaments expressed during mouse development (Casanova *et al,* 1995). While heterogenous and weak expression of keratins is often found in many SCLC lines, they are readily detectable by western blot analysis (Elias *et al,* 1988). Western blot analysis failed to detect keratin expression in DLKP, until immunoprecipitation with very high cell numbers was used ($10^7$ cells) (Section 3.1.4.3). Treatment with BrdU, however, induced about 10-15% of DLKP cells to express the simple keratins 8 and 18, as determined by both immunocytochemistry (Section 3.1.3.2) and immunoprecipitation (Section 3.1.4.3). The immunocytochemical staining of BrdU-treated DLKP was strong and shows the formation of keratin filament structures radiating through the cytoplasm. It was necessary to develop immunoprecipitation techniques to quantify keratin expression in DLKP due to the fact that BrdU induced expression in only about 10-15% of these cells (Section 3.1.4.2). The extremely low-level keratin expression found in untreated DLKP suggests either leaky expression or degradation products that do not form filaments detectable by immunocytochemistry. The more differentiated cell line, A549

expresses moderate to high levels of keratin protein, which is upregulated upon exposure to BrdU. This was shown using immunocytochemistry (Section 3.1.3.1) and western blot analysis (Section 3.1.4.1).

Prolonged exposure of both cell lines to 10μM BrdU results in further morphological changes and increased staining intensity for both K8 and K18 in DLKP, with slight but not highly significant increases in the percentage of cells induced (Section 3.1.3.4). These findings were verified when immunoprecipitation was extended to 21 days (Figure 3.1.4.3.2). In order to demonstrate that this was not a selective "cloning" of BrdU-responsive cells, two of the clonal populations identified by McBride et al (1998) were treated with 10μM BrdU and assessed for keratin expression using immunocytochemistry. Both DLKP-SQ and DLKP-I clones proved to be inducible (Section 3.1.3.5). In the case of A549, while treatment resulted in further morphological changes in these cells, becoming more stretched and beginning to "bridge" one another, the apparent increased staining intensity of these cells for K8 and K18 (Figure 3.1.3.4) was not observed in western blot analysis (Figure 3.1.4.1), which suggested that K8 and K18 expression in these cells reaches a maximum after only 3-4 days. While expression remained elevated throughout the study, the increase reached a maximum within a few days. A549, unlike DLKP, already express keratins and an explanation for this effect is offered in section 4.2.3.3.3. The relative extent of differentiation of both cell lines is highlighted and discussed in Section 5.0.

In addition, immunocytochemical analysis of DLKP treated with BrdU extended over three months showed a weak induction of K8 expression in approximately 70% cells, with a small percentage still exhibiting strong keratin induction (Figure 3.1.3.3.3). More interestingly, immunocytochemical analysis of DLKP initially treated with BrdU for 7 days and then grown in its absence for 3 months (Section 4.2.1) showed that these cells retained the pattern of keratin expression (Figure 3.1.3.3.4) observed in BrdU-treated cells. These results suggest that BrdU is an “irreversible” maturational inducer of DLKP, in agreement with findings reported by Feyles et al (1991) using a SCLC cell line, NCI-H69. It would appear therefore, that the induction of differentiation and growth inhibition

upon exposure to BrdU (Section 4.2.1), at least in these cells, may occur by separate mechanisms. BrdU may be capable of inducing a specific pathway(s) that commits these cells to an irreversible differentiation, but induces additional growth-arresting or cytostatic effects that can be reversed upon withdrawal of BrdU. A decrease in cyclin A and increase in p21 expression in human melanoma cells upon exposure to BrdU suggested that BrdU is capable of arresting these cells at a $G_1$ transition point in the cell cycle (Rieber *et al,* 1996). Attempts to detect p21 expression in BrdU-treated DLKP and A549 failed due to problems with the antibody used (Santa Cruz) (data not shown).

Induction of keratin intermediate filament proteins K8 and K18 is indicative of simple epithelial differentiation (Section 1.2). The pattern of expression of keratin intermediate filaments (IFs) has been shown to alter with the differentiation status of the epithelial cell type. For example, as the dermal layer of the skin develops, the pattern of keratin filament expression changes in epithelial cells as they migrate to the surface of the skin, reflecting their change in function and differentiation status (Tseng *et al,* 1982; Fuchs and Byrne, 1994). A non-epithelial leukaemic cell line, HL60 was used to determine the specificity of induction of keratin expression in epithelial versus non-epithelial cell types. Figure 3.1.4.4 shows that there was no detectable keratin expression in either treated or untreated HL60s using immunoprecipitation, suggesting that keratin induction by BrdU may be epithelial-specific.

#### 4.2.2.1 BrdU Induces Keratin Expression at a post-transcriptional level:

To examine the level at which keratin expression was induced in these cells, Northern blot and PCR analysis was performed to measure transcript levels for both K8 and K18. Surprisingly, in both DLKP and A549, despite significant changes in protein expression, there was no change in the levels of message for either K8 or K18 in either cell line (Figure 3.1.5). This indicated that the level of induction of simple keratin expression in these epithelial cells was post-transcriptional. Even more surprisingly, both K8 and K18 transcripts were detectable in DLKP, albeit at a lower level than that for A549. This

suggested that proteolytic instability due to the absence of one partner keratin (Section 1.2) was unlikely to be a factor, unless one of the transcripts was not being translated.

The majority of research into the regulation of keratin expression during differentiation has focused at the level of transcription. A number of researchers have identified elements in numerous keratin genes that appear to be important in cell-specific regulation of their expression. Oshima et al (1990) identified an AP-1 site involved in the regulation of K18 expression by c-Jun and c-Fos (Section 1.5.2.1). In addition, it was suggested that low levels of K18 expression in undifferentiated F9 cells may be due to low levels of both AP-1 factors, that are increased during Retinoic Acid-induced differentiation. The K18 gene has been reported to contain a RA-responsive Alu element (Vansant and Reynolds, 1995). Seven expression-specific DNA sites for protein binding were identified in the K18 gene (Neznanov and Oshima, 1993), two of which were identified as subject to differential methylation in expressing and non-expressing tissues. An enhancer element suggested to regulate cell type and differentiation-specific expression was located 3' to the human K1 gene (Huff *et al,* 1993; Rothnagel *et al,* 1993) that conferred calcium sensitivity. The promoters of K5, K14 and K17 were shown to contain elements that were direct targets for transcriptional regulation by both Retinoic Acid and thyroid hormone receptors (Tomic-Canic *et al,* 1996). The complexity of keratin regulation in mammalian tissues is highlighted by the fact that at least five DNA sites that specifically bind nuclear proteins have been identified in the K5 gene alone (Ohtsuki *et al,* 1992). Of these, two activate transcription, one inhibits it, and the remaining two are of unknown function. The cell type- and differentiation-specific expression of keratins is more than likely an extremely complex affair, involving the interaction of both negative and positive regulatory elements. Expression will depend on the cellular complement of enhancers that bind to these regions. In the type II keratin, K8, important control elements are thought to lie within the body of the K8 gene itself (Casanova *et al,* 1995). In addition, methylation (Casanova *et al,* 1995) and labile repressors of transcription (Cremisi and Duprey, 1987) have been implicated in cell-specific expression of keratins.

A number of post-transcriptional mechanisms regulating keratin expression have been proposed (Section 1.2.1), including post-transcriptional down-regulation of K5 (type II) mRNA levels (Paine *et al,* 1992). Since both K8 and K18 mRNAs are detectable this suggests that such mechanisms are not involved in the inhibition of keratin filament formation in DLKP, especially in light of the fact that there are no changes in mRNA levels to accompany the induction of keratin expression upon exposure to BrdU. In addition, proteolytic degradation, reported by Kulesch et al (1989), is not likely to play a role since both transcripts are present, and if translated efficiently stable filaments should form. In agreement with this, treatment of DLKP with a cocktail of protease inhibitors at 0.5, 1 and 2x concentrations recommended for such studies (Roche, #1-697-498) could not induce keratin expression in these cells (Dr. Noel Daly).

The absence of keratin expression in poorly differentiated cell lines such as DLKP may represent very early developmental mechanisms regulating keratin expression in lung epithelia or a mechanism whereby tumours downregulate their differentiated phenotype. To further investigate the induction of keratin expression in both DLKP and A549, the effects of BrdU on the translational apparatus of the cell were investigated.

### 4.2.3 BrdU and its effects on Translation:

In light of the mechanism by which Bromo-deoxyuridine exerts its effects on cells it is not surprising that research to date has focused on the transcriptional effects of this drug. BrdU is a Thymidine analogue that competes with naturally occurring Thymidine for incorporation into DNA, where it alters promoter behaviour and ultimately the transcription of targeted genes (Section 1.3.2). However, recent reports on the role of translation in the regulation of differentiation and development (Luis *et al,* 1993; Wormington, 1993; Curtis *et al,* 1995) highlight the possible importance of translational regulation of gene expression in the control of such processes. To date, no-one has extended studies with differentiating agents such as BrdU to determine the downstream effects of transcriptional activation upon the translational apparatus of the cell. In addition, few studies have investigated the role of translation in the regulation of processes such as lung cancer differentiation, particularly in poorly differentiated lung cancers. This is despite the suggestion that, in type II cells of the lung, growth factors might regulate the translation rather than the mRNA abundance of at least some growth-related genes and that this ability to respond to translational control may be developmentally regulated (Clemens *et al,* 1990).

#### 4.2.3.1 BrdU and its effects on translation Initiation Factor eIF-4E:

eIF-4E (Section 1.6.3.2.4.3) is the only specific mRNA-binding protein of the translation initiation complex and provides the cap-binding specificity of the eIF-4F complex. It is present in limiting concentrations and is a key factor in translation initiation, the primary target for regulation of translation rates. This regulation occurs through complexity in mRNA structure and binding of repressors proximal to the cap. While much is now understood regarding the roles of such RNA complexity and eIF-4E expression/activity in the regulation of cellular growth, little is known about their roles in differentiation.

#### 4.2.3.1.1 Altered eIF-4E expression in differentiating cell lines:

Examination of the levels of eIF-4E in BrdU-treated epithelial lines showed a marked increase in the expression of this protein over time (Figure 3.2.2(a)&(b)). The induction in response to BrdU-treatment is very rapid with significant changes in expression within one day. This makes it very difficult to pin down exact timing of induction of eIF-4E in relation to other factors described in this study, since the costs to examine induction within hours of treatment would be prohibitive and extremely labour intensive. In any case, the sensitivity of methods used to detect the expression of both mRNAs and proteins may not be precise enough to determine with certainty which factor appears first, with this short timeframe.

Immunocytochemical analysis of eIF-4E in both A549 and DLKP revealed some differences in the pattern of expression of this protein. While in A549 there is a universal increase in eIF-4E distributed amongst all of the cells (Figure 3.2.1.1), in BrdU-treated DLKP there appears to be a percentage of cells that stain far more intensely than other cells (Figure 3.2.1.2). Due to the morphological changes in BrdU-treated cells it is difficult to assess by immunocytochemistry alone if all cells have increased expression of eIF-4E. Many of the treated DLKP cells exhibit a similar staining intensity to those of untreated cells, but they are significantly larger than the untreated cells. Does this mean that there are elevated levels of eIF-4E in these cells but that it is simply distributed over a larger area in the cell, or is there no change? It is difficult to say.

Either way, immunocytochemistry tells us that a percentage of DLKP cells very similar to that induced to express keratins, are also induced to express very high levels of eIF-4E. Co-immunocytochemistry has so far failed (perhaps due to incompatibility of co-incubation of the antibodies available) to tell us whether or not this effect on both keratins and eIF-4E is coincidental or actually indicative of a relationship between the two. It is not unreasonable to imply that it is indeed those cells induced to overexpress eIF-4E that are also induced to express keratin filaments, especially in light of the fact that eIF-4E is universally upregulated in BrdU-treated A549 cells, also reflecting the

pattern of upregulation of keratins in these cells. It is hoped that continued modification of conditions for co-immunofluorescence will help to answer this question. It will, however, not establish a direct link between keratin expression and eIF-4E levels. More direct evidence was provided by the induction of keratin expression in DLKP when transfected with the eIF-4E cDNA (Section 3.5.1). A mechanism by which eIF-4E induces keratin expression is proposed in section 4.2.3.3.3.

In the leukaemic cell line HL60, the levels of eIF-4E dramatically decreased upon exposure to BrdU (Figure 3.2.2(d)). This may correlate with a decrease in translation as this cell line commits to differentiation. Bloume et al (1999) reported significant decreases in both transcriptional and particularly translational efficiency, together with decreased size during erythrocyte differentiation. This is reflected in a visual decrease in the size and protein content (Figure 3.2.8) of BrdU-treated HL60s, attributable most likely to similar transcriptional and translational decreases in these cells.

Overexpression of eIF-4E has been shown to selectively upregulate the translation of mRNAs with complex 5' UTR secondary structures (Rosenwald *et al,* 1995; Kevil *et al,* 1996; Rousseau *et al,* 1996). In an attempt to assess eIF-4E activity during epithelial differentiation, the expression of one of these, Ornithine Decarboxylase (ODC), was examined by western blot analysis. Unfortunately, the antibody (Sigma) constantly produced high levels of background non-specific binding, making it impossible to decipher any sort of true result (data not shown). A role for elevated eIF-4E expression during the differentiation of DLKP is outlined in section 4.2.6.1.

#### 4.2.3.1.2 Transcriptional induction of translation initiation factor, eIF-4E?:

To date, both RT-PCR (Section 3.2.3) and Northern blotting (3.2.4) analyses suggest that there is little change in eIF-4E mRNA levels in the BrdU-treated epithelial lung cancer cell lines, DLKP and A549. However, DLKP expresses high levels of eIF-4E, evidenced by both Western blot (Figure 3.3.2) and Northern blot signals (Section 3.2.4), while it appears to be only induced to express significantly higher levels of the protein in

approximately 10-15% cells by immunocytochemistry (Figure 3.2.1). This may mean that changes in expression in this system are beyond the sensitivity of conventional methods for the determination of transcript levels within cells (RT-PCR/ Northern Blotting). In A549, in which the response appears more universal, RT-PCR analysis suggests that there may be an increase in eIF-4E transcript levels in BrdU-treated cells (Section 3.2.3). It is possible that analysis of gene transcription using more sensitive techniques (Nuclear Run-on/ RNase protection assays) may provide evidence for altered transcription of the eIF-4E gene in BrdU-treated cells. However, conventional methods suggest that induction of eIF-4E by BrdU is not at the transcript level. An alternative mechanism by which eIF-4E may be induced, at a post-transcriptional level, is outlined in the following section (Section 4.2.3.1.3).

