A Study of c-myc Translational Regulation in Multiple Myeloma

by

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Abstract

A study of \textit{c-myc} translational regulation in multiple myeloma
Fiona E M Paulin

In cell lines derived from patients with multiple myeloma (MM), a B-cell neoplasia, a 10-20 fold elevation in the level of \textit{c-myc} protein has been observed. This was not accompanied by a concomitant increase in \textit{c-myc} mRNA levels and there was no alteration in the half life of the protein suggesting that a translational mechanism could be responsible for the elevated \textit{c-myc} protein levels.

Sequence analysis of \textit{c-myc} exon 1, a region previously implicated in translational control, revealed no gross sequence abnormalities of the \textit{c-myc} gene in the MM cell lines. However, in 4 out of 5 MM cell lines examined, a consistent C\textrightarrow{}T transition at position 2756 was observed. This mutation was not seen in any control cell lines or in peripheral blood samples derived from healthy patients implying that the sequence alteration is not merely a polymorphism of this locus and may be associated with the malignant phenotype. Additionally, the MM cell line which did not possess the mutation displayed atypical antigenic expression.

Examination of proteins binding to the \textit{c-myc} 5'UTR revealed a large number of proteins with this capacity. In addition, the mutant sequence displayed enhanced binding affinity to proteins, specifically polypeptides of 98 and 38 kD. Differences in RNA binding factors were also detected between the control and MM cell lines.

\textit{c-myc} exon 1 was found to inhibit the translation of heterologous reporter genes \textit{in vitro}. However, this region was also found to be capable of promoting the internal initiation of ribosomes in dicistronic reporter constructs. Moreover, the mutant sequence displayed an augmented capacity for internal initiation of translation.

Both the mutation and altered protein factors in the multiple myeloma cell lines thus appear to affect the translational efficiency of \textit{c-myc} mRNA and their combinatorial effects may be sufficient to account for the elevated levels of c-Myc observed.
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### Abbreviations

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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt's lymphoma</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMSC</td>
<td>bone marrow stromal cell</td>
</tr>
<tr>
<td>BR</td>
<td>basic region</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CE</td>
<td>cell extract</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>diaminoethanetetra-acetic acid</td>
</tr>
<tr>
<td>eEF</td>
<td>eukaryotic elongation factor</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>eRF</td>
<td>eukaryotic release factor</td>
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<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
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<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HLH</td>
<td>helix-loop-helix</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry segment</td>
</tr>
<tr>
<td>kD</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>LCL</td>
<td>lymphoblastoid cell line</td>
</tr>
<tr>
<td>LZ</td>
<td>leucine zipper</td>
</tr>
<tr>
<td>Met-tRNA</td>
<td>methionyl-tRNA</td>
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MM  multiple myeloma
MOPS  3-[N-morpholino]propanesulfonic acid
mRNA  messenger RNA
mRNP  messenger ribonucleoprotein particle
Mt  mutant
NAD  nicotinamide adenine dinucleotide
NP40  nonylphenyl-polyethylene glycol
OAF  osteoclast activating factor
ORF  open reading frame
PAGE  polyacrylamide gel electrophoresis
PB  peripheral blood
PBMC  peripheral blood mononuclear cell
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PE  phycoerythrin
PTB  polypyrimidine tract binding protein
RNA  ribonucleic acid
rNTP  ribonucleoside triphosphate
SDS  sodium dodecyl sulphate
TCA  trichloroacetic acid
TE  Tris-EDTA
TEMED  N,N,N',N'-tetramethyl-ethylenediamine
TMEV  Theiler's murine encephalomyocarditis virus
TNF-β  tumour necrosis factor-β
Tris  tris(hydroxymethyl)aminomethane
Tween 20  polyoxyethylene sorbitan monolaurate
uORF  upstream open reading frame
UTR  untranslated region
Wt  wild type
X-GAL  5-bromo-4-chloro-3-indolyl-β-D-pyranoside
CHAPTER 1

Introduction
Introduction

1.1 General Overview

The growth, division and finite lifespan of eukaryotic cells are highly regulated processes, all ultimately governed at a molecular level by complex interactions of innumerable genes and gene products. Correspondingly, gene expression is precisely controlled at a variety of points. Broadly, these encompass the processes of transcription, mRNA metabolism, including post-transcriptional modifications and mRNA stability, and translation. Perturbation to any of these exquisite controls resulting in deregulated gene expression, particularly in growth control genes i.e. oncogenes and tumour suppressor genes, can therefore lead to neoplastic transformation.

Deregulation of the c-myc proto-oncogene is commonly observed in B-cell malignancies and consequently is thought to play a pivotal role in development of these diseases. Preliminary data, presented herein, indicate that this may be in part at the level of translation in the plasma cell disease multiple myeloma. This observation is of particular significance because, to date, no consistent molecular alterations have been associated with multiple myeloma. Long term prognosis for multiple myeloma patients is extremely poor and the identification of novel therapeutic targets e.g. inhibition of translation of the c-myc gene is of paramount importance.

1.2 Multiple Myeloma

1.2.1 Epidemiology

Multiple myeloma (MM) is a malignancy of terminally differentiated B-cells in which there is a proliferation of plasma cells within the bone marrow. The disease is relatively rare accounting for 1% of all malignancies in the USA and approximately 15% of haematological malignancies (reviewed in Nieszvizky et al., 1993). The incidence of occurrence and the mortality rate however have recently been increasing (reviewed in Riedel & Pott, 1992). Males are affected more than females with the annual rate per 100,000 being 4.7 for men compared to 3.2 in women (Riedel & Pott, 1992). There are also variations in ethnic origin, with blacks having almost twice the incidence rate of whites (McWhorter et al., 1989). MM predominantly affects people over 65 years of age, the median age at diagnosis being 70 years (reviewed in Riedel & Pott, 1992). From the time of diagnosis the median survival is around 36 months (Nieszvizky et al., 1993).
1.2.2 Clinical symptoms of multiple myeloma

MM is frequently difficult to detect as early stages of the disease are often asymptomatic. However, the combined symptoms of bone pain, anaemia and fatigue are commonly indicative of multiple myeloma (Dimopoulos et al., 1992). Three clinical attributes are employed in diagnosis of the disease (Figure 1.1). The first is the presence of a characteristic monoclonal M protein in serum and/or urine, arising as a result of overproduction of a particular immunoglobulin subtype. In the majority of cases this is IgG or IgA with IgD and IgE being relatively rare. In addition, 80% of myeloma cases produce an excess of immunoglobulin light chains, with k being more prevalent than λ (Niesvizky et al., 1993).