#### 4.2.3.1.3 Investigation of eIF-4E phosphorylation levels:

Vertical slab iso-electric focusing (IEF) (Section 2.11) of samples from both DLKP and A549 revealed a very interesting shift in the phosphorylation status of eIF-4E in BrdU-treated cells from predominantly non-phosphorylated towards and possibly even in favour of the phosphorylated form of the protein (Figure 3.2.5). This indicates that not only is there an increase in eIF-4E expression in differentiating lung cancer lines DLKP and A549, but there is also a shift in the phosphorylation ratio towards the more active form of eIF-4E. These cells exhibit elevated expression of integrins (Meleady and Clynes, in preparation), which are signalling and attachment factors involved in metastasis and differentiation. Integrins signal through both Rho and Ras kinase pathways (Schlaepfer and Hunter, 1998). Examination of ERK, the downstream kinase of Ras, showed a small but significant increase in the levels of active, phosphorylated ERK in A549 upon exposure to BrdU (Figure 3.2.6.). ERK exhibits a high degree of autoregulation (Cook *et al,* 1997; Frost *et al,* 1997), even in the presence of activated upstream kinases, that may prevent it from becoming excessively phosphorylated in situations where upstream signalling from integrins or other signalling factors is elevated. Serum stimulation studies (Section 4.3) revealed that ERK was indeed capable of autoregulation in A549, despite the fact that A549 has been reported to harbour Ras

mutations (Mitsudomi *et al,* 1991) (Section 1.6.4). In the case of DLKP, no significant increases in ERK activity were detected in BrdU-treated cells (Figure 3.2.6). We attribute this to the suspected high level expression of autocrine growth factors in this cell line (Section 4.3), in which case the additional stimulus of increased integrin expression may not have significant/detectable effects on ERK activity. It is possible that the increase in integrin expression in BrdU-treated DLKP and A549 (Meleady and Clynes, in preparation) may result in stimulation of eIF-4E phosphorylation, which in turn enhances the translation of eIF-4E mRNAs, which are complex and suggested to be heavily dependent on the availability of phosphorylated, active eIF-4E (Lavoie *et al,* 1996). This may explain the failure of both RT-PCR and Northern blotting to detect significant changes in eIF-4E transcript levels (4.2.3.1.2).

Interestingly, it has been suggested by eIF-4E overexpression studies that the kinase involved in eIF-4E phosphorylation has the catalytic capacity to accommodate higher amounts of eIF-4E, but that the phosphatase that controls its dephosphorylation does not (DeBenedetti and Rhoads, 1990). Therefore, increased signalling may not be necessary to achieve the observed effects on eIF-4E phosphorylation in eIF-4E overexpressing lines, and may not be the initiator of these events. We have provisional evidence for the increased expression of the translation initiation factor, eIF-2$\alpha$ (Section 3.4.1.1). Induction of this factor, a regulator of global translation (Kimball *et al,* 1998) may result in translational increases in eIF-4E, initiating the increase in eIF-4E which is then phosphorylated, allowing selective translation to begin, including further and selective increases in eIF-4E itself.

The leukaemic line, HL60 showed a predominance of non-phosphorylated eIF-4E in control cells (Figure 3.2.5). However, upon differentiation, the decrease in eIF-4E observed by western blot appeared to be predominantly in the non-phosphorylated form of the protein. This may reflect a basal requirement of the cell for phosphorylated and active eIF-4E for survival, or may simply imply that the high levels of eIF-4E in undifferentiated HL60s are largely redundant or surplus to cellular requirements for general household translation. These results would indicate that, while there is probably a

decrease in translation initiation rates in these cells, it is not likely to be as dramatic as western blotting would predict (Figure 3.2.2 (d)).

#### 4.2.3.2 eIF-4F complex formation:

eIF-4F complex formation (Section 1.6.3.2.4) is determined by competition between eIF-4G and 4E-BPs for association with eIF-4E. The levels of eIF-4G do not appear to change significantly in DLKP (Figure 3.2.7.2). However, there appears to be a slight increase in eIF-4G in differentiating A549s (Figure 3.2.7.2). Detection of these slight changes may be more difficult in DLKP if, as suspected, only a small percentage of cells are reacting so dramatically to BrdU exposure. To date, a commercial antibody (Santa Cruz) has failed to satisfactorily detect 4E-BP1 (Section 1.6.3.3.4.4) expression in these cells. However, a private source (Dr. Nahum Sonenberg, Montreal) of 4E-BP1 antibody has shown more promise (Section 3.2.7.3). Conditions are currently being refined to improve the quality of blots obtained using this antibody, by using extended gel systems to allow improved resolution. Preliminary western blotting analyses, while unclean, suggest that 4E-BP1 levels and phosphorylation remain unaffected during BrdU-induced differentiation (Figure 3.2.7.3). This is in agreement with serum stimulation studies using the specific FRAP/mTOR kinase inhibitor, Rapamycin (Section 4.3), which suggests that 4E-BPs may not play a significant role regulating eIF-4E phosphorylation in A549. A number of others have reported such findings (citations within Fraser *et al,* 1999), suggesting that 4E-BPs may not regulate eIF-4E phosphorylation in all cell systems or under all circumstances. 4E-BPs may play roles under different circumstances, but it may be that some cell types, perhaps poorly developed cells in particular, show little dependence on 4E-BPs for eIF-4E regulation. More detailed examinations of the levels of 4E-BP1 expression and activity, in addition to FRAP (the 4E-BP1 regulatory kinase) activity in differentiating cells would be an important future direction for the research program.

Correlations have been reported between phosphorylation of eIF-4E, association of eIF-4E with eIF-4G protein, and enhancement of RNA helicase activity (Fukuchi-Shimogori

*et al,* 1997). While phosphorylation of eIF-4E is not a direct indicator of eIF-4F formation (Dr. Simon Morley, personal correspondence), it is a good indication thereof (see also; Section 4.2.3.1.3).

#### 4.2.3.3 *In-Vitro* Translation:

*In-vitro* translation is a technique used to closely examine the regulation of translation (Bablanian and Banerjee, 1986; Wu *et al,* 1993; Ohlmann *et al,* 1996). The advantage of these systems is that they allow a greater control of the environment, and while they are artificial in the sense that the experiments are removed from the cellular environment, they are a useful tool to investigate the finer details of translation control. *In-vitro* translation has been used to confirm the inhibitory effects of proteins on the translation of specific mRNAs (Bhasker *et al,* 1993; Ostareck-Lederer *et al,* 1994) and examine the roles of factors in controlling translation initiation (Rau *et al,* 1996; Svikin *et al,* 1996). These systems were used to further pursue the possibility that simple keratin protein expression may be regulated at the translational level during early lung development. A number of developmentally important genes appear to display a high degree of translational regulation.

##### 4.2.3.3.1 Development of *in-vitro* Translation (IVT):

The *in-vitro* translation systems most commonly used are Rabbit Reticulocyte Lysate and Wheat Germ extracts; Commercial sources (Promega and Boehringer) were used (Section 3.3). It was decided to use RNA from A549 to develop this technique, as A549 cells express relatively high levels of K8 mRNA that is translatable *in-vivo,* and as such should also be translatable *in-vitro*. Initial attempts to translate total RNA isolated from A549 cells in both systems for subsequent analysis by western blotting proved problematic (Section 3.3.1.1). It was decided to adapt the immunoprecipitation procedure developed to detect keratin expression in DLKP (Section 3.1.4.2) as a "clean-up" step to try to reduce this background interference (Section 3.3.1). Having determined that RRL systems produced the best results, an optimal concentration of 1μg total RNA per

reaction mixture was determined (Section 3.3.2) for use in further studies on keratin regulation.

#### 4.2.3.3.2 A Putative Translational Repressor of Keratin Synthesis in DLKP:

RNA from A549 cells, known to be translatable both *in-vivo* and *in-vitro* (Section 4.2.3.3.1), was significantly inhibited in its ability to synthesise K8 protein in the presence of cytoplasmic extracts from DLKP (Figure 3.3.3.1). On the other hand, the level of K18 synthesis did not appear to be significantly affected by DLKP extracts (Figure 3.3.3.2). Reactions were set up from "master-mixes" to ensure equality in all reactions. This suggested that K8 mRNAs may be translationally repressed in DLKP. These crude cytoplasmic extracts (section 2.19.3) are basically the entire contents of the cell, with the nucleus centrifuged out. If a translational repressor is present it should be present in the cytoplasm to exert its effect.

In these systems, the RNA template would be expected to be stable since the cytoplasmic extraction protocol used was an adaptation of an older protocol used for the isolation of cytoplasmic RNA for use in Northern Blotting. Combined with the addition of RNase-inhibitor during preparation of the extracts and again prior to *in-vitro* translation, this should eliminate the possible influence of RNases in the inhibition observed. In addition, the failure to affect K18 in these reactions provides very strong evidence that, as suspected, RNAses are not active in these cytoplasmic extracts. To further investigate the possible repression of K8 synthesis in DLKP, we demonstrated that the K8 transcripts present in DLKP itself were functional and translatable (Figure 3.3.3.4). These results suggest that the absence of keratin expression in DLKP is due to a repressor of K8 synthesis at the translational level and not due to non-translatable/non-functional transcripts in this cell line. Previous work in this laboratory (Meleady, 1997) demonstrated that cyclohexamide could weakly induce keratin expression in about 5% DLKP. At the time it was speculated that this was due to the inhibition of the synthesis of a "destabilising" factor in DLKP that degraded keratin proteins more rapidly in DLKP

than A549, thought not to express this factor. We now propose that this effect is due to the inhibition of the synthesis of a repressor of K8 translation.

The presence of a repressor of simple keratin translation has been suggested from previous *in-vivo* tissue studies (Tyner and Fuchs, 1986), and has led to speculation that keratin mRNAs may be either masked (Winter and Schweitzer, 1983) or translationally repressed (Su *et al,* 1994). Here we provide additional evidence and propose a mechanism by which such repression regulates the temporal expression of simple keratin K8/18 filaments during early lung development. Very little is known about the regulation of K8 gene expression in comparison with that of its partner, K18 (Casanova *et al,* 1995). We suggest that K8 expression may be significantly regulated at the level of translation. Numerous reports have suggested that type II keratins (K8) are involved in the induction of their type I partners (K18) and that the regulation of both can be uncoupled (Section 1.2.1) (Darmon, 1985). Comparison of the gene sequences encoding the partner keratins K5 and K14 offers no insight into their co-ordinate expression (Ohtsuki *et al,* 1992). Such findings have led to suggestions that stricter regulation of overall filament formation occurs through tight regulation, at as yet unidentified levels, of the expression of the type II partner of a keratin filament. This probably allows a more co-ordinate and rapid response to differentiation signals than does induction of two or more separate genes. We suggest that translational repression of the simple type II keratin, K8 is a simple and precise means of regulating overall K8/18 filament formation until definite moments in early development.

Section 3.3.4 highlights some unusual properties of the K8 mRNA observed during data-base searches. The 3' UTR of K8 is significantly longer than that of its partner, K18. Sequences within the 3' UTR are thought to be critical in the developmental "unmasking" of repressed mRNAs (Standart and Jackson, 1994; Spirin, 1996) (Section 1.6.1). The 5' UTR of the K8 mRNA spans only 60 nucleotides, while that of K18 is 52 nucleotides in length. However, within the 60 nucleotides of the K8 5' UTR, a 10-nucleotide sequence is repeated, separated by only a single nucleotide, and is located +2 to the AUG start codon. According to data-base searches this motif is unique to the K8

mRNA in humans and we speculate that it may represent a binding site for the putative repressor of K8 synthesis suggested by *in-vitro* translation studies (Section 3.3.3). This is the first solid evidence of such repression.

#### 4.2.3.3.3 A role for eIF-4E in early development?

Roles for eIF-4E in differentiation (Jaramillo *et al,* 1991) and maturation (Beretta *et al,* 1998) have been suggested. While increased eIF-4E availability during development may simply act by increasing the ability of normally uncompetitive, poorly expressed differentiation-related mRNAs to avail of eIF-4E to begin translation, we suggest that eIF-4E may play a role in the relief of translational repression. Developmental "cues or signals" have been proposed to initiate unmasking events during development but as yet no such "signals" have been identified. We propose that one such "signal" may be increased eIF-4E availability. In agreement with this, overexpression of eIF-4E in DLKP was capable of inducing K8 expression (Section 3.5.1), strongly suspected to be translationally repressed in this cell line (Section 4.2.3.3.2). eIF-4E/4F increases the helicase activity of cells (Rebagliati and Melton, 1989; Klein and Melton, 1994) and can displace proteins associated with the 5' UTR (Svitkin *et al,* 1996), both possible means of relieving translational inhibition (Figure 4.1). 5'/3' UTR-bound repressors and increased eIF-4E availability may form the basis of a mechanism for global repression and unmasking of repressed mRNAs at critical points in development. This would be a rapid and reversible means of cellular differentiation in response to stimuli and could possibly be the means by which rapid de-differentiation, thought to be a mechanism involved in recovery from tissue damage (Emura *et al,* 1997), could occur in the lung. We suggest that transcription and translation may combine during early development to regulate the precise cell-specific and timed expression of developmental genes, respectively. The extended 3' UTR of K8 may mean that polyadenylation during very early development is the "true" signal to relieve repression of K8 synthesis. Sequences within the 3' UTR of masked mRNAs are thought to recruit factors that polyadenylate the mRNA (reviews; Wormington, 1993; Vassalli and Stutz, 1995). Developmental polyadenylation of mRNAs is thought to recruit PAB (Poly(A)-binding proteins) (Figure 1.15; Section

1.6.3.4.2), which in turn recruits eIF-4E/4F to the 5' end of mRNAs. BrdU may have simply by-passed or mimicked this event by increasing eIF-4E availability by alternative means. Thus the proposed 5' UTR repression element and significantly longer 3' UTR of K8 (in comparison to K18) may act in concert to both repress and bring about unmasking of the K8 mRNA in early development (Figure 4.1). Alternatively, the "developmental signal" proposed to unmask mRNAs may be elevated availability of eIF-4E itself, and polyadenylation may be a result of activation of translation. The actual timing and role for developmental polyadenylation remains unclear, speculated to be either a cause or consequence of unmasking.

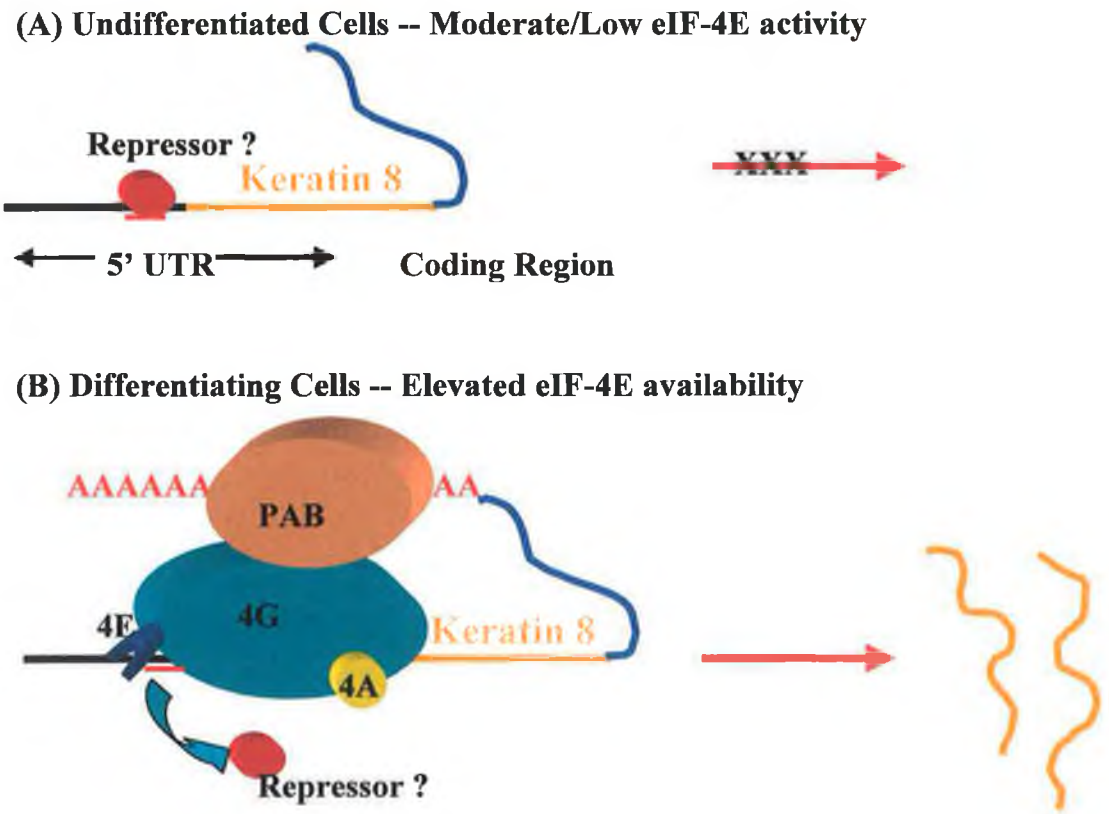



**Figure 4.1: A possible mechanism for both repression of simple keratin K8 synthesis and its induction.** (A) Under normal circumstances K8 mRNAs are translationally repressed in DLKP. (B) Increased availability of eIF-4E/4F during differentiation displaces the putative 5'-bound repressor of K8 translation. Increases may be due to increased eIF-4E levels/activity, or through recruitment to repressed mRNAs due to developmental polyadenylation, which recruits PAB/eIF-4F complexes to RNAs. In undifferentiated cells (A), RNAs are not polyadenylated and so compete for eIF-4E poorly.

The very low level expression of keratins in DLKP may represent slight "leakiness" in this regulation, known to occur in distally-placed translational repressors (the putative repressor binding site is located –36 nucleotides from the cap site; Figure 3.3.4). Of interest, in A549 the synthesis of K8 reaches a maximum after only 3-4 days, suggesting that these RNAs simply reach maximum translational efficiency at this point (Section 3.1.4.1). The strong increase in expression over the 3 days probably represents the very high level of transcript present in these cells. To date we have not been able to confirm the presence or absence of this repressor in A549 due to the high levels of K8/18 transcripts and proteins in cytoplasmic extracts from these cells (Figure 3.3.3.2).

Translational repression has been shown to play important roles in both early development and in somatic cells committing to terminal differentiation. As erythrocytes develop into enucleated reticulocytes transcription is inactive, and important nuclear breakdown events are catalysed by the appearance of an enzyme called 5'-Lipoxygenase (LOX). The LOX mRNA is produced in the bone marrow but translationally repressed (Ostareck-Lederer *et al,* 1994) until the very final stages of mammalian erythropoiesis (Standard and Jackson, 1995; Sun and Funk, 1996). 5'UTR-based translational regulation has been shown to control the levels of the iron detoxification protein, ferritin, and repressor-binding activity is directly regulated by iron levels within the cell (review; Altmann and Tracshel, 1993). Specific 3'UTR-based repression has been shown in the tra-2 (review; Sonenberg, 1994; Curtis *et al,* 1995) and the Lin-14 (Arasu *et al,* 1991; Lee *et al,* 1993) mRNAs of *C.elegans*, whose correct temporal expression is critical for normal development (Austin, 1994). In addition, elements within the 3' UTR of activin, a potent mesoderm inducing factor, have been identified in its translational repression (Klein and Melton, 1995). Overexpression of eIF-4E was shown to induce expression of this factor and development of mesoderm in *Xenopous laevis* embryos (Klein and Melton, 1994). These results are very reminiscent of our own findings in relation to the regulation and induction of simple keratin expression in the poorly differentiated epithelial line, DLKP.

The exact nature of this translational repressor is unknown. With 97% of the human genome not coding for proteins or RNAs with clear functions (Nowak, 1994) and the identification of novel functions for RNAs such as small RNAs, antisense and ribozymes (Wickens and Takayama, 1994), this factor could be a protein, small RNA or antisense, all of which should be stable in these extracts. Translation of the lin-14 mRNA is actually regulated by a repressor encoded by the lin-4 gene, which encodes a small RNA and not a protein (Arasu *et al,* 1991; Lee *et al,* 1993). As yet to be proven, relief from this repression is presumably due to loss of lin-4 gene expression (encoding the antisense RNA) or increased helicase activity in developing cells, known to be associated with increased eIF-4E availability (Rebagliati and Melton, 1989; Klein and Melton, 1994). Further characterisation and isolation of this putative novel translational repressor of K8 synthesis will require considerable effort and expertise, but we have provided additional evidence for its existence and propose a mechanism by which repression occurs and is relieved to allow simple keratin filament formation during early lung development.

### 4.2.4 Transcription Factors involved in BrdU-induced Differentiation:

#### 4.2.4.1 c-Myc Expression:

Despite intensive investigation for almost two decades, c-*myc* remains a fascinating and enigmatic subject. A large and compelling body of evidence indicates that c-*myc* is a transcription factor with central roles in the regulation of cell proliferation, differentiation and apoptosis, but its exact function remains elusive (Sakamuro and Prendergast, 1999). Some authors, however, still retain the narrow view that oncogenes simply promote cell growth (or death, acting as "life-or-death signals") while blocking differentiation. This view is not compatible with the results presented in this thesis.

##### 4.2.4.1.1 BrdU and c-Myc expression:

Western blot analysis showed a dramatic increase in the levels of c-Myc protein (MW 67 kDa) during the differentiation of both epithelial lines (Figure 3.4.1). Increased expression of c-Myc protein in A549 and DLKP upon exposure to BrdU may contribute to the induction of eIF-4E expression in these systems (Section 4.2.3.1.1). eIF-4E has been identified as one of the few known transcriptional targets of the c-*myc* proto-oncogene (Jones *et al,* 1996).

BrdU has been reported to induce differentiation of the leukaemic cell line, HL60 (Yen and Forbes, 1990) and melanoma lines (Valyi-Nagy *et al,* 1993) resulting in/from decreased levels of c-*myc* mRNA transcript. In order to investigate the downstream effects of modulating c-*myc* expression during BrdU-induced differentiation, human leukaemia cells, HL60s were cultured and BrdU-treated under the same conditions as described in the literature by Yen and Forbes (1990). Decreased c-*myc* expression upon differentiation of these cells was confirmed by PCR. The analysis for c-*myc* was only performed on a single occasion, to confirm that conditions were reproducing those of Yen and Forbes (1990). Unfortunately the data was lost due to poor development of film. Western blot analysis repeatedly failed to detect c-Myc expression, most likely due to the

fact that c-Myc levels in this system were decreasing. However, we repeatedly demonstrated that the expression of its downstream target, eIF-4E, decreased in BrdU-treated HL60s (Figure 3.2.2(d)).

It may appear contradictory that both c-Myc and eIF-4E are upregulated during epithelial lung cancer differentiation, while, as might be more readily expected (given their importance in growth regulation), they are observed to decrease during leukaemic differentiation. The widespread reports of decreased c-*myc* expression during differentiation (Yen and Forbes, 1990; Valyi-Nagy *et al,* 1993; Shimizu *et al,* 1994; Warner *et al,* 1999) have led to suggestions that c-*myc* downregulation is a signal for growth arrest (Bennett *et al,* 1994). However, the roles of oncogenes such as c-*myc* and eIF-4E in differentiation are very poorly understood. Much work has focused on their roles in transformation, but, as stated by Ryan and Birnie (1996), "it would be naïve to assume that the only transcriptional targets of c-*myc* are those involved in transformation". In addition, it is equally naïve to assume that effects in one cell type are universal, and in fact, it has been stated that the effects of BrdU itself are cell-specific (Valyi-Nagy *et al,* 1993). Pre-commitment to differentiation, which involves changes in protein expression to accommodate differentiation, have been suggested to require an upregulation of growth regulatory genes, such as c-*myc* (Yen *et al,* 1987). In fact, the analysis of cell cycle arrest in adipocyte differentiation reveals that c-*myc* levels actually increase (Reichert and Eick, 1999). The growth arrest induced in these cells was attributed to the expression of the cdk (cyclin dependent kinase) inhibitors p21 and p27 (see also; Section 4.2.2). c-*myc* has been shown to promote differentiation of human epidermal stem cells (Gandarillas and Watt, 1997) and constitutive expression of c-*myc* promotes terminal differentiation by driving keratinocytes from the stem cell compartment into the transit amplifying compartment (Watt, 1998) (Figure 1.1, Section 1.4). c-*myc* antisense has been shown to inhibit the neuroendocrine differentiation of SCLC lines (van Waardenburg *et al,* 1998) and transformation of human bronchial epithelial cells with c-*myc* induced multidifferentiated carcinomas in nude mice (Pfeifer *et al,* 1991), implying a role for elevated c-*myc* expression during lung cancer differentiation. We feel that a major problem at present is the preoccupation of many

studies with the levels of c-*myc* mRNA expression in cells. Current understanding in relation to c-Myc isoform expression show that Myc1 and Myc2 have different properties and that their expression is regulated at the translational level (Section 4.2.4.1.2).

#### 4.2.4.1.2 A role for c-Myc expression during Lung Cancer Differentiation:

We propose a role for increased expression of both c-*myc* and eIF-4E during epithelial lung cancer differentiation based on the work presented here and on very recent understandings in relation to the expression of c-Myc isoforms and their functions. The c-*myc* gene is transcribed from four promoters (Figure 4.2), with the vast majority of transcripts originating from the P1 and P2 promoters, which encode 75-90% and 10-25% of c-*myc* mRNAs, respectively (Ryan and Birnie, 1996), while the P0/P3 promoters are relatively inactive. Within the extended 5' UTR of the c-*myc* mRNAs, except for those few originating from the P3 promoter (Bodescot and Brison, 1996), there is an alternative translation initiation site, encoded by an upstream CUG (also recognised by initiator tRNAs) (Figure 4.3).

Translation initiation at the AUG encodes the p64 (Myc 2) form of the c-Myc protein, while initiation at the alternate CUG encodes an N-terminally extended form of the protein, Myc 1 (p67) (Figure 4.3). Both Myc 1 and Myc 2 are capable of transactivating Myc E-Box elements, which are the DNA-recognition sequence for c-Myc binding (Section 1.5.2.2.1) (Blackwood *et al,* 1994). The N-terminal transactivation domain of the c-Myc protein is essential to Myc biology (Luscher and Larsson, 1999) and its extension in Myc 1 been suggested to be the cause of c-Myc1's ability to transactivate an additional subset of genes (Onclerq *et al,* 1988; Hann *et al,* 1994) through an additional non-canonical E-box element. The overexpression of c-Myc 1, but not c-Myc 2, is significantly inhibitory to cell growth (Hann *et al,* 1994), suggesting that c-Myc 1 may be necessary to keep the c-Myc 2 protein "in check" (Hann *et al,* 1995). In agreement with this, numerous reports of cancers are associated with translocations or mutations in exon 1 of the c-Myc gene (Saito *et al,* 1983; Hann *et al,* 1984; Hann *et al,* 1995), which result in the loss of c-Myc 1 expression.

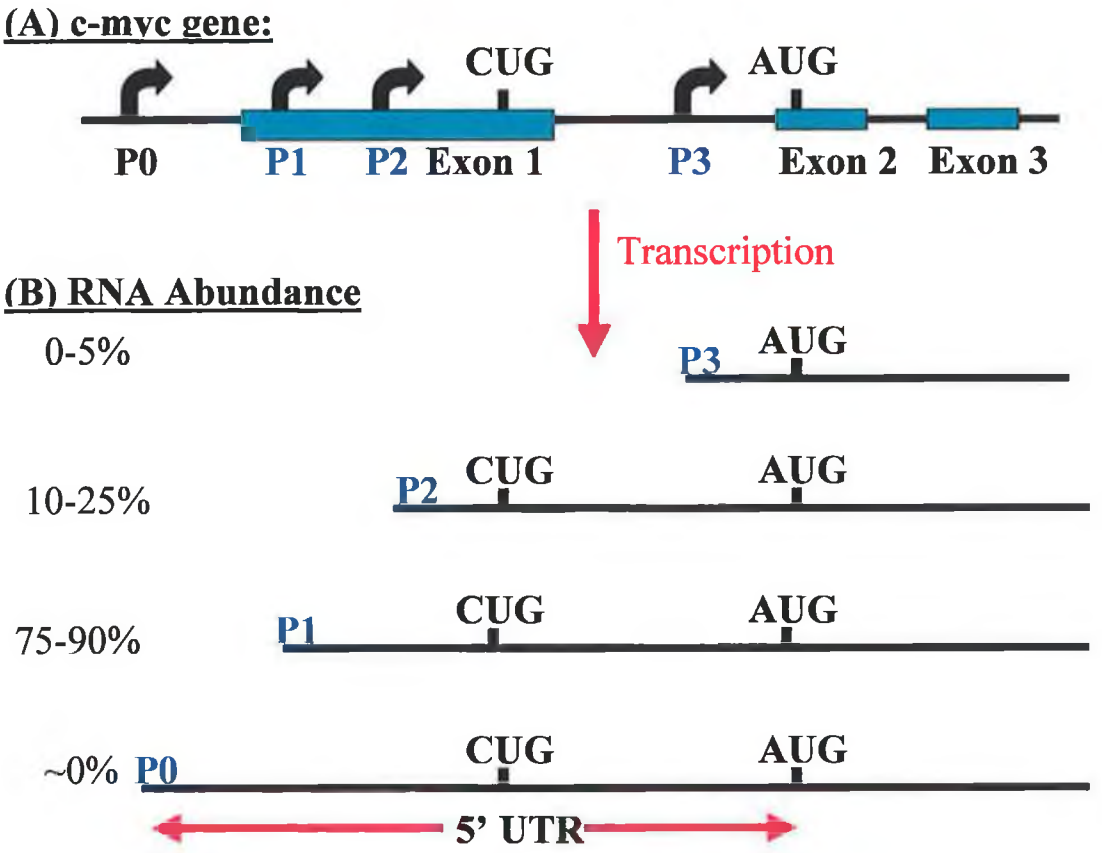



**Figure 4.2: The c-*myc* gene (A) produces four mRNA transcripts (B) from four distinct promoters (P0-P3).** The overwhelming majority of c-*myc* mRNAs originate from the P1 and P2 promoters. The other promoters (P0 and P3) are relatively inactive and their functional significance is unknown.