The second characteristic feature is that within the bone marrow greatly increased numbers of plasma cells are found, constituting 10% or more of the nucleated cells present (Kyle, 1992). These myeloma cells, distinguishable by the presence of an acentric nucleus, are frequently accompanied by atypical morphological features including variations in cell size and cytoplasmic staining. Nuclear abnormalities and asynchronism, whereby the cytoplasmic maturity is disproportionate to the degree of nuclear differentiation, are also widespread (Boccadoro & Pileri, 1995).

Osteolytic lesions, primarily in regions of haematopoietically active marrow (skull, vertebrae, ribs, pelvis and long bones), sometimes concurrent with diffuse osteoporosis, completes the diagnostic triad. These lesions arise as a result of complex interactions between the bone marrow microenvironment and myeloma cells which stimulate the production of a number of osteoclast activating factors, notably interleukin 1 (IL-1) and tumour necrosis factor (TNF-β), which are responsible for bone destruction (Durie, 1988).

A clinical staging system based on these parameters, and others, is used for prognosis of multiple myeloma and correlates with tumour mass and survival (Durie & Salmon, 1975). However, this system in some cases is unreliable and more recently other factors including serum levels of β-2-Microglobulin (β2M) (Cuzick et al., 1985), interleukin 6 (IL-6) (Reibnegger et al., 1991) and expression of common acute lymphoblastic leukaemia antigen (CALLA) (Durie & Grogan, 1985) have been employed.

Treatment of the disease, in virtually all cases, involves chemotherapy often in combination with bone marrow transplantation (reviewed in Barlogie et al., 1989). More recently biological response modifiers such as interferon (IFN) (Petrutti et al., 1994) and anti-IL-6 antibodies (Klein et al., 1990) have been tried with differing success rates. Such treatments have tended to be palliative rather than curative with patients typically suffering from multiple fractures, renal failure, anaemia and recurrent infections due to
Figure 1.1: Diagnostic triad of multiple myeloma. (A) Serum electrophoresis of immunoglobulin levels showing a normal polyclonal desitometer trace and a monoclonal paraprotein peak characteristic of MM. (B) Roentgenogram of the skull displaying a large osteolytic lesion of the frontal bone. (C) Trephine bone marrow biopsy stained with May Grunwald Giemsa (MGG) (x400). Large numbers of atypical plasma cells are visible. Panels A & B taken from Kyle & Bayrd, 1976.
impaired immunological status. Death as a result of haemorrhage and/or infection is usually inevitable, occurring within a period of a few months to several years from diagnosis (Holbert, 1988). Better prognostic factors for MM are therefore required, but with elucidation of the cellular and molecular mechanisms underlying the disease advances in therapy should be forthcoming (reviewed in Kyle, 1994).

1.2.3 The cellular basis of multiple myeloma

Plasma cells are terminally differentiated cells of the B-cell lineage (Figure 1.2). These cells normally circulate within the lymphopoeitic system and typically display an elevated RNA content and high rates of protein synthesis, presumably reflecting their antibody secretory function (Barlogie et al., 1989). Myeloma cells also possess these characteristics but have either retained or regained a proliferative capacity, albeit low, more reminiscent of stem cells (Drewinko et al., 1981). In addition, myeloma cells show heterogeneous expression of a diverse array of non plasma cell specific B-cell lineage antigens as well as some B-cell unrelated antigens (Boccadoro & Pileri, 1995) which would tend to suggest that the lesion giving rise to MM occurred at an early stage in B-cell morphogenesis. Also, complications of the disease sometimes involve secondary myeloid abnormalities eg. acute myeloid leukaemia (AML) and myeloid dysplasia (MDS) corroborating this idea (Bergasel et al., 1979).

The widespread dissemination of the disease throughout the body, advocates the necessity for a motile element, either in the form of a pre-plasma cell precursor or a mature myeloma cell. Indeed, peripheral blood lymphocytes bearing the unique myeloma clone idiotypne have been identified (Berenson et al., 1987). Expression however was restricted to the B-cell lineage, implying that the myeloma progenitor arose after divergence of the two lymphocytic pathways. More recently an allele specific oligonucleotide PCR (ASO-PCR) method for the complementary determining regions (CDRs), which are B-cell clone specific, has been utilised both to define the neoplastic clone and determine the level of maturation (Billadeau et al., 1992; Bakkus et al., 1994). This identified the clonogenic cell in MM to be a pre-switched, somatically mutated B-cell that had undergone antigenic selection, thereby designating the B-cell memory compartment as the most likely candidate (Bakkus et al., 1994). However, other reports have found no evidence of different myeloma clone Ig isotypes circulating within the bloodstream, suggesting that the myeloma precursor is derived from post-class switched cells (Vescio et al., 1994). Such apparent discrepancies though may be accounted for by the sensitivity of the PCR approach employed (Bakkus et al., 1994).

Notwithstanding, the detection of clonally related but idiotypically diverse cells could itself imply the existence of an altered pre-B cell population but with which additional
Figure 1.2: B-cell differentiation pathway.
changes are required for full myelomagenesis (Epstein et al., 1995). Although evidence is therefore mounting to eliminate stem cell involvement as the progenitor of myeloma cells, the locality of the malignant transformation still remains unclear. The most favoured hypothesis is that of a malignant progenitor developing in the bone marrow, becoming mobilised into the lymphoid system, and returning to the bone marrow microenvironment where a variety of combinatorial factors induce the proliferation and differentiation of the myeloma clone (Epstein, 1992).

1.2.4 The bone marrow microenvironment

Bone marrow consists of a multilayered adherent network, composed of a mixed population of endothelial cells, reticular cells, adipocytes, macrophage, osteoclasts and fibroblasts, collectively termed bone marrow stromal cells (BMSC) (Lichtman, 1981). These can be divided into three distinct classes based on morphology and expression of adhesion molecules (Table 1.1) (Caligaris-Cappio et al., 1992). The major components are fibroblast-like elements which secrete an extracellular matrix of fibronectin, laminin and collagen IV (Caligaris-Cappio et al., 1991). Interactions with this extracellular component, and also intercellular associations, are dictated by the differential expression of adhesion molecules within the stromal cell network. This is further complicated by additional interactions of haematopoietic cells, which is again dependent on the ligands they exhibit (Funk et al., 1994; Segat et al., 1994).