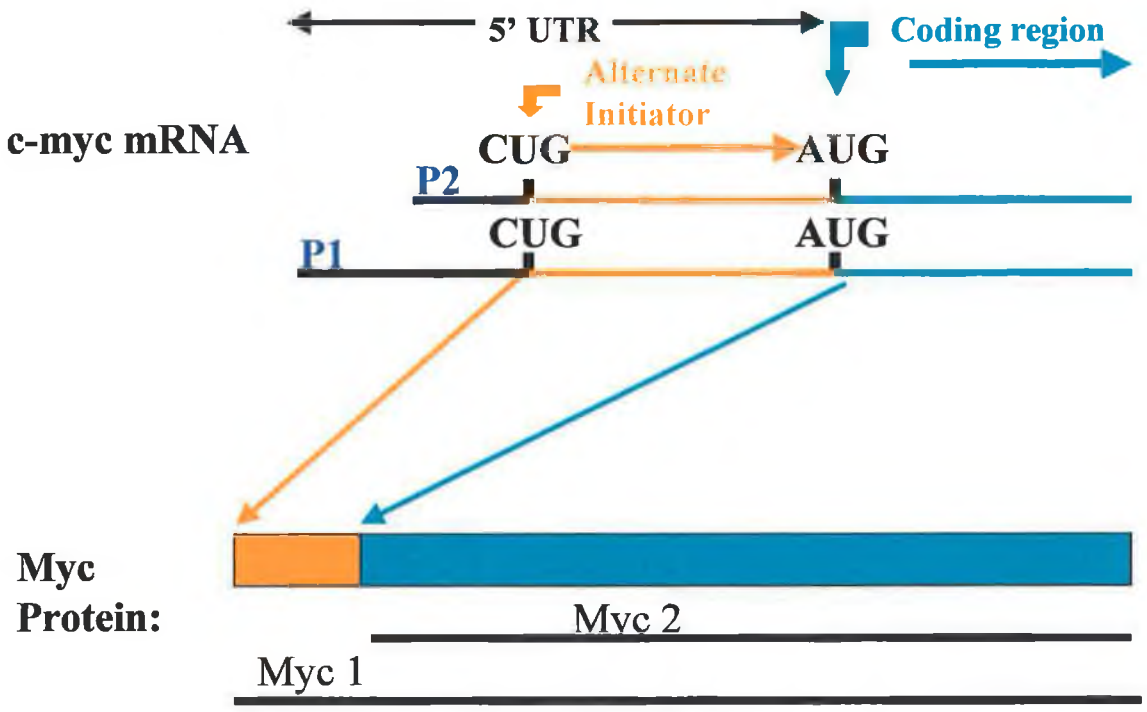



**Figure 4.3: Alternate usage of an internal CUG in the 5' UTR of c-*myc* mRNAs.** Translation normally initiates at the AUG start codon, resulting in c-Myc 2 (p64) expression. However, under certain conditions, initiation switches to the CUG initiator upstream of the AUG, resulting in an N-terminally extended form of the Myc protein, c-Myc 1 (p67).

Reports of alternate, non-AUG intiated translation in c-myc (Hann and Eisenmann, 1984) have suggested that Myc isoforms may be reciprocally synthesised under conditions such as methionine deprivation (Hann, 1994; Hann, 1995). Attempts to explain the phenomenon of alternate translation initiation in c-*myc* mRNAs produced reports that c-*myc* mRNAs contain an IRES (Internal Ribosome Entry Site) that allows cap-independent translation (Nanbru *et al,* 1997) (Section 1.6.3.2.4.2.1). However, IRES do not explain the mechanism by which alternate usage of AUG and CUG codons occurs in c-*myc* mRNAs and their existence in c-myc is now heavily disputed (Carter *et al,* 1999).

Evidence for a scanning mechanism of translational regulation of Myc isoform expression was provided by Hann et al (1994). The exact mechanism of differential translation was very recently shown to be dependent upon the availability of eIF-4E/4F

(Carter *et al,* 1999) (Figure 4.4). Under normal conditions the ribosome scans along the 5' UTR of the c-*myc* mRNA, subject to translational regulation due to the complexity of the 5' UTR. At a particular point, termed the Internal Ribosome Repositioning Element (IRPE) (Carter *et al,* 1999), the scanning ribosome is positioned close-and "jumps" across-to the AUG initiator to begin translation. However, under conditions of elevated eIF-4E, initiation at the alternate CUG codon begins due to unwinding of the 5' UTR and the IRPE itself. In this situation, while both AUG and CUG initiation occurs, the relative increase in CUG recognition is significantly stronger than that of AUG. Because the overwhelming majority of c-Myc transcripts contain the AUG/CUG initiators, it is primarily eIF-4E/4F levels and activity that determine the induction of c-Myc 1 expression. Alternate uses for upstream CUG initiation codons that result in expression of isoforms of FGF-2 have also been reported (Kevil *et al,* 1996). Breast carcinomas expressing elevated eIF-4E also exhibit the larger isoforms of FGF-2, which could play an important role in tumourigenesis (Nathan *et al,* 1997).

We propose that the induction of differentiation in lung epithelial, and also possibly in other cell types may revolve at least in part around the complex inter-relationship between eIF-4E and c-Myc 1 expression, highlighted in figure 4.5. Differentiating lung cancer cell lines showed elevated eIF-4E expression, in addition to a shift towards the phosphorylated and more active form of the protein (Section 3.2). When c-Myc 1 levels were examined, very strong induction of p67 expression was detected (Section 3.4.1). The antibody used (Santa Cruz) is p67-specific, and as such we have not determined the levels of c-Myc2 expression in our systems. Since both isoforms activate Myc E-box elements (Blackwood *et al,* 1994), while c-Myc 1 can activate an additional subset of genes and repress growth (Hann *et al,* 1994) and is translationally enhanced by increased eIF-4E availability, we suspect that it is c-Myc 1 expression that is more important in early development. We are currently seeking an antibody that detects both forms of this protein to allow direct comparison of expression during differentiation. This is the first direct demonstration during cellular differentiation of both p67 induction and an increase in the factor (eIF-4E) that regulates c-Myc isoform ratios. In addition, overexpression of

eIF-4E induced both K8/18 and c-Myc1 expression, as well as a larger form of YY1 in DLKP (Section 3.5.1).

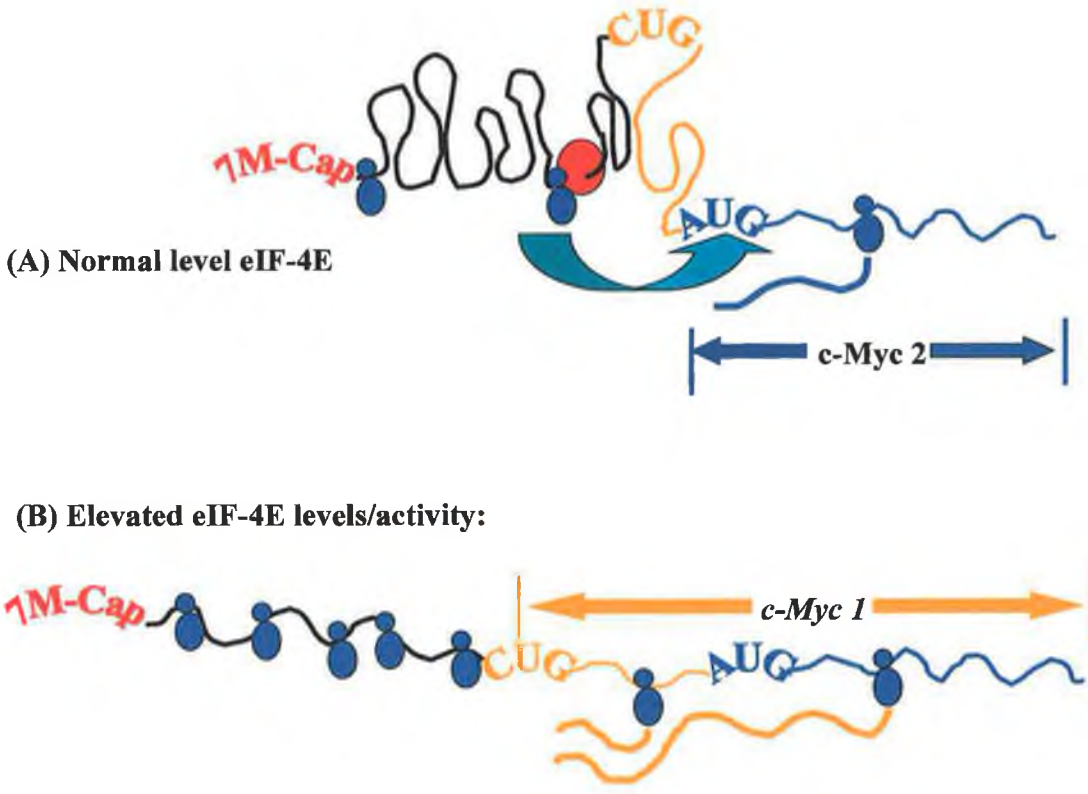



**Figure 4.4: Alternate initiation from the CUG in c-*myc* mRNAs is dependent upon the availability of eIF-4E/4F.** (A) Translation normally initiates at the AUG start site due to IRPEs (Internal Ribosome Repositioning Elements) (Red Circle). (B) On occasions of increased eIF-4E availability, both the complex 5' UTR and the IRPE itself are unwound, shifting the balance of translation toward the upstream CUG codon and enhancing translational efficiency of the mRNA itself.

We propose that c-Myc 1 induction may be the mechanism by which eIF-4E uncouples its own growth stimulatory effects to allow it to function in the induction of developmental mRNAs and new protein synthesis required for differentiation (Section 4.2.3.3.3). The close inter-dependency between eIF-4E (transcriptionally enhanced by Myc1/2) and Myc 1 (selectively translated during increased eIF-4E availability) has led us to suspect that these two proteins may form the core of a differentiation pathway that is particularly important in early development (Section 4.2.6), establishing a feedback

loop that drives the cell towards differentiation (Figure 4.5). Nature has always proven to be very complex, and such interaction and "feedback" communication between regulatory factors is probably a truer reflection of reality than single-step, unidirectional cascades and pathways. The complexity of such pathways is further elaborated upon in figure 4.7 and 4.8.

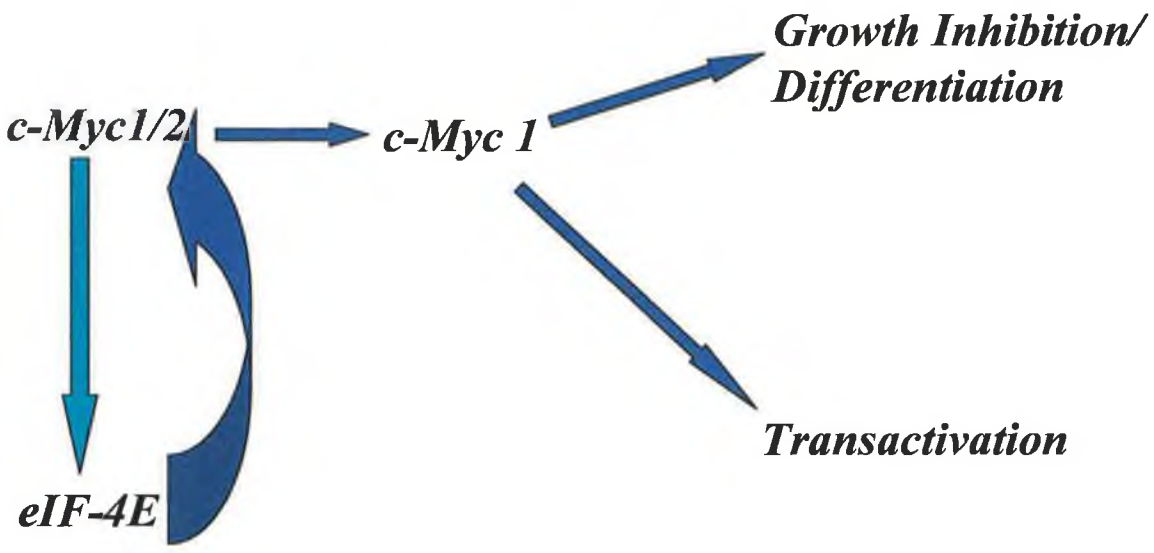



**Figure 4.5: Differentiation of epithelial lung cancer cells revolves around a complex relationship between eIF-4E and c-*myc*.** While the eIF-4E gene is a target for activation by c-Myc, increasing eIF-4E gene transcription, increased availability of eIF-4E results in selective translation of the growth inhibitory c-Myc 1 isoform. Since c-Myc 1 can still transactivate eIF-4E, a feedback loop is established that continues to increase both eIF-4E and selective c-Myc 1 isoform expression, driving the cell towards differentiation.

#### 4.2.4.1.3 eIF-2α expression in differentiating lung cancer lines:

When levels of eIF-2α protein were examined they were shown to increase in both A549 and DLKP upon exposure to BrdU (Figure 3.4.1.1). It is suspected that this reflects the activity of the BrdU-induced upregulation in c-Myc expression in these cells (Section 4.2.4.1.1) (Rosenwald *et al,* 1993). Unfortunately these western blots could not be repeated due to the scarcity of the antibody (a kind gift of Dr. Simon Morley). eIF-2α and its regulator, the GTP exchange and recycling factor, eIF-2B (Section 1.6.3.2.2) display a very complex inter-relationship (Section 1.6.3.3.2 & briefly reviewed below).

After AUG start codon recognition by the met-tRNA eIF-2 is released as an inactive, GDP-bound molecule. Recycling to an active, GTP-bound form requires GTP-GDP exchange, catalysed by eIF-2B. eIF-2B activity is regulated by eIF-2 itself. Phosphorylation of the eIF-2α sub-unit on $Ser^{51}$, by kinases such as PKC and HRI, results in eIF-2B becoming sequestered in an inactive eIF-2-GDP-eIF-2B complex. Elevated eIF-2α expression in the non-phosphorylated form could increase global translation rates. Alternatively, increased expression and phosphorylation of eIF-2α could result in a global decrease in translation by providing excess phosphorylated eIF-2α to "mop-up" available eIF-2B within the cell. A very interesting observation is that the 3' UTR of differentiation-related mRNAs, such as tropomyosin, activate PKR (Protein Kinase RNA activated; Section 1.6.3.2.2.2) activity (Davis and Watson, 1996), decreasing translational efficiency (Rastinejad *et al,* 1993) and inducing differentiation (Rastinejad and Blau, 1993; L'Ecuyer *et al,* 1995) (Figure 4.6).