One of the initiating events, responsible for evolution of MM, is therefore thought to involve an altered repertoire of cell surface antigen expression on myeloma cells which in turn modifies BMSC interaction. Concurrent with this is the fact that binding of plasma cells to BMSC results in a release of cytokines. Notably these include interleukins 1, 6 and 8 (IL-1β, IL-6, IL-8), granulocyte macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor beta (TNF-β) (Klein & Bataille, 1992; Merico et al., 1993). These have pleiotropic effects but many result in the release of further cytokines which feedback to the original cells, serving to amplify the signal, culminating in a self-perpetuating mechanism that may contribute towards propagation of the malignant clone (Figure 1.3).

Co-culture studies using BMSCs and autologous peripheral blood mononuclear cells (PBMC) or myeloma cells lines have proved indispensable in delineating myeloma cell growth due to the reproducibility of the assay system. Three distinctive events are observed after 3 weeks of co-culture. 1) Myeloma cell proliferation is observed. 2) Increasing numbers of osteoclasts are produced. 3) IL-6 levels are dramatically upregulated (Caligaris-Cappio et al., 1992). However, only BMSC obtained from MM patients have this growth stimulatory property (Caligaris-Cappio et al., 1992), though
<table>
<thead>
<tr>
<th>Adhesion Molecule</th>
<th>Fibroblast-like cells</th>
<th>Macrophage</th>
<th>Osteoclasts</th>
</tr>
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<tbody>
<tr>
<td>β1 integrin</td>
<td>CD29</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β2 integrin</td>
<td>CD18</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>β3 integrin</td>
<td>CD61</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>αL integrin</td>
<td>CD11a</td>
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</tr>
<tr>
<td>N-CAM</td>
<td>CD56</td>
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<td>+</td>
</tr>
</tbody>
</table>

Table 1.1: Expression of adhesion molecules on bone marrow cell types. Adapted from Caligaris-Cappio et al., 1992.
Figure 1.3: Schematic representation of the bone marrow/plasma cell microenvironment found in multiple myeloma patients highlighting the cytokine interactions thought to be important in development of the disease.
binding of MM cell lines to BMSC from both normal and MM patients has been shown to stimulate IL-6 secretion (Uchiyama et al., 1993). This function has been shown to require intimate cell to cell contact as it can be partially blocked by antibodies to β1 and β2 integrins, VLA4, CD29 and LFA-1 (Uchiyama et al., 1993; Lokhorst et al., 1994). Similar physical association has also been shown to be required for IL-6 production between MM cell lines and osteosarcoma cell lines (Barille et al., 1995) indicating that adhesion molecules are of key importance.

The rationale for these observations lies firstly in the fact that MM BMSCs are actively growing, compared to controls, and only they release significant amounts of IL-6 and IL-8 into the culture (Merico et al., 1993). Secondly, adhesion of myeloma cells induces a transient increase in IL-6 transcription in BMSCs (Chauhan et al., 1996). Thirdly, IL-6 is a potent osteoclast activating factor (OAF) which in concert with other OAFs produced by BMSCs (IL-1β, TNF-β, M-CSF & IL-3) stimulates the production of osteoclasts responsible for bone destruction (Caligaris-Cappio et al., 1992).

1.2.5 Interleukin-6 : The major growth factor of MM

Interleukin-6 has been identified as the major cytokine responsible for propagation of the plasmablastic clone. Its effects have been postulated to occur in one of three different ways. 1) To act as a stimulus for proliferation on a myeloma progenitor cell type. 2) To act simply as a differentiation factor. In this connection IL-6 is normally required for the maturation of activated B cells into immunoglobulin secreting cells (Hirano et al., 1985). 3) To influence both proliferation and differentiation of myeloma cells (Hata et al., 1993). In addition to this, IL-6 more recently has been found to inhibit apoptosis of myeloma cells but this is not mediated by Bcl-2 (Kawano et al., 1995; Lichtenstein et al., 1995; Xu et al., 1995). The exact mechanism by which IL-6 exerts these effects is not fully known. Signal transduction via the Interleukin-6 Receptor (IL-6R) induces tyrosine phosphorylation and association with gp130 the signal transducing subunit of IL-6 (Murakami et al., 1993). The ligand activated receptor/gp130 complex is then thought to activate either a Src kinase or Janus kinase which then activates p21 ras, via She phosphorylation (Ogata et al., 1995; Neumann et al., 1996).

Controversy still remains over whether IL-6 is an autocrine or paracrine growth factor for MM. Kawano et al originally proposed that myeloma cells produced IL-6. The main reasons were fourfold. 1) 50% of patient samples showed a proliferative response to IL-6 in vitro. 2) Myeloma cells expressed IL-6R. 3) Addition of antibodies to IL-6 inhibited proliferation. 4) Small amounts of IL-6 were detected in culture supernatants (Kawano et al., 1988). This was substantiated by work of Hata et al. who demonstrated a correlation of the expression of IL-6 and IL-6R transcripts in pre-plasma cells (Hata et al., 1993).
Several MM cell lines have also been shown to produce and be responsive to IL-6 (Hitzler et al., 1991; Zhang et al., 1994). Furthermore treatment of myeloma cells with interferon α and β has been reported to induce expression of both IL-6 and IL-6R (Epstein, 1992).

However, conflicting reports indicate that BMSCs are the major source of IL-6 (Klein et al., 1989; Caligaris-Cappio et al., 1991; Epstein, 1992; Lokhorst et al., 1994; Chauhan et al., 1996). This is consistent with the fact that murine plasmacytoma cell lines are all dependent on exogenous IL-6 for proliferation. Moreover, in MM cell lines, expression of IL-6 mRNA is detected only in a small number of cases even though IL-6R mRNA distribution is more universal (Hitzler et al., 1991). More recently IL-6, specifically produced by stromal cells, has been shown to be required for early plasma cell differentiation (Kawano et al., 1995).

IL-6R expression was found to be absent in mature myeloma cells derived from patient samples suggesting a reduced requirement for IL-6 (Hata et al., 1993). It has been postulated that the growth of these cells could be sustained by circulating IL-6 and the soluble form of the IL-6 receptor (Epstein, 1992). This is also consistent with the fact that high IL-6 serum levels in MM patients were associated with poor prognosis (Ludwig et al., 1991). Contradictory reports, however, indicate that IL-6 expression is highly variable and not predictive of the disease state (Emile et al., 1994; Ballester et al., 1995). The main conclusion is therefore that myeloma cells show variable dependency on exogenous IL-6 which may be correlative with their maturation state (Epstein et al., 1995). Thus it appears that both autocrine and paracrine mechanisms function for IL-6 production in myeloma cells. Initially immature myeloma cells display a paracrine requirement for IL-6 allowing differentiation. Upon maturation this is gradually lost and the sustained prerequisite for IL-6, to prevent apoptotic cell death, is met by the autocrine production of IL-6.

1.2.6 Antigenic characteristics of myelomatous plasma cells

There is a large heterogeneity in the expression of cell surface antigens both between normal and myelomatous plasma cells but also between patient material and MM cell lines. In addition, individual cells within each of the aforementioned classes display a diverse array of phenotypic characteristics possibly reflecting development of these neoplastic cells. To date, no one antigen has been ascribed as unique and singular to multiple myeloma, therefore myeloma cells tend to be distinguished on the basis of multiple parameters.

In general MM plasma cells express surface antigens that can be broadly classed into four groups i) B-cell lineage related antigens ii) B-cell lineage unrelated antigens iii)
intercellular adhesion molecules and iv) intercellular signalling molecules (Boccadoro & Pileri, 1995). Again, there is considerable variability in the expression of these molecules, which is summarised in Table 1.2. Normal plasma cells also possess many of these characteristics (Table 1.3) but in general can be distinguished on the basis of increased CD56 expression and reduced CD11a reactivity (Ahsmann et al., 1992; Pellat-Deceunynck et al., 1995). It may be of significance in the development of the disease that both these molecules are involved in adhesion, indicating the necessary involvement of bone marrow stromal cell network.

Ordinarily MM plasma cells can be isolated from whole blood samples simply by using the criteria of strong CD38 expression and low to intermediate levels of CD45 expression (Billadeau et al., 1996; Witzig et al., 1996). Although this technique would also identify normal plasma cells idiotypic examination attests to the myelomatous origin of these cells, presumably due to their inflated numbers. Indeed, it has been shown that these two parameters are an accurate and reliable means of detecting and quantifying circulating malignant plasma cells in multiple myeloma patients (Witzig et al., 1996).

CD38

CD38 is a 45 kD transmembrane glycoprotein, consisting of a short N-terminal cytoplasmic tail and a large C-terminal extracellular loop (Jackson & Bell, 1990). It is widely expressed particularly on lymphocytes specifically being associated with immature and activated cells (Malavasi et al., 1994). CD38 is thought to be an important immunoregulatory molecule as monoclonal antibodies which cross react to it have been shown to have diverse effects. These include both induction of proliferation in B-cells and rescue from apoptosis in mature lymphocytes (Santos-Argumedo et al., 1993; Yamashita et al., 1995). However, ligation of CD38 also induces apoptosis of B cell precursors providing additional stromal cells or stroma-derived cytokines are present (Kumagai et al., 1995).

It has recently been shown that in immature B cells, activation of CD38, by dimerisation, causes a rapid induction of tyrosine phosphorylation, activation of syk tyrosine kinase and induced phosphorylation of phospholipase C-γ and the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) (Silvennoinen et al., 1996). In addition PI3-K associated with the cbl gene product (Kitanaka et al., 1996). All of the above effects however were found not to involve a Ca2+ flux (Kirkham et al., 1994; Silvennoinen et al., 1996). Moreover, in myeloid HL60 cells stimulated to differentiate with retinoic acid, thereby resulting in an induction of CD38, cbl was also identified as a prominent tyrosine phosphorylated protein (Kontani et al., 1996).
## Table 1.2: Cell surface antigen expression of myeloma cells

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Associated</th>
<th>Reactivity (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B-cell lineage related</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD10</td>
<td>Pre-B</td>
<td>20-58</td>
<td>2, 6, 7</td>
</tr>
<tr>
<td>CD19</td>
<td></td>
<td>20-60</td>
<td>1, 6, 7</td>
</tr>
<tr>
<td>CD20</td>
<td></td>
<td>20-36</td>
<td>5, 6</td>
</tr>
<tr>
<td>CD23</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CD24</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CD38</td>
<td>PC</td>
<td>100</td>
<td>3, 6, 7</td>
</tr>
<tr>
<td><strong>B-cell lineage unrelated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>TC</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CD4</td>
<td>TC</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CD14</td>
<td>Myeloid</td>
<td>0-6</td>
<td>1, 5, 6</td>
</tr>
<tr>
<td>CD33</td>
<td>Myeloid</td>
<td>65</td>
<td>2</td>
</tr>
<tr>
<td><strong>Intercellular adhesion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD49e</td>
<td>VLA-5</td>
<td>0-100</td>
<td>3</td>
</tr>
<tr>
<td>CD49d</td>
<td>VLA-4</td>
<td>90</td>
<td>3, 4</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>95</td>
<td>4</td>
</tr>
<tr>
<td>CD56</td>
<td>NK</td>
<td>0-40</td>
<td>1, 8</td>
</tr>
<tr>
<td><strong>Intercellular signalling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td>0-95</td>
<td>7</td>
</tr>
<tr>
<td>CD45</td>
<td></td>
<td>58</td>
<td>6</td>
</tr>
<tr>
<td>HLA-DR</td>
<td></td>
<td>35</td>
<td>5, 6</td>
</tr>
</tbody>
</table>

References:
1) Duperray *et al.*, 1989
2) Epstein *et al.*, 1990
3) Kawano *et al.*, 1993
4) Kim *et al.*, 1994
5) San Miguel *et al.*, 1995
6) Terstappen *et al.*, 1990
7) Tong *et al.*, 1994
8) Uchiyama *et al.*, 1992
<table>
<thead>
<tr>
<th>Surface antigen</th>
<th>Normal PC</th>
<th>Myelomatous PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a (LFA-1)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CD38</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>CD44</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD45</td>
<td>-</td>
<td>-/+</td>
</tr>
<tr>
<td>CD49d (VLA-4)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD49e (VLA-5)</td>
<td>+</td>
<td>-/+ (immature/mature)</td>
</tr>
<tr>
<td>CD54</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD56</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>CD58 (LFA-3)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 1.3**: Phenotypic expression of cell surface antigens of normal plasma cells (PC) and myelomatous plasma cells. Parenthesised term in first column denotes the associated complex.

- : negative expression  
+ : weak expression  
++/++++ : strong expression
CD38 is also a bifunctional ectoenzyme capable of catabolising nicotinamide adenine dinucleotide (NAD+) to cyclic ADP-ribose (cADPR) and then hydrolysing cADPR to adenosine diphosphoribose (ADPR) (Takasawa et al., 1993). Recent studies have also suggested that nicotinamide adenine dinucleotide phosphate (NADP+) can also be utilised as a substrate, particularly in acidic conditions (Aarhus et al., 1995). This may be of particular significance due to the fact that the CD38 receptor is internalised by a process of receptor mediated endocytosis (Funaro et al., 1990).