While levels of keratin mRNAs do not change during BrdU-induced differentiation and probably do not possess the above activity, we speculate that other differentiation-related mRNAs may be increased during BrdU treatment, possibly as part of the unique subset of genes activated by Myc 1 that inhibit cellular growth (Section 4.2.4.1.2). In this scenario c-myc also provides PKR with its substrate by transcriptionally increasing the levels of eIF-2α. It would be of interest to examine the possible influence of increased eIF-2α, in either the active or inactive, phosphorylated form during differentiation. eIF-2α levels

and activity are thought to regulate global translation (Kimball *et al,* 1998), and as such may play a role in regulating the overall protein synthesis and growth rates during differentiation. Under conditions of elevated eIF-4E expression complex or repressed RNAs are initiated more frequently, and can compete more efficiently for eIF-2α, becoming translated at the same rate as "normal" mRNAs. The levels of global protein synthesis then become dependent upon eIF-2α availability. This would be an interesting approach to pursue and should form the basis of an extensive study of the function and interaction of both eIF-4E and eIF-2α during differentiation.

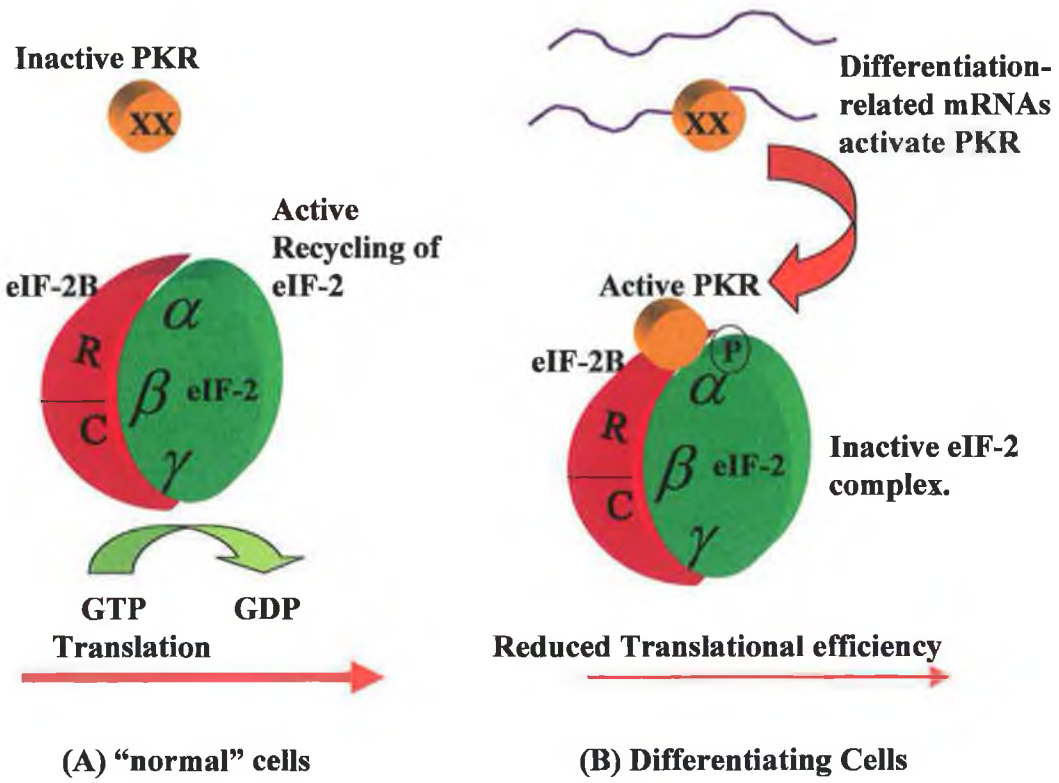



**Figure 4.6: A role proposed for eIF-2α induction during differentiation.** Activation of PKR activity by the 3' UTR of differentiation-related mRNAs results in eIF-2α phosphorylation and reduction in global translation rates.

### 4.2.5 YY1 Expression:

The Yin-Yang1 (YY1) transcriptional regulator is thought to be of critical importance in the control of normal development (Riggs *et al,* 1991). The unusual properties exhibited by this transcription factor allow it to regulate the expression of different genes in opposing fashion (Section 1.5.2.3), making it a pivotal factor in the regulation of developmental gene expression. Normally it acts as a negative transcriptional element, downregulating the expression of genes possessing the YY1-binding motif within their 5' promoter element (Lee *et al,* 1994; Zhou *et al,* 1995). However, certain genes are actually transcriptionally upregulated by the binding of this factor, among these being the c-myc gene (Riggs *et al,* 1993; Lee *et al,* 1994). It was therefore decided to examine the levels of YY1 expression in BrdU-treated cells to determine if there was any effect, and if so, if there was any correlation between YY1 and c-Myc expression in both epithelial and leukaemic lines.

#### 4.2.5.1 BrdU and YY1 expression:

When the levels of YY1 were investigated in DLKP and A549, they showed elevated expression of YY1 protein upon treatment with BrdU (Figure 3.4.2). A role for YY1 in inducing and regulating c-Myc, initiating a cascade of events leading to epithelial differentiation is discussed in section 4.2.6. In contrast to the situation in DLKP and A549, the levels of YY1 in BrdU-treated HL60s actually decrease (Figure 3.4.2), further implying a role for YY1 in the regulation of c-*myc* expression during BrdU-induced differentiation.

Bromo-deoxyuridine has been shown to upregulate the levels of YY1 in embryonic myoblasts (Lee *et al,* 1992), in agreement with our observations in epithelial lines. It seems very likely that YY1 plays a central role in the control of normal differentiation and development, due to its unusual ability to differentially regulate the expression of various genes. YY1 plays a role in the development of both B-cells and fibroblasts (Riggs *et al,* 1991) and in myogenesis (Lee *et al,* 1992; Shrivastava and Calame, 1994). The

modulation of YY1 expression by BrdU may be central to the ability of this drug to influence the differentiation status of cells.

YY1 has been shown to increase transcription from the P1 and P2 promoter of the c-*myc* gene (Riggs *et al,* 1993). In addition, YY1 has also been shown to associate with the c-Myc protein itself (Shrivastava *et al,* 1993) and their association inhibits the transactivation properties of both proteins. YY1 is thought to compete with Max (Section 1.5.2.2.1), excluding it from association with c-Myc (Shrivastava *et al,* 1994). Myc does not block the binding of YY1 to DNA, in agreement with previous findings (Shrivastava *et al,* 1993), and association studies suggest that c-Myc interferes with the ability of YY1 to contact the basal transcription factors TATA-binding protein (TBP) and TFIIB (Section 1.5.1.1). Changes in c-Myc levels, which accompany mitogenic stimulation or differentiation of cultured cells, affect the ratio of free to c-Myc-associated YY1 (Shrivastava *et al,* 1996). This has led to speculation that one function of the c-Myc oncoprotein is to modulate the expression of YY1-dependent developmental genes by virtue of its association with YY1 (Liu *et al,* 1995). In co-transfection experiments, c-*myc* expression was able to reduce YY1 activating function from eight-fold in the absence of co-expressed c-*myc* to two-fold in its presence (Shrivastava *et al,* 1993). In light of YY1s ability to activate c-*myc* gene transcription, association between these two proteins may form the basis of an autoregulatory mechanism that controls the expression and activity of both proteins (Grignani *et al,* 1990), preventing excessive loss of growth control during periods of elevated c-myc expression. In light of the very recent discoveries regarding the functions and regulation of c-Myc isoforms (Section 4.2.4.1.2), it would be of great interest to determine if there is a preferential association of YY1 with either p64 or p67. Does association with YY1 inhibit only the transactivating properties of c-Myc? Does this actually enhance the additional growth-inhibitory effects of c-Myc 1? Unfortunately the antibodies used in these studies (Santa Cruz) were not suited to co-immunoprecipitation to determine the relative rates of association between these two factors during the BrdU-induced differentiation process. The complexity of their inter-relationship is highlighted in Figure 4.7. A possible role for YY1 in initiating epithelial differentiation in response to BrdU is outlined in Section 4.2.6.

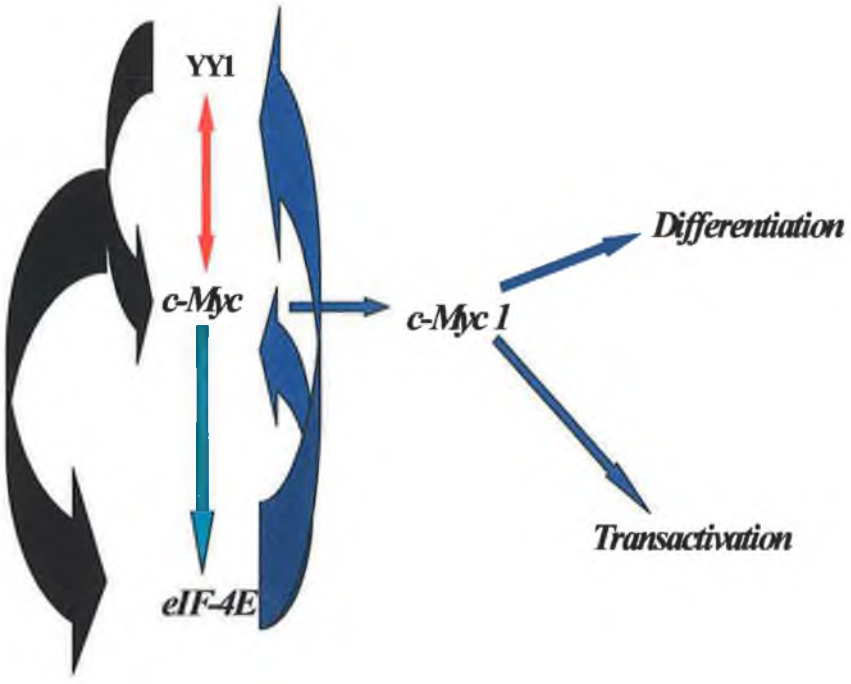



**Figure 4.7: The inter-relationship between YY1, c-Myc and eIF-4E.** YY1 can both activate transcription of the c-myc gene and interact with the c-Myc protein to inhibit the transactivating potential of both proteins (red arrow). c-Myc is a regulator of eIF-4E gene activity (green arrow), while both YY1 and c-Myc are targets for translational regulation by eIF-4E (blue arrows). YY1 regulates c-Myc transcription, but may also regulate the expression of the TATA-less eIF-4E2 gene (black arrows).

It has recently been shown that humans possess two forms of the eIF-4E gene (Gao *et al,* 1998). While the protein products are the same, the eIF-4E1 gene contains a c-Myc activated E-box element (Section 1.5.2.2.1), thought to regulate growth-inducible eIF-4E expression, while eIF-4E2 lacks a TATA-box (Section 1.5.1.3) and is thought to be constitutively expressed. This raises the possibility that eIF-4E2 may be a target for activation by YY1 (Section 1.5.2.3.3). An intriguing possibility is that eIF-4E activation occurs as part of a YY1-induced developmental cascade to induce early differentiation events, including a switch to c-Myc1 protein synthesis. Since both eIF-4E genes encode the same protein, the induction of eIF-4E expression observed in these studies cannot be attributed to either YY1 or c-Myc activation, and may even be attributable to both (Figure 4.7). TATA-less genes are commonly "house-keeping" genes whose ubiquitous expression may be attributed to the ubiquitous expression of YY1. However, it is possible that elevated expression of YY1 may also form the basis of a mechanism whereby cells increase the expression of such genes to fulfil new protein synthesis and growth requirements during early development. The complex interactions between the regulatory

factors in figure 4.7 steps away from the old view of single-step, unidirectional cascades that are non-interacting. In reality, nature's regulatory pathways are likely to be very complex, depending upon "feedback" mechanisms and "communication" between factors to regulate processes such as differentiation. This complexity is highlighted by the differentiation observed when either eIF-4E or YY1, key regulatory factors in the proposed "differentiation cascade", were overexpressed in DLKP using cDNA constructs (Section 3.5).

#### 4.2.5.1.1 cdc2 expression in BrdU-treated cells:

When the levels of cdc2 were examined in BrdU-treated DLKP, the levels of cdc2 protein were elevated in treated cells as determined by western blot analysis (figure 3.4.2.1). These experiments were performed to examine the possible role of YY1 in regulating the expression of cdc2, based on a recent report on the characterisation of the murine cdc2 gene (Jun *et al,* 1998). Interestingly, no consensus sequence for a TATA box exists at an appropriate position within the promoter region of the cdc2 gene. The TATA sequence motif forms the most common transcription factor binding site for RNA polymerase II transcribed mRNAs and is usually located around 30 bp from the transcriptional start site (Watson *et al,* 1992a) (Section 1.5.1.1). YY1 can promote transcription from TATA-less promoters (Section 1.5.2.3.3). Very similar to the c-*myc* sequence known to be activated by YY1 due to the presence of SP1 and E2F binding sites proximal to the YY1 site (Riggs *et al,* 1991), the cdc2 promoter has a major positive regulatory sequence between –188 and –38 (Jun *et al,* 1998) containing several putative transcription factor binding sites, including those of YY1, SP1, and E2F. cdc2 would therefore be a prime candidate for YY1 regulation based on its promoter sequence.

While the observed increase in cdc2 expression in differentiating epithelial lung cancer cells is very likely due to the BrdU-induced upregulation in YY1 expression in these cells, the fact remains that BrdU may also affect the levels of other factors involved in the expression of cdc2. Inappropriate expression of cell-cycle related proteins such as cyclin D, cdc2, and cdk2 have been associated with growth arrested rat fibroblasts overexpressing c-Fos (Balsalobre and Jolicoeur, 1995). However, BrdU has been used to increase the levels of YY1 protein, in place of transfection of the YY1 cDNA (Lee *et al,* 1992). This would seem to indicate that BrdU is accepted as a direct, or very nearly direct, modulator of YY1 expression. In light of the results presented here it would appear, therefore, that cdc2 is a likely candidate for positive activation by YY1, joining c-*myc* and the ribosomal protein delta sites (Riggs *et al,* 1993; Lee and Lee, 1994). YY1 appears to be a positive regulator of a number of growth-related genes (Shrivistava and Calame, 1994), and cdc2 may be another of these.

### 4.2.6 BrdU and a possible Differentiation Cascade:

BrdU is a synthetic halogenated thymidine analogue and, unlike RA (Section 4.4), it does not elicit a "true" receptor-mediated differentiation in exposed cells. The uniqueness of BrdU would appear to lie in its ability to modulate the levels of only a few, if not a single regulatory gene that may, once it is discovered, hold the key to the control (both positive and negative) of a whole host of regulatory pathways involved in the differentiation, development and proliferation of eukaryotic cells. Our results suggest that BrdU activates at least two events in DLKP, a reversible growth inhibitory effect (Section 4.2.2) and a differentiation-inducing effect. Our work has focused on determining key factors in the latter. Based on the results presented here, we propose a cascade of events in differentiating lung cells upon exposure to BrdU (Figure 4.8). While speculative at this stage, further experiments will either confirm this cascade or lead to modifications, redefining the proposed cascade in an attempt to more accurately determine the nature of such complex regulatory mechanisms. Section 4.2.6.1 breaks this cascade down, highlighting the roles of key factors (A-E) identified by this study. This cascade may offer an insight into alternate interactions and functions of what are commonly taken to be growth-promoting factors, the roles of which are poorly characterised in situations such as differentiation.