The function of these two products in B cells however is not clear. cADPR is a known second messenger that regulates intracellular Ca\textsuperscript{2+} mobilisation in an inositol 1, 4, 5 - triphosphate (IP\textsubscript{3}) independent manner, instead functioning via the co-factor calmodulin. ADPR can either covalently attach to proteins in a non-enzymatic way or in the presence of an ADP-ribosyl transferase enzymatically ADP ribosylate proteins (reviewed in Lund et al., 1995). It is conceivable therefore that one or both of these moieties could alter molecules within close proximity and affect signalling and/or adhesion. In this regard, CD38 has been shown to have the ability to bind hyaluronate, a large glycosaminoglycan of the extracellular matrix and mediate adhesion between endothelial cells and lymphocytes (Dianzani et al., 1994).

A potential 120 kD ligand for CD38 has recently been identified (Deaglio et al., 1996). It was found to be predominantly expressed on endothelial cells but interestingly co-localised with CD38 molecules on the surface of several lymphocytic cell lines, suggesting a functional significance. Direct ligation with CD38 in T cell lines did result in an increase in the cytoplasmic Ca\textsuperscript{2+} concentration (Deaglio et al., 1996).

In conclusion, CD38 has a number of diverse functions and although seemingly unrelated all play an immunoregulatory role (Figure 1.4). The fact that signalling via the CD38 receptor results in different outcomes depending on the differentiation status of the cell would tend to suggest that accessory molecules may play a vital part in diversification of the signal. The high expression of CD38 in myeloma cells may therefore not be coincidental but rather instrumental in development of the disease. Indeed, recently a homologue of CD38, BST-1 has been found expressed on stromal cell lines (Kaisho et al., 1994).

**CD45**

The leukocyte common antigen CD45 consists of a family of transmembrane proteins which are widely expressed on cells of haematopoietic origin (reviewed in Thomas & Lefrancois, 1988). Different isoforms arise as a result of alternative splicing of three variable exons and encode the extracellular domain, enriched in O-linked glycosylation
Figure 1.4: Schematic diagram of the human CD38 cell surface antigen and its multifunctional roles including; adhesion to endothelial cells, signal transduction and ADP-ribosyl cyclase and cADPR hydrolase enzymatic activities. Adapted from Malavasi et al., 1994.
Four different isoforms predominate, having molecular weights of 180, 190, 205 and 220 kD. These are further classified into either low molecular weight isoforms (180 kD) termed CD45RO or high molecular weight isoforms (205/220 kD) termed CD45RA as determined by antigenic recognition and exon inclusion (Jensen et al., 1989).

During differentiation, both B cells and T cells undergo a transition in CD45 isoform expression. Initially in precursor cells the higher CD45RA isoform is expressed but upon activation there is a gradual change to the lower CD45RO isoform expression (Jensen et al., 1989). Pre-plasma cells and early plasma cells therefore express only CD45RO with all expression being lost in mature plasma cells. The variable expression of CD45 on myeloma cell lines reflects this differentiation process.

Moreover, in MM patients there is a decrease in the number of circulating B-cells expressing CD45RA, possibly reflecting the general phenomenon of reduced lymphopoiesis and the normal propensity of mature plasma cells to migrate out of the bloodstream (Pilarski & Jensen, 1992). This circulating population is also accompanied by greatly elevated levels of CD45RO B-cells which corroborates the idea that myeloma cells arise from a stage late in the B-cell differentiation pathway (Jensen et al., 1991). The pleomorphic nature of CD45 expression in myeloma cells has also been postulated to indicate that these cells undergo differentiation comparable to normal, eventually resulting in mature myeloma cells as found within the bone marrow (Pilarski & Jensen, 1992).

CD45 has been shown to be required for antigen receptor function on both T and B lymphocytes and in T cell development (Pingel & Thomas, 1989; Justement et al., 1991). Critical to this is the protein tyrosine phosphatase (PTPase) activity found in the cytoplasmic domain, instigating its involvement in signal transduction, specifically activating src family protein kinases (Justement et al., 1991). This activity may be modulated by the CD45 associated protein (CD45-AP) which is linked to CD45 via its transmembrane domain and is expressed solely in leukocytes (Kitamura et al., 1995).

The ligand of CD45 is, as yet, undefined although recently a population of adherent cells found within the thymus was identified to express a potential candidate peptide (Seavitt & Thomas, 1996). Monoclonal antibodies to CD45 have been shown to induce apoptosis in both T and B lymphocytes and act via the PTPase activity, as inhibitors of both protein tyrosine kinases and protein tyrosine phosphatases blocked cell death (Klaus et al., 1996). Furthermore, in CD45 defective cells, engagement of antigen receptors with a soluble antigen induces a weak signal that is sufficient to promote the maturation and/or survival of B cells (Cyster et al., 1996). Taken together these results would tend to indicate that CD45 is involved in cell death and loss of this molecule in myeloma cells could prolong
survival of the clonogenic cells. Presumably, in normal circumstances, mechanisms other than CD45 would be responsible for apoptotic cell death.

1.2.7 The causes of multiple myeloma

The causes of multiple myeloma are largely unknown although exposure to ionising radiation is probably the most cogent risk factor. Studies on atomic bomb survivors and those in radiation related occupations all indicate an increased frequency in occurrence of MM (reviewed in Riedel & Pottern, 1992). A number of studies have also linked occupational exposure to an elevated risk of MM. These include those involved in agriculture, metal workers and hairdressers exposed to hair dyes. In all cases the causal agents are undefined but possible candidates include pesticides, benzene and other organic chemicals, some of which are known to be powerful carcinogens (reviewed in Riedel & Pottern, 1992).

In addition to environmental factors there appears to be some genetic component involved in multiple myeloma. There are several lines of evidence to substantiate this hypothesis. Firstly, the male and ethnic predispositions to the disease would tend to corroborate this idea. There are also several reports of familial multiple myeloma (Shoenfeld et al., 1982) amongst siblings and concurrence of the disease in identical twins (Snowden & Greaves, 1995). In addition murine plasmacytomas, a similar disease which can be induced by pristane, shows very different responses depending on the genetic background of mice used (Potter et al., 1994). Indeed, susceptibility and resistance genes have been localised to the distal end of mouse chromosome 4 (Mock et al., 1993). Much effort has therefore been employed into finding human genetic markers for the disease.

1.2.8 Cytogenetic analysis of multiple myeloma

A number of cytogenetic and karyotypic studies have been carried out on MM (Ferti et al., 1984; Gould et al., 1988) but no consistent chromosomal abnormalities have been found. Between 20-50% of MM patients however are aneuploid (Garcia-Sanz et al., 1995; Tabemero et al., 1995) but such aberrations are thought to be as a consequence, rather than a cause of the disease (Haak et al., 1995).

Chromosomal translocations are commonly found in a variety of B-cell neoplasia and often involve the immunoglobulin loci, presumably reflecting the chromosomal rearrangements occurring within these regions (Korsmeyer, 1992). Two cases are particularly well documented. In the first, Burkitt's lymphoma (BL) rearrangements between chromosome 8 and either chromosomes 2, 14 or 22 are responsible for all known cases of the disease (Cory, 1986). As a result, the c-myc oncogene is juxtaposed to an
immunoglobulin locus and c-myc expression becomes deregulated. Similarly the Philadelphia chromosome (Ph) t(9;22) is the cytogenetic hallmark of chronic myeloid leukaemia (CML). This balanced translocation creates a chimeric BCR-ABL gene, expressing a stable gene product which functions to inhibit apoptosis, prolonging survival of the progenitor cells (Bedi et al., 1994).

However, chromosomal translocations in MM are relatively rare (<10%) and have been shown to involve a variety of chromosomes although most frequently on #1, #3, #6, #8 and #14 (Table 1.4) (Ferti et al., 1984). It is interesting to note that the translocation t(8;14) (q24;q32) has been statistically associated with IgA myeloma, with the breakpoint on chromosome 14 again being coincidental with the heavy chain immunoglobulin locus (Ferti et al., 1984; Gould et al., 1988). The significance of this in development of the disease however is not clear, nor is it unique to MM. More recently the 19p 13 band was also delineated as a recurrent breakpoint involved in translocations in MM (Taniwaki et al., 1994). This region maps to the E2A gene, which encodes the enhancer binding proteins E12/E47 (Mellentin et al., 1989).

Dysregulation of cyclin D1 in two multiple myeloma cell lines has also been proposed to occur by translocation into an IgH γ- switch region (Chesi et al., 1996). Furthermore, in a recent study 12/12 multiple myeloma cell lines were found to contain an illegitimate switch recombination site (Bergsagel et al., 1995). It has therefore been hypothesised that an error in the lymphocyte specific mechanism of gene rearrangement, can result in tumourogenesis, and the resultant neoplasia is independent of the chromosomal locus involved but determined by whether VDJ joining segments or switch recombination sites are employed (Chesi et al., 1996). The main premise of this theory relies on the involvement of either an oncogene or tumour suppressor gene which as a consequence of the translocation will become deregulated. However, chromosomal translocations are only one possible mechanism by which oncogenic expression can be altered.

1.2.9 Oncogene involvement in multiple myeloma

Many oncogenes have been examined both in patients and multiple myeloma cell lines, however the frequency with which mutations occur varies considerably between studies. Mutations have been observed in both K-ras and N-ras, although point mutations at codon 61 in N-ras are most commonly found (Neri et al., 1989; Corradini et al., 1993). The frequency in patient samples varied between 10-50%, with the highest percentages found being associated with advanced stages of the disease (Neri et al., 1989; Portier et al., 1992; Corradini et al., 1993; Van Ness et al., 1995). Approximately one quarter of MM cell lines were found to contain ras mutations (Portier et al., 1992), however, 80% of myeloma cell lines contained p53 mutations (Mazars et al., 1992). Mutations in the
Table 1.4: Chromosomal translocations most commonly observed in multiple myeloma.

<table>
<thead>
<tr>
<th>Chromosomal translocation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>t (8 : 22) (q24 : q11)</td>
<td>Sole et al., 1994</td>
</tr>
<tr>
<td>t (8 : 14) (q24 : q32)</td>
<td>Gould et al., 1988</td>
</tr>
<tr>
<td>t (11 : 14) (q13 : q32)</td>
<td>Haak et al., 1995</td>
</tr>
<tr>
<td>t (14 : 18) (q32)</td>
<td>Haak et al., 1995</td>
</tr>
</tbody>
</table>
tumour suppressor gene *p*53 though occur at low frequencies (20%) in patient samples and again correlate with clinically aggressive forms of the disease (Portier et al., 1992; Neri et al., 1993). Mutations in both *ras* and *p*53 genes are therefore unlikely to represent the initiating mutation in MM but later stages in the process of multistep carcinogenesis. Indeed tumour progression in multiple myeloma has been shown to require deregulation of both oncogenes and tumour suppressor genes (Brown et al., 1994).

52% of patients have deletions of the tumour suppressor gene *Rb-1* (Tricot et al., 1994) and *bcl-X* has been shown to be expressed in both myeloma cells and cell lines (Tu et al., 1995). The *bcl-2* gene is translocated in 80% of human follicular lymphomas resulting in high expression of the gene product. Similar levels of *bcl-2* expression have been observed in multiple myeloma cell lines, but it is unclear whether this as a result of deregulation or merely reflects *bcl-2* levels in normal plasma cells (Pettersson et al., 1992).

### 1.2.10 c-myc involvement in multiple myeloma

The other oncogene to have been extensively studied in multiple myeloma is *c-myc*. It is commonly involved in many B-cell neoplasia eg. Burkitt's lymphoma, murine plasmacytoma, lymphomas (Potter, 1990). Deregulation of the *c-myc* gene in MM, presumably by inappropriate transcription, is principally thought to occur by three mechanisms; 1) translocation, 2) point mutations within regulatory sequences 3) amplification of the gene (Greil et al., 1991). Chromosomal translocations however are rare both in patient material and in cell lines (Gazdar et al., 1986; Selvanayagam et al., 1988) and similarly point mutations have been observed only in a minority of cases (<5%) (Selvanayagam et al., 1988; Pegoraro et al., 1989). Amplifications of the *c-myc* gene are also not commonplace, unlike plasma cell leukaemia where 66% of cases display an abnormal karyotype for this locus (Sumegi et al., 1985).

consistent with this, increased expression of *c-myc* protein levels have been observed in many MM cell lines (Jernberg-Wiklund et al., 1992), but these were unaccompanied by any gross structural abnormalities of the *c-myc* gene. Likewise, two recently derived MM cell lines displayed high Myc levels without rearrangements of the *c-myc* gene (Hollis et al., 1988; Pegoraro et al., 1989). In this case, abnormally sized transcripts, leading to an increased stability accounted for this observation, although these mechanisms occur infrequently. However, there is growing evidence to suggest that the dysregulation of *c-myc* may be prevalent among human myelomas, consistent with its murine counterpart.