It must be noted that it is not known whether eIF-4E is directly involved in the increase in β-integrin expression observed in BrdU-treated A549 and DLKP (work by Dr. Paula Meleady) (Figure 4.8). $\beta_1$-integrin subunits are known to be heavily regulated at the post-transcriptional level (Zutter *et al,* 1992) and findings with BrdU treated cells agree with this (Meleady and Clynes, in preparation). $\beta_1$-integrin dimerises with, among others, $\alpha_2$-integrin subunits to form an active attachment and signalling cell surface complex. c-Myc-regulated α-integrin subunits have been identified (Liu *et al,* 1998), and in agreement $\alpha_2$-integrin expression has been found to be increased in BrdU-treated cells at both transcript and protein levels (Meleady and Clynes, in preparation). Results with $\alpha_2$-integrin expression independently confirm, by implication, that Myc is both upregulated and actively involved in the regulation of gene expression in BrdU-treated epithelial

cells. However, similar to keratins (Section 1.2.1), $\beta_1$-integrin subunit expression may simply depend on *myc*-regulated $\alpha$-integrin expression for proteolytic stability, but they are included in the diagram to illustrate their post-transcriptional induction by BrdU.

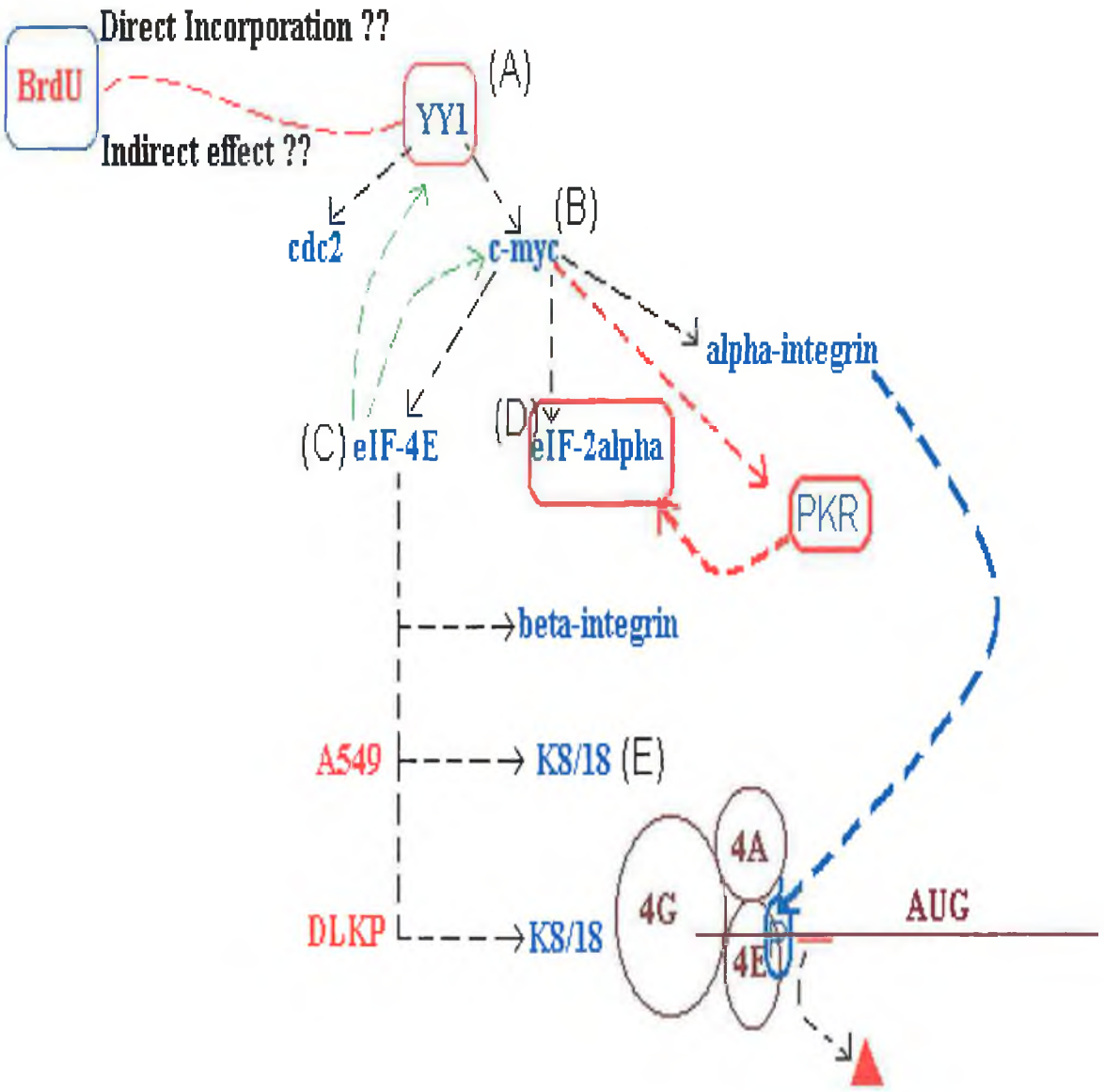



**Figure 4.8: A full cascade incorporating findings to date in BrdU-treated epithelial lines.** YY1 is proposed as a possible master target for initiating the cascade. BrdU-induced activation of integrin expression is proposed to initiate eIF-4E phosphorylation (Blue lines). Activated eIF-4E results in altered isoform expression in c-Myc, and possibly YY1 (Green Lines) establishing a "feedback communication" within the cascade. In addition, eIF-4E is proposed to relieve translational repression (the putative repressor of K8 synthesis is represented by the red triangle) or increase the translation of poorly competitive mRNAs. Induction of eIF-2α may be involved in the induction of eIF-4E. Induction of PKR during differentiation, however, may result in the phosphorylation of eIF-2α and the down-regulation of global translation (Red lines), allowing eIF-4E to selectively increase the translation of poorly competitive mRNAs while global translation is controlled separately by eIF-2α.

#### 4.2.6.1 The Cascade and how it works:

At first sight this cascade probably appears very complex. However, in comparison with the overall control of differentiation, this cascade more than likely represents a single, incomplete "branch" of a complex regulatory "tree", very similar to the branching and interaction of the regulatory kinase cascades that control cell signalling (Figure 1.18; Section 1.6.4). Each of the factors involved in this cascade have already been introduced and their roles discussed. This section briefly summarises findings to highlight the intricacy of the proposed cascade and the individual and interactive contributions of each key factor (Figure 4.8; A-E).

**(A)** **Yin-Yang 1** (Section 1.5.2.3):

1. **Initiates** and **Regulates** the **cascade** through its ability to both transcriptionally activate **c-*myc*** gene transcription and interact with the c-Myc protein (Section 4.2.5.1).
2. We suspect that YY1 may also influence **eIF-4E** levels directly, as well as via c-*myc* (Section 4.2.5.1).

**(B)** **c-Myc** (Section 1.5.2.2):

1. The proposed **target for YY1** initiation and regulation of this cascade (A.1 above).
2. The complex inter-relationship between **Myc1 isoform** expression and **eIF-4E** forms the basis of a differentiation-inducing feedback loop (Section 4.2.4.1.2).
3. **Myc 1** expression is proposed to **keep growth "in check"** to allow elevated eIF-4E activity during early development without loss of growth control.

**(C)** **eIF-4E** (Section 1.6.3.2.4.3):

1. Selectively **increases the translation** of poorly competitive or **repressed mRNAs** involved in differentiation (Section 4.2.3.3.3).

2. **Selective translation** of **c-Myc 1** forms the basis of a **"safeguard"** against aberrant growth during periods of elevated eIF-4E activity (B.3 above).

**(D)** **eIF-2α** (Section 1.6.3.2.2):

1. We have provisional evidence that **eIF-2α** levels increase during BrdU-induced differentiation and suggest a role for this induction in allowing eIF-4E to operate in inducing differentiation-related mRNAs without stimulating **global translation** (Section 4.2.4.1.3).

**(E)** **Keratin 8/18** (Section 1.2 & 4.2.2):

1. We have provided additional **evidence** for the existence of **translational control** in the regulation simple keratin expression in poorly differentiated epithelial lines (Section 4.2.3.3.2).
2. Little is known about the regulation of K8 expression (Casanova *et al,* 1995). Based on our results we suggest that **translation** may be an important control point in the **regulation of K8** expression, particularly in early development.
3. We propose a **mechanism** by which simple keratin synthesis is regulated at the level of translation in DLKP, **dependent upon elevated eIF-4E** availability (Section 4.2.3.3.3 & C.1 above).

The events proposed in this cascade probably occur very rapidly, since strong induction of eIF-4E is observed within one day (Figure 3.2.2). Unfortunately determination of the exact timing and sequence of induction of YY1, c-Myc and eIF-4E to hours and minutes is difficult to achieve practically. The cascade rapidly becomes very complex as the three factors central to the overall induction of differentiation (YY1, c-myc and eIF-4E) display an intricate inter-regulatory relationship. YY1 is a transcriptional activator of c-*myc* gene transcription, while interaction between YY1 and the Myc protein downregulates the activity of both factors. c-Myc is a transcriptional activator of eIF-4E expression, while eIF-4E is the key determinant in Myc isoform expression.

In addition, YY1, Myc and eIF-4E have all been predicted to exhibit a high degree of translational regulation and dependence upon elevated eIF-4E (Safrany and Perry, 1993; West *et al,* 1998; Lavoie *et al,* 1996, respectively), confirmed by changes in both YY1 and c-Myc in eIF-4E overexpressing DLKP (Section 3.5.1). In eIF-4E overexpressing CHO cells, c-*myc* transcription was found to shift to the P1 promoter, in addition to enhancing translation of c-myc mRNAs (DeBenedetti *et al,* 1994). Could this be due to the effect of eIF-4E overexpression on YY1 (Section 3.5.1.3)? YY1 regulates transcription from both the P1 and P2 promoters of the c-*myc* gene (Riggs *et al,* 1991). As such, while changes in protein levels (the functional effectors of the gene) are very obvious in BrdU-treated cells, we have had some difficulty in demonstrating precise changes in mRNA levels for these factors. We suspect that BrdU initiates this cascade at the transcriptional level, but that the translational enhancement of the synthesis of these key regulators by eIF-4E may overshadow the contribution of transcription, particularly in DLKP, in which induction does not appear to be a universal phenomenon. However, the critical factor is the level of the functional protein within the cell, not the RNA, unless an antisense or ribozyme is involved. This is highlighted by the regulation of c-Jun expression (Figure 1.5; Section 1.5.2.1.1) and the finding that overexpression of eIF-4E can increase the levels of secretable vaso-permeability factor (VPF) 130-fold at the translational level (Kevil *et al,* 1996).

Might the failure to detect changes in keratin mRNAs be questioned? To induce such a strong protein expression either keratins are strongly transcriptionally induced, which would have been detected, or they show a high degree of translational dependence upon eIF-4E, as for complex mRNAs. Since the 5' UTR of keratins is very short and uncomplicated (Section 3.3.4), there must be a factor bound to this region that simulates complexity (by impeding ribosomal progression under normal levels of eIF-4E expression). This would then act as a target for translational relief by eIF-4E, as suggested by our studies (Section 4.2.3.3.3).

Application of this model to other systems will test its validity and possibly explain some as yet unexplained results. For example, BrdU-treated human melanoma cells exhibited

decreased Endothelin A receptor expression despite an increase in $ET_A$ mRNA levels (Ohtani *et al,* 1997). This was attributed to a decrease in either receptor stability or translational efficiency. Levels of c-*myc* mRNA have been reported to decrease upon exposure to BrdU in human melanoma cells (Valyi-Nagy *et al,* 1993). Applying this to the model we have developed, a decrease in eIF-4E similar to that of HL60s could be predicted. mRNAs with complex 5' UTRs, such as many receptors, are particularly sensitive to eIF-4E levels and as such BrdU-mediated modulation of eIF-4E in these cells may explain this anomaly.

#### 4.2.6.2 A potential "master-target" for BrdU .... YY1?

From Figure 4.8, YY1 may be considered as a "master target" for BrdU-activation and initiation of the differentiation cascade proposed. The ability of YY1 to interact with numerous other proteins (Section 1.5.2.3.2), influencing its activity as a repressor or activator of transcription, suggests that it may also serve to regulate and respond to additional cascades. Alternatively, this ability to interact with other proteins may imply that it is simply an important downstream target of other, as yet unidentified developmental proteins induced by BrdU. BrdU may directly influence the expression of a number of key developmental genes, and the YY1-based cascade proposed here is undoubtedly only one cascade of many induced by BrdU. These cascades more than likely "cross-talk" to influence one another, making elucidation of the exact molecular mechanisms by which BrdU operates very complex. As stated by Watt (1991), "The idea of a single master gene that encodes a transcription factor that binds to a common motif upstream of all genes that are activated during differentiation turns out to be an oversimplification". Identification of key factors and tying-together of "cascades" such as those proposed here will enable more advanced models for lung differentiation to be developed.

The fact that transient transfection of YY1 (Section 3.5.2.3) induces K8 and c-Myc1 expression in DLKP, showing elevated eIF-4E (the speculated effector for YY1 in this induction), only serves to enforce the idea that YY1 is a key factor/regulator of the

proposed cascade. In addition, BrdU has been used in place of transfection of the YY1 cDNA (Lee *et al,* 1992), further suggesting that YY1 is a primary target for activation by BrdU. While suspected, however, we have as yet to provide conclusive evidence of a contribution from transcription to the regulation of these key factors.

#### 4.2.6.3 BrdU and Cytotoxicity:

Due to the synthetic nature and mechanism of action of BrdU (substitution into DNA) the issue of cytotoxicity and selection has been raised (Alexander *et al,* 1992). This was answered, in part, by the fact that a toxic chemotherapeutic agent, Adriamycin, used at numerous concentrations cannot induce or select for keratin positive cells (McBride *et al,* 1999). Using a clonal neural crest stem cell line that exhibited remarkable similarity to our own poorly differentiated clonal lung cell line, DLKP, Ross et al (1995) were able to show that BrdU could induce differentiation but did not select for any one particular clone. DLKP contains SQ-, I-, and M-type subclones (McBride *et al,* 1998), all of which prove keratin negative and at least two of which can be induced to express keratins by BrdU (Section 3.1.3.5). BrdU has been described as an irreversible maturational inducer of a SCLC cell line (Kidson and DeHaan, 1990; Feyles *et al,* 1991), in agreement with our findings (Section 4.2.2). These findings suggest that BrdU is not acting via a selection process, and suggests that BrdU is a potent and perhaps irreversible inducer of differentiation, at least in these cells.