19% of MM patients have also been reported to have a 5-12 fold overexpression of *c-myc* mRNA, but the rationale for this observation is unclear (Selvanayagam et al., 1988).
Similar studies examining both c-myc protein and mRNA levels have detected elevated mRNA levels in 80-90% of MM patients with considerable heterogeneity in expression (Greil et al., 1991). However, it was noted that the protein and mRNA levels did not correlate, particularly for IgA secretory clones i.e. although these clones contained the highest percentage of cells expressing high levels of protein, they contained the lowest percentage of cells expressing increased message. This observation may corroborate the fact that co-transformation of mice using an ABL-MYC retrovirus induces predominately IgA tumours and elevated levels of c-myc expression in a patient study of 180 cases, correlate with IgA myelomagenesis (Largaespada et al., 1992; Skopelitou et al., 1993). Furthermore, c-myc expression coincides with malignancy stage and the tumour burden (Greil et al., 1991; Skopelitou et al., 1993).

More recently detailed studies have reported that 70% of MM patients and 60% of MM cell lines display a larger transcript size than normal (3.1 kb), thought to arise from the P0 promoter region (Hoover et al., 1994; Travis et al., 1994). In addition, due to the presence of both IL-2 and IL-6 response elements within the promoter region, transcription from P0 is specifically induced by those cytokines (Epstein et al., 1995). It is possible therefore that factors within the bone marrow microenvironment could specifically affect c-myc transcription, giving cells either a growth advantage, or in combination with accessory proteins e.g. high levels of bcl-2, prevent programmed cell death. The large heterogeneity of c-myc expression between cells substantiates the fact that c-myc may have an influential role in myelomagenesis.

1.3 The c-myc Oncogene

1.3.1 The myc family of oncogenes

c-myc belongs to a small family of related proto-oncogenes which have been implicated in a variety of neoplasia. The most extensively studied member, c-myc, was originally identified as the cellular homologue of v-myc, the transforming determinant of avian myelocytomatosis virus MC29 (Vennstrom et al., 1982). The other two members, N-myc and L-myc, which displayed a high degree of homology to c-myc, were identified as amplified sequences in human neuroblastoma and small cell lung carcinomas respectively (Schwab et al., 1983; Nau et al., 1985).

All three genes have the same characteristic 3 exon / 2 intron structure and encode for homologous polypeptides containing highly conserved domains separated by blocks of amino acid displaying less similarity (Marcu et al., 1992). Indeed it is these functional domains that impart their congruent characteristic properties of cellular transformation
and involvement in cell cycle progression (Luscher & Eisenman, 1990). The three members however, are differentially expressed during mammalian development with \( N-myc \) and \( L-myc \) displaying strict spatial and temporal specificity, whereas \( c-myc \) expression is almost ubiquitous, being found in proliferating cells of both embryonic and adult tissues (Marcu et al., 1992).

Two other genes \( B-myc \) and \( S-myc \) have also been identified. These genes have small regions of homology to \( c-myc \) but in contrast seem to inhibit malignant transformation (Sugiyama et al., 1989; Resar et al., 1993). In addition, other cellular sequences bearing only limited homology to \( c-myc \) have been found but these are thought to represent pseudogenes (DePinho et al., 1991).

1.3.2 The roles of \( c-myc \)

From an early stage \( c-myc \) was implicated in cell cycle control, as its deregulation was often intimately associated with neoplastic transformation. Examination of \( c-myc \) protein and mRNA levels indeed revealed that it was expressed throughout the cell cycle, suggesting that it may be required for continuous cell cycle progression (Thompson et al., 1985). In quiescent cells there is a rapid induction of \( c-myc \) expression upon mitogenic stimulation as cells traverse the G0-G1 transition (Kelly et al., 1983). As this occurs prior to \textit{de novo} protein synthesis \( c-myc \) is classified as an immediate early response gene. Overexpression of exogenous \( c-myc \), under the appropriate conditions, though, is sufficient to drive quiescent cells into the cell cycle without the concomitant expression of other immediate early genes (Eilers et al., 1991). Similarly, antisense oligonucleotides inhibiting \( c-myc \) expression causes a block to cell cycle progression with cells arresting in G1, both in serum stimulated and exponentially growing cells (Heikkila et al., 1987; Loke et al., 1988). \( c-myc \) expression is therefore essential for cell proliferation. Accordingly, differentiation of cells is accompanied by a down regulation of \( c-myc \) expression (reviewed in Marcu et al., 1992; Meichle et al., 1992).

In direct contrast it was also observed that in cells expressing high levels of \( c-myc \) protein, under suboptimal growth conditions, there was an increased incidence of apoptosis (Evan et al., 1992). This apparent paradox was reconciled into the "dual signal" hypothesis whereby \( c-myc \) plays a pivotal role in both cell proliferation and cell death, the final outcome being governed by other gene products and external factors (Figure 1.5) (Evan et al., 1992; Evan & Littlewood, 1993; Harrington et al., 1994). Coupling these contradictory pathways thereby acts to tightly regulate cell numbers but also could function to suppress tumour progression by inducing apoptosis of a mitogenically stimulated clone (Evan & Littlewood, 1993). This concurs with the fact that deregulated \( myc \) expression, by itself, is insufficient to evoke malignant transformation, instead acting
Figure 1.5: The “dual signal” hypothesis which incorporates the pivotal role of the c-myc protein in governing cell proliferation or cell death. Adapted from Evan & Littlewood, 1993. Abbreviations: IFN, interferon; TGFβ, transforming growth factor β; PDGF, platlet derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor.
to immortalise primary cells. Only in combination with secondary events ie. deregulation of other oncogenes or tumour suppressor genes is a tumourigenic phenotype observed (Land et al., 1983).

c-myc has also been proposed to have a direct role in DNA replication (Ariga et al., 1989). However the results obtained from a number of studies indicate a tentative connection rather than definitive proof of c-myc involvement in synthesis of DNA (reviewed in Marcu et al., 1992).