The most convincing experiment that BrdU does not select for reactive or pre-differentiated cells was that of Rauth and Davidson (1993). Treating mouse melanoma cells with BrdU, and the two related analogues IdU (Iododeoxyuridine) and FdU (Flourodeoxuridine), the authors demonstrated an inhibition in cell growth of 60%, 78% and 96% respectively. When the authors correlated growth inhibition with Tyrosinase activity, both IdU and FdU are cytotoxic, since they inhibited cell growth and tyrosinase activity proportionally. However, BrdU was shown to inhibit tyrosinase activity significantly more than it inhibits cell growth, proving that BrdU is exerting its effects at a gene expression level and not at a cytotoxic level.

## 4.3 The ERK Kinases and eIF-4E Phosphorylation in NSCLC:

Results in BrdU-treated epithelial cells (Section 3.2.6 & 4.2.3.1.3) suggested that in the lung cancer cell lines examined, ERK retains its ability to regulate its own activity (Cook *et al,* 1997). To further investigate this, serum starvation experiments were used to quickly assess the roles of various kinase pathways in the regulation of eIF-4E activity in these lung cancer cell lines (Section 3.6).

### 4.3.1 Serum stimulation studies in A549 NSCLC:

Interestingly, serum-starvation of DLKP proved to be difficult (data not shown). This may be due to the fact that DLKP is categorised as a very poorly differentiated NSCLC-NE/SCLC-V that grows quite aggressively. This, combined with the fact that DLKP grows quite happily in serum-free medium (SFM) (Meleady and Clynes, 1995), would seem to indicate that DLKP is capable of producing autocrine growth factors or harbours mutations which render intracellular pathways independent of extracellular signals.

On the other hand, serum starvation of A549 for 48 hours proved sufficient to achieve the desired de-phosphorylation of both ERK and eIF-4E. Initial results demonstrated that, upon withdrawal of serum, ERK becomes dephosphorylated in A549 cells (Figure 3.6.1). Even protein loading was shown by the use of anti-ERK antibodies, showing that the levels of ERK per lane are the same, while the decrease in phosphorylation was evidenced using phospho-specific antibodies (a kind gift of Dr. John Lyons). The rephosphorylation of ERK upon serum stimulation is paralleled by a concomitant rephosphorylation of eIF-4E (Figure 3.6.1), as assessed by iso-electric focusing (IEF).

When the re-phosphorylation profiles of both ERK and eIF-4E in serum-stimulated A549 were examined (Figure 3.6.2) they appear "normal", compared to other cell lines (Foschi *et al,* 1997). The 'peak or spike' in the phosphorylation profile is due to the "hyperphosphorylation" of kinases, caused by the sudden re-exposure of inactivated kinases to mitogens that stimulate their rephosphorylation. There is a peak in eIF-4E

phosphorylation (30 min.), reflecting the ERK phosphorylation (15 min.) profile. This agrees with the theory that ERK is the major kinase of Mnk, the only know direct kinase of eIF-4E (Waskiewicz *et al,* 1997) to date. The slower return to "normal" levels of phosphorylation in eIF-4E probably reflect the sluggish activity of the eIF-4E phosphatase, suggested by DeBenedetti and Rhoads (1990).

In "normal" cells, ERK can regulate its own activity through the phosphorylation of SoS and the induction of MKP (Cook *et al,* 1997) (Figure 4.9). Phosphorylation of SoS (Son of Sevenless) results in dissociation of the SoS-Grb2 complex, breaking the signal emanating from surface receptors and downregulating ERK activity. MKP (MAP-Kinase Phosphatase) is a family of dual specificity phosphatases that dephosphorylate ERK1 and ERK2 (Cook *et al,* 1997). ERK is a downstream activation target of the Ras signalling molecule, which in turn is itself a target for activation by SoS (Figure 4.9). Theoretically, the dephosphorylation of Ras is not possible in A549, reported to harbour a K-12 mutation (Mitsudomi *et al,* 1991) (Section 1.6.4), thereby "short-circuiting" the SoS-mediated autoregulation of ERK activity (Figure 4.9). Assuming the Ras mutational status of A549 reported by Mitsudomi et al (1991) is maintained in these cells, these studies confirm the presence SoS-independent autoregulation of ERK activity alluded to previously (Cook *et al,* 1997; Greulich and Erickson, 1998). Further analysis using "GST-Raffette" (a fragment of Raf interacting with Ras) may be required to ensure the Ras mutational status of the current stock of cells. However, Ras mutated or not, the results of these phosphorylation studies confirm the autoregulatory capacity of ERK in the stock of A549 used for BrdU differentiation studies (Section 4.2).

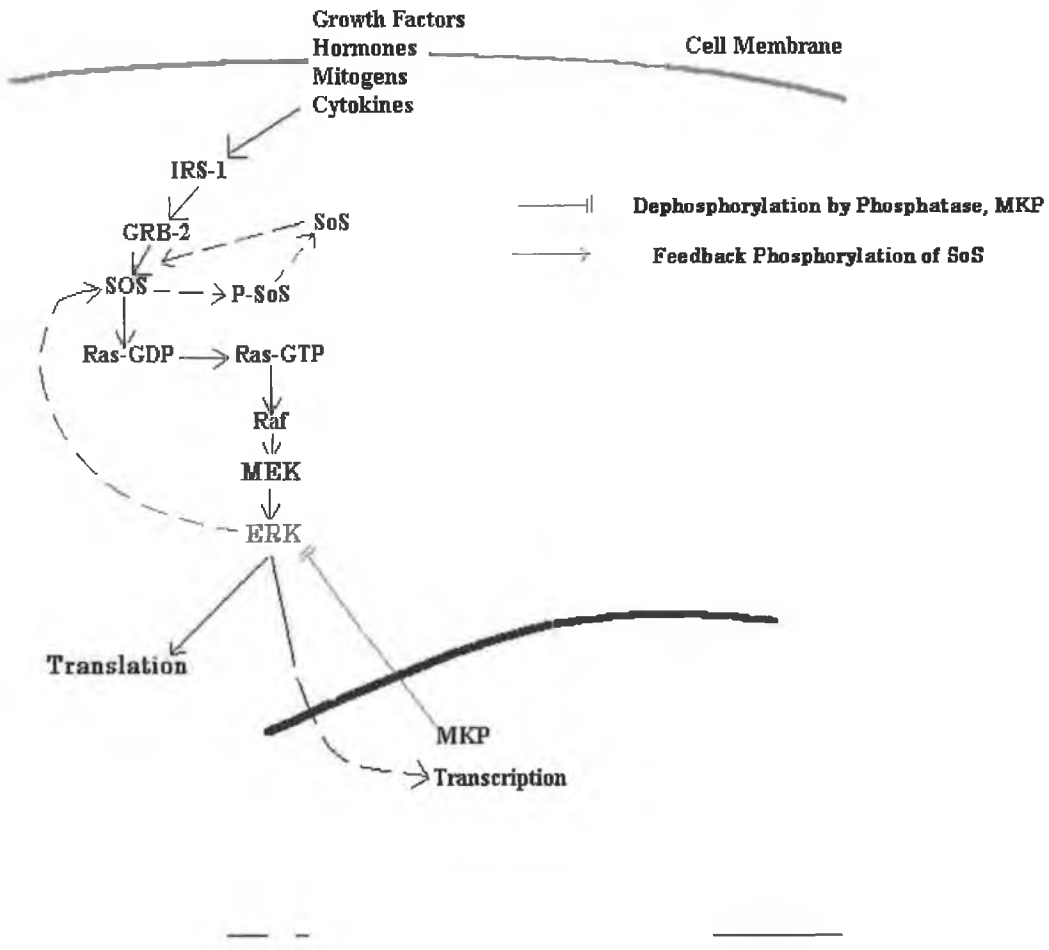



**Figure 4.9: The autoregulatory capacity of ERK.** Induction of ERK activity rapidly induces two mechanisms by which shuts down its own activation. (A) ERK phosphorylates SoS, resulting in its dissociation from the SoS/Grb-2 complex and breaking the signal cascade from the surface of the cell. (B) ERK activity induces the expression of MKP, a family of phosphatases the inactivate both ERK1 and ERK2.

### 4.3.2 Specific Kinase inhibitors in A549 serum studies:

Specific kinase inhibitors were used to investigate the roles of various key kinases in the regulation of eIF-4E activity in A549 (Figure 4.10) (Section 2.12). Results of serum stimulation of A549 in the presence of specific kinase inhibitors are presented in section 3.6.3 (A-E).

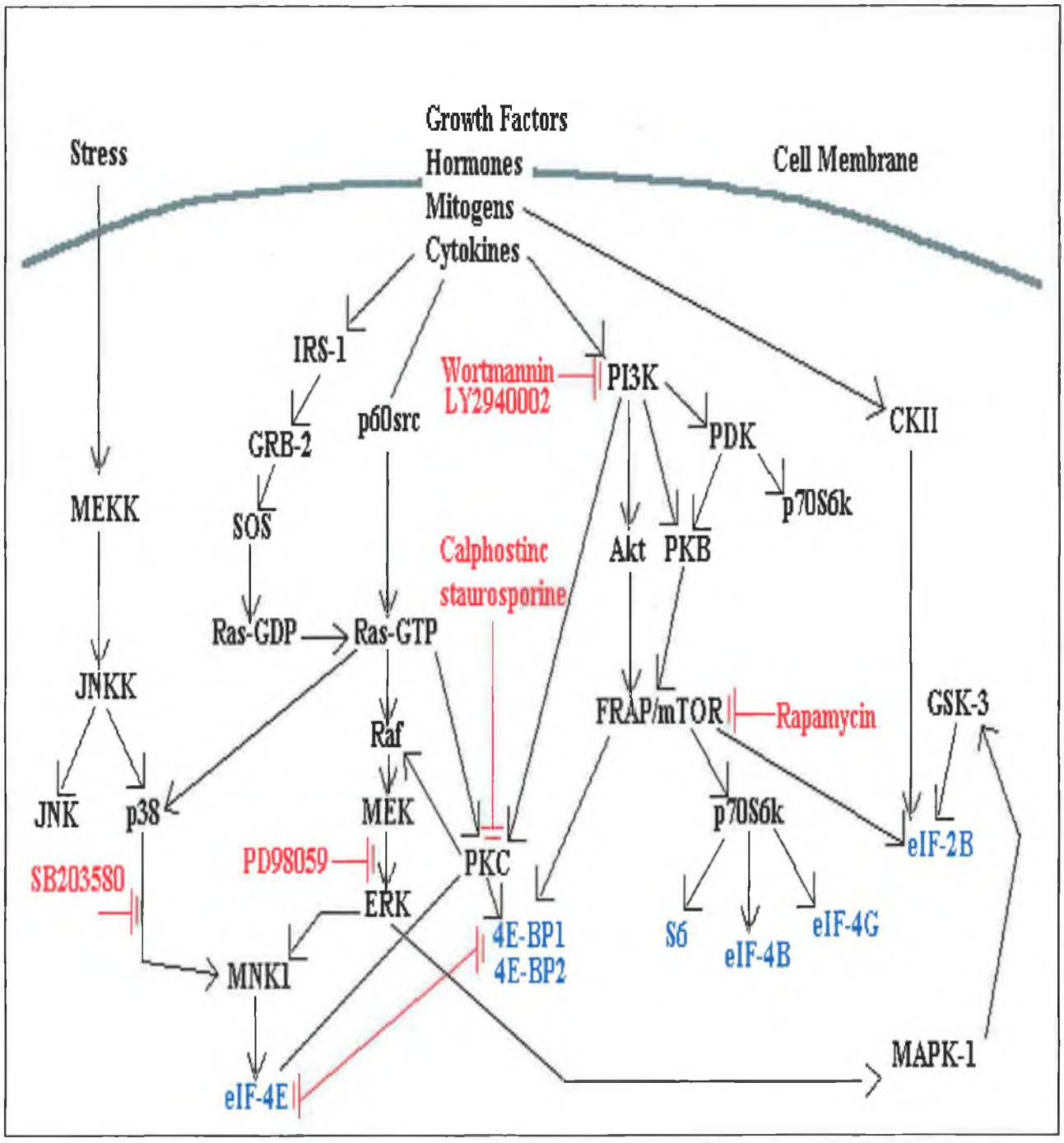



**Figure 4.10: Specific Kinase Inhibitors.** Points at which inhibitors operate are illustrated by the red T-bars. Notably, Rapamycin inhibits the phosphorylation of 4E-BPs, enhancing their ability to associate with and inhibit eIF-4E. Therefore, the use of Rapamycin, unlike the other inhibitors mentioned, inhibits eIF-4E phosphorylation indirectly.

The most significant inhibition of eIF-4E rephosphorylation upon re-stimulation with serum was achieved with PD98059, a direct inhibitor of ERK activity. eIF-4E phosphorylation was also mildly affected by SB203580, which is a specific inhibitor of the stress-related p38 kinase. Both ERK and p38 regulate the activity of the eIF-4E kinase, Mnk (Waskiewicz *et al,* 1997). The contribution of the stress-related kinase, p38 is not as significant as that of ERK, and results from both PD98059 and SB203580 suggest that ERK is the major kinase of Mnk/eIF-4E in these cells. This may be the reason why ERK exhibits such a strong autoregulatory capacity, as it plays a critical role as a downstream regulator of mitogenic activation of both eIF-4E and a number of transcription factors (Frost *et al,* 1997; Sugden and Clerk, 1997). A lesser but significant inhibition of eIF-4E phosphorylation occurred in the presence of Wortmannin, a fungal PI-3K inhibitor that will interfere with PI-3K-Raf-mediated ERK activation, as well as the FRAP/mTOR-mediated phosphorylation of 4E-BPs. However, there was no inhibition by the FRAP/mTOR inhibitor, Rapamycin, suggesting that the effects observed using Wortmannin are probably due to its ability to interfere with PI-3K-mediated activation of the MEK/ERK pathway. It would appear unusual to say that eIF-4E phosphorylation was not noticeably affected by the inhibition of the 4E-BP-regulatory FRAP/mTOR pathway. However, it has been reported previously in a number of cell systems that eIF-4E phosphorylation was not prevented by rapamycin (citations within Fraser *et al,* 1999), suggesting that 4E-BPs may not play a major role in eIF-4E regulation in all cell types. Cumulatively, the results of these inhibitor studies suggest that ERK, p38 and PI-3K/PKC directly regulate eIF-4E activity in A549, but that the 4E-BPs are not involved.

## 4.4 Retinoic Acid and its effects on Epithelial Lung Cancer Differentiation:

Retinoic Acid (RA) has been shown to regulate the differentiation of skin epithelial (Peehl *et al,* 1993) and lung cancer (Doyle *et al,* 1989) cell lines, and modulate the expression of keratins in RA-responsive cells at both transcriptional and post-transcriptional levels (Gilfix and Eckert, 1985; Tomic *et al,* 1990; Stellmach *et al,* 1991; Jing *et al,* 1996). Retinoic Acid is one of the Retinoids (Vitamin-A derivatives) that has been extensively used in clinical applications of "Differentiation Therapy" for the treatment of various cancers and has shown the most potential to date (Section 1.3.1). Vitamin-A deficiency has been associated with an increased risk of cancer development, while Vitamin-A deficiency-induced squamous cell characteristics in small cell carcinomas are reversed by the addition of Retinoic Acid to the medium (Terasaki *et al,* 1987). The topical addition of RA has been shown to reduce papilloma formation (Tennenbaum *et al,* 1998). Both A549 and DLKP were exposed to *all-trans* Retinoic Acid in culture to assess their ability to respond to physiological differentiating agents.

### 4.4.1 Retinoic Acid induces differentiation in A549 but not in DLKP !!

As can be seen from the growth profiles, both cell lines exhibited significant growth inhibition (Figure 3.7.1) when exposed to 20μM Retinoic Acid. A549 cells grew in colonies exhibiting minimal cell-cell contact (Figure 3.7.2.1), similar to that reported for other RA-treated cell types (Kopan *et al,* 1987). Morphologically DLKP did not exhibit significant changes (Figure 3.7.2.2). The growth inhibitory effect observed in DLKP is more than likely due to a very different mechanism than that of A549 and is discussed later (Section 4.4.2.).

In A549, treatment with RA results in the downregulation of K18 protein expression (Figure 3.7.3.1.). This is in agreement with findings that RA downregulates K18 transcript levels in the same cell line (Ledinko and Costantino, 1990). Surprisingly the partner filament protein of K18, K8 decreased over 24-48 hours before recovering and

even increasing in expression (Figure 3.7.3.1). Despite the continued decrease in K18 protein, its partner, K8 was often elevated after 48-72 hours of RA treatment. There was no detectable change in transcript levels for K8 in the experiments presented here (Figure 3.7.4). The initial decrease in K8 protein expression is probably a reflection of the proteolytic instability of K8 in the absence of its partner, K18 (Kulesh *et al,* 1989). The increase in K8 after 24-48 hrs would, therefore, seem to contradict the whole concept of keratin filament pairing and proteolytic stability. However, K8 is also capable of partnering K19 (Section 1.2). It has been shown that, under these conditions, A549 is induced to express K19 by Retinoic Acid (Meleady, unpublished data) (Section 4.4.2). With the induction of K19 expression, K8 can form new filaments and regain proteolytic stability. The pattern of keratin expression would therefore appear to reflect a switch in keratin partnering, and may explain the fact that no changes in transcript levels were observed for K8. It is thought that the majority, if not all keratin genes respond to Vitamin-A, whether it be induction or repression. However, there is very little known about K8 gene regulation compared to its partner, K18 (Casanova *et al,* 1995).

On the other hand, the very poorly differentiated NSCLC-NE/SCLC-V cell line, DLKP showed no detectable induction of keratin expression (Figure 3.7.3.2). When immunoprecipitation of keratins was increased to $5x10^7$ cells, the very low level of "leaky" keratin expression observed in this cell line was not altered in any way (Figure 3.7.3.2). Immunocytochemistry showed no detectable levels of keratin expression in either treated or untreated cells (3.7.2.2.) (there is slight background staining, an artefact of film development). The results for both A549 and DLKP suggest that RA induces an alternative differentiation pathway to that observed using BrdU (Section 4.2). RA is known to induce AP-1 complexes (Pankov *et al,* 1994) (Section 1.5.2.1) that transcriptionally regulate simple keratin gene expression (Oshima *et al,* 1990). The RA-response probably does not overlap with the pathway proposed for BrdU-mediated differentiation. In agreement, one-off western blot analysis showed no significant changes in either c-Myc or eIF-4E expression (data not shown).

cRABPs (cellular Retinoic Acid Binding Proteins) (Section 1.3.1) have been shown to be involved in the regulation of cytoplasmic RA levels (Zou *et al,* 1994) and cRABPII levels are elevated during the differentiation of human skin (Eller *et al,* 1994). Unfortunately, cRABPI proved undetectable in both A549 and DLKP by western blot analysis. It is possible the effects are attributable to cRABPII, which appears to be more widespread in human squamous cell carcinomas (Eller *et al,* 1994; Zhou *et al,* 1994). However, an alternative explanation for the effects and differences observed between the two cell lines is proposed in section 4.4.2.

### 4.4.2 Retinoic Acid Receptor (RAR) expression:

Examination of the expression of two RARs, RAR-α and RAR-β, yielded some interesting results. RAR-α was present in both cell lines (Figure 3.7.5.1), while RAR-β is expressed in A549 but appears to be absent or truncated in DLKP (Figure 3.7.5.2). The lack of RAR-β in DLKP may explain the inability of RA to modulate keratin gene expression in these cells. The modulation of keratins, and perhaps altered morphology and general differentiation induced by RA in A549 cells, may be largely attributable to the presence of RAR-β in these cells.

The retinoic acid receptors belong to a family of nuclear hormone receptors (Giguere *et al,* 1987; Petkovich *et al,* 1987). The RA-responsive receptors consist of two subfamilies, the Retinoic Acid Receptors (RARs) and the Retinoid X Receptors (RXRs), of which there are three family members, the α, β, and γ forms of each. The receptors have been shown to mediate the RA response and influence the expression of several developmentally important genes, including keratins (Tomic *et al,* 1990) (Section 1.3.1.1). K19 is upregulated in K8/18 positive luminal epithelial cell lines, with a concomitant loss of K5/14 expression and the expression of RAR-β closely correlated with K19 mRNA levels (Ivanyi *et al,* 1993). Treatment of a pluripotent human germ cell tumour-derived cell line, NCCIT with RA resulted in the induction of both K8 and K19 expression in these cells (Damjanov *et al,* 1993). Positive and negative recognition elements in these genes may explain the decrease in K18, while K19 levels increase

under the same conditions. Modulation of K8 levels would then be attributable to a post-transcriptional proteolytic stabilisation of K8 as it switches partner.

The role of RARs in the control of differentiation and development has been shown using truncated RARs that can block differentiation and promote features of malignancy (Aneskievich and Fuchs, 1995) and overexpression of dominant-negative RARs could inhibit skin development in transgenic mice (Saltou *et al,* 1995). The RAR-β subtype of these receptors appears to be particularly important to the control of differentiation and development. The loss of RAR-β expression has been associated with aggressive, metastatic and poor clinical diagnosis of cancers. In-situ hybridisation studies have shown that approximately 58% of NSCLC have lost the expression of RAR-β, while 25% have lost RAR-γ and 5% have lost RAR-α expression (Xu *et al,* 1997). Geradts et al (1993) showed that abnormalities in RAR-β expression are common in human lung cancer cell lines, with 75% of SCLCs and approximately 50% of NSCLC failing to show RAR-β induction after treatment with RA. Loss or truncation of RAR-β in DLKP may help to explain its lack of response to RA as well as its characterisation as a very poorly differentiated, aggressive NSCLC-NE/SCLC-variant.

DLKP does, however, exhibit a growth inhibition in the presence of 20μM RA. Beyond a simple toxicity of RA, the presence of RAR-α may play a role in this response. Rat tracheobronchial epithelial cells that express RAR-α, RAR-γ, and RXR-β (but lack RAR-β), were shown to be induced into growth arrest and apoptosis by retinoids via RAR-α-dependent pathways (Zhang *et al,* 1995). The response in DLKP may be a more simple RAR-α-mediated growth arrest or apoptotic induction without a true commitment to differentiation. Such tumours would be difficult to treat, but may be potential targets for combination therapies using drugs, such as BrdU at low levels, that may induce RAR-β expression in these cells, allowing subsequent treatment with RA. Such therapies, however, could only truly be designed once the differentiation of such cells is more clearly understood.

Section 5.0

# *Conclusions and Future Work*

## Section 5.0 Conclusions and Future Work

This project began as an investigation into the mechanism by which the differentiating agent, BrdU is able to alter the differentiation status of epithelial lung cells. Results suggested a possible role for translation in regulating simple keratin filament formation, a rather unexpected mode of action for such a drug. Our results highlight novel roles for the small cap-binding translation initiation factor, eIF-4E in our *in-vitro* systems, with possible relevance for early lung development and lung carcinogenesis. In addition, we propose a differentiation-regulating cascade based upon the observed changes in the levels of both c-Myc and YY1 transcription factors, which we suspect may be involved in the induction of eIF-4E-dependent differentiation mechanisms. We suspect that YY1 is an initiator of this cascade, and may be a direct target for BrdU activation. The fact that this cascade appears to be downregulated in HL60 cells suggests that we may be looking at an epithelial-specific pathway.

The oncogenic properties of eIF-4E have been established (DeBenedetti and Rhoads, 1990; Lazaris-Karatzis et al, 1990). However, we propose that one of the "natural" roles of eIF-4E is to regulate the translational efficiency of differentiation-related mRNAs during early development. Poorly expressed or repressed mRNAs are unable to efficiently compete for available eIF-4E. Increased availability of eIF-4E during early development may be one of the as yet unidentified developmental "signals" to begin translation of these mRNAs. It has been show that overexpression of eIF-4E can induce mesoderm formation in *Xenopous laevis* embryos (Klein and Melton, 1994), increasing expression of activin, known to be translationally repressed in these cells (Klein and Melton, 1995). Similarly, we provide strong evidence for the translational repression of simple keratin expression in the poorly differentiated, stem cell-like cell line, DLKP, and propose a model whereby increased expression of eIF-4E in this cell line can induce K8/18 proteins, an indication of simple epithelial differentiation. The majority of studies into the regulation of keratin filament formation during epithelial differentiation have focused on the transcriptional regulation of their expression. However, very little is known about the mechanism(s) by which they are regulated, particularly in poorly differentiated cell types. In addition, in comparison to its partner keratin, K18, the mechanisms regulating K8 expression are relatively unknown

(Casanova et al, 1995). We suggest that translation may be a significant regulatory point in the expression of K8, and perhaps overall K8/18 filament formation, particularly in early development. Translation is now becoming more widely accepted as a critical regulator in the correct timing of developmental gene expression.

In both BrdU-treated and eIF-4E overexpressing epithelial cell lines, we have shown the induction of c-Myc1 expression. The expression of this Myc isoform has been shown to be heavily dependent upon the availability of eIF-4E (Carter et al, 1999). We suggest that the induction of this growth-inhibitory form of the Myc protein may be a mechanism by which eIF-4E can prevent aberrant growth of cells during periods of elevated eIF-4E expression in the early stages of development. In agreement with this hypothesis, loss of c-Myc1 expression due to mutation or translocation has been associated with numerous cancers (Saito et al, 1983; Hann et al, 1984; Hann et al, 1995), commonly thought of as a disease of "abnormal differentiation" (Sporn and Roberts, 1983).

We suspect that YY1 may be an important initiator of the cascade proposed to be activated by BrdU (Section 4.2.6). It is an important developmental regulator that has been shown to be a target for BrdU-induced transcriptional activation in myoblasts (Lee et al, 1992). YY1's unusual nature and ability to alter its properties as an activator or repressor of transcription through interaction with other proteins may highlight YY1 as an extremely important "cross-talk" factor, allowing this cascade to respond to and influence the activity of other factors/cascades within individual cell types. This may form at least part of the basis of cell-specific differences during differentiation. Different cell types will have a different complement of transcription factors and as such will have different responses to and effects upon the activity of both YY1 and this cascade as a whole. In addition, there may be an autoregulatory mechanism within the cascade itself, through interaction between YY1 and c-Myc, controlling the activity of these factors themselves and the overall activity of this cascade. Such cascades, while complex in appearance, are probably a truer representation of the natural "communicating" regulatory pathways that control cellular processes such as differentiation than "single-step" models.

BrdU ultimately acts at the transcriptional level. Its ability to increase the availability of eIF-4E and therefore the selective translation of complex, repressed and poorly competitive mRNAs is likely to be a major factor in its ability to influence the differentiation status of cells. Our studies suggest that BrdU may induce eIF-4E activity through changes in signalling activity during differentiation (altering phosphorylation levels of eIF-4E) and/or via small increases in transcription of the eIF-4E gene (via changes in c-*myc* expression, a regulator of eIF-4E transcription); a combination of both may strongly influence eIF-4E availability. The induction of c-Myc1 expression is due to alternate translation initiation at an upstream codon, a process dependent upon eIF-4E availability, further confirming the increased activity of eIF-4E in BrdU-treated epithelial cells and highlighting the possibly underestimated importance of translation in the regulation of processes such as differentiation. Our results with eIF-4E overexpressing DLKP suggest that YY1 may also be subject to translational regulation of isoform expression in a manner similar to that shown for c-Myc. Such N-terminal extension of transcription factors by eIF-4E may be a means of altering the transcriptional capacity of the cell during periods of elevated eIF-4E activity.

It is difficult to directly compare the effects observed in DLKP and A549. While they are both epithelial lung cancer cell lines, they are also very different. DLKP is a poorly differentiated, NSCLC-NE/SCLC-V, while A549 has been categorised as a diffuse, glandular adenocarcinoma. The activation of the YY1 cascade in A549 may represent activation of a "dormant relic" of a cascade required for earlier development. Alternatively, its activation may drive A549 towards another differentiated state. Their differences are highlighted by their response to the physiological differentiating agent, Retinoic Acid (RA). While RA is capable of altering the differentiation status of A549, it appears to be unable to do so in DLKP. Our results suggest that the lack of expression, or expression of a truncated form of, RAR-β is a major factor in the failure of DLKP to respond RA, and may hold the key to the poorly differentiated nature of this cell line. The elevated levels of eIF-4E in DLKP may represent a developmental function of eIF-4E in poorly differentiated cell types, downregulated at later stages of differentiation, and may not be directly related to its "cancer state".

**Future Work:**

The work described in this thesis has identified a number of key factors and mechanisms that may be critical in the control of simple keratin filament formation in differentiating lung epithelial cells. Avenues that would be of value to further pursue are briefly outlined:

1) Examine mRNA levels and gene activity of eIF-4E, eIF-2α, YY1 and c-myc using more advanced techniques (Nuclear Run-on/ Rnase protection) to determine the relative contribution of transcription to the regulation of the proposed cascade.
2) Examine the levels of c-Myc2 expression in BrdU-treated and eIF-4E overexpressing cells.
3) Transfection studies: Examine the individual effects of each of the key factors identified by this study on epithelial differentiation by cDNA overexpression studies: eIF-4E, YY1, eIF-2α and c-*myc* (distinguishing between c-Myc1 and c-Myc 2 isoform expression) would be prime candidates for critical regulatory factors in this process.
4) Further develop *in-vitro* translation investigations to determine more exactly the specificity of translational repression in DLKP and possibly identify the nature of this repression.
5) DNA array technology may be a means of "mass-screening" for BrdU-activated genes, by comparing RNA extracts from BrdU-treated and untreated cells.
6) Immunocytochemical and *in-situ* hybridisation studies using tissue sections from early lung and cancer samples may be used to identify the *in-vivo* relevance of factors, such as eIF-4E, shown to be involved in differentiation in our in-vitro system.

Section 6.0

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