### 1.3.3 The c-myc gene structure

The human c-myc gene, as previously mentioned, lies on chromosome 8 and spans a region of more than 8 kb. It is composed of three exons; the first of which is predominantly non-coding; the second and third encoding the 2 nuclear phosphoproteins Myc 1 and Myc 2 (Watt et al., 1983a) (Figure 1.6A). Human exon 1 however also has the coding potential for an unrelated polypeptide of 188 amino acids, myc HEXI (Gazin et al., 1986). This 32 kD protein dimerises readily to give a 58 kD species, which was detected immunologically both in HeLa cell extracts and murine cell extracts, despite the fact that mouse c-myc exon 1 does not have this coding potential (Gazin et al., 1986; Dedieu et al., 1988). The function of this protein however remains unknown.

### 1.3.4 c-myc protein: structure/function

Myc 1 and Myc 2, of 453 and 439 amino acids respectively are short lived nuclear phosphoproteins (Beimling et al., 1985; Watt et al., 1985). They have apparent molecular weights of 64 kD and 67 kD (Hann et al., 1988), but such anomalous migration on SDS-PAGE gels is common for nuclear transforming genes (Miyamoto et al., 1985). Multiple phosphorylation sites are present, including both glycogen synthase kinase (GSK III) and casein kinase II (CKII) sites (Marcu et al., 1992). Threonine 58 and serine 62 are also known to be phosphorylated, as mutations at these sites, commonly observed in Burkitt's lymphoma, interfere with p107-mediated phosphorylation resulting in an enhanced transforming property (Hoang et al., 1995).

Characterisation of the Myc polypeptides revealed a number of structural motifs which imparts upon them their characteristic functional attributes (Figure 1.6B). These include a nuclear localisation signal, defined to the nonopeptide sequence PAAKRVKLD, a non-specific and specific DNA binding domain and a transactivation domain (reviewed in Kato & Dang, 1992). Within the acidic transactivation domain (TAD) are glutamine/proline rich regions which are responsible for its anomalous molecular weight (Kato & Dang, 1992).
Figure 1.6: (A) Schematic diagram of the human c-myc gene. (B) Schematic diagram of the c-Myc proteins and their functional domains. Adapted from Marcu et al., 1992.

NL: Nuclear localisation  LZ: Leucine zipper
BR: Basic region  GSK III: phosphorylation by glycogen synthase kinase
H-L-H: Helix-loop-helix  CK II: phosphorylation by casein kinase II
Sequence specific DNA binding is mediated by the C-terminal basic region (BR) which lies immediately N-terminal to the helix-loop-helix (HLH) and leucine zipper (LZ) dimerisation motifs. These motifs are commonly found in other transcription factors and collectively constitute the specific DNA binding domains (BR/HLH/LZ) as dimerisation is obligatory for binding function (Voronova & Baltimore, 1990). In conjunction with the presence of the transactivation domain this strongly implicated a role for Myc polypeptides as transcription factors controlling gene expression.

1.3.5 Max: A dimerisation partner for Myc

Paradoxically Myc homodimers were found not to bind DNA at physiological concentrations (Dang et al., 1989). However, an explanation for this was provided by the discovery of Max, and its murine counterpart Myn, which are capable of dimerising with Myc due to a complimentary BR/HLH/LZ motif (Blackwood & Eisenman, 1991; Prendergast et al., 1991).

Max is widely expressed in both proliferating and non-proliferating cells and unlike c-myc protein levels, its abundance does not vary with the cell cycle (Blackwood et al., 1992). During erythroid differentiation, but not myelomonocytic differentiation, however, there is a down-regulation of both max mRNA and protein (Delgado et al., 1995). Similarly, concomitant blocking of c-myc and max expression leads to terminal differentiation of myeloblastic leukaemia cells (Nguyen et al., 1995). Two forms of Max protein (Max; 21 kD/Max 9; 22 kD) differing by 9 amino acids at the N-terminus have been identified. Both are very stable with a half life of greater than 24 hours (Blackwood et al., 1992). This is unaltered, as is that of Myc, on dimerisation (Blackwood et al., 1992).

Myc/Max heterodimers preferentially form over Max/Max homodimers due to the kinetics of the interactions (Leff et al., 1995). Both are capable of binding the 6 bp CACGTG consensus site, or E-box, although binding to CATGTG and other non-canonical sequences (CA—TG) has been observed (Blackwell et al., 1993). However, as Max lacks a transactivation domain binding of Max homodimers represses transcription (Blackwood & Eisenman, 1991; Amati et al., 1992; Kato et al., 1992; Kretzner et al., 1992; Amati et al., 1993a). Myc/Max heterodimers also have a higher affinity of binding this site and phosphorylation of Max, by casein kinase II (CKII), has been shown to inhibit binding of Max/Max homodimers but not Myc/Max heterodimers (Blackwood et al., 1992). Other reports have implicated that the sequence context of the CACGTG motif can selectively discriminate against binding of Max homodimers (Fisher et al., 1993). Max dimerisation to Myc is therefore critical to functioning of c-myc as a transcription factor and correspondingly Max is a prerequisite in both cell cycle progression and apoptosis, paralleling its oncogenic potential (Amati et al., 1993a; Amati et al., 1993b).
1.3.6 Other proteins binding Max

The role of Max in controlling Myc expression has been further complicated by the discovery of other proteins, bearing the BR/HLH/LZ hallmark, capable of binding Max (Figure 1.7). These include Mad, Mad3, Mad4 and Mxi1 (Ayer et al., 1993; Zervos et al., 1993; Hurlin et al., 1995). These proteins can all bind the same CACGTG motif but in conjunction with mSin3, the mammalian homologue of a yeast transcription repressor, form ternary complexes that repress transcription (Ayer et al., 1993; Hurlin et al., 1995; Schreiber-Agus et al., 1995). Overexpression of these proteins can inhibit c-Myc dependent cell transformation, implying they can antagonise Myc functions and are therefore potential tumour suppressors (Lahoz et al., 1994; Hurlin et al., 1995) (Figure 1.8).

1.3.7 Cellular targets of Myc

Although discovery of the E box motif facilitated the search for Myc regulated genes the ability of other factors, including upstream stimulatory factor (USF), transcription factors E3 and E4 (TFE3, TFE4) and associated protein 4 (AP4) to also bind this sequence, hampered their detection (Beckmann et al., 1990; Carr & Sharp, 1990; Gregor et al., 1990; Hu et al., 1990). To address this question a fusion of Myc and a portion of the oestrogen receptor (Myc ER) was generated, thereby allowing constitutive c-myc expression only in the presence of β-oestradiol (Eilers et al., 1991). Comparative cDNA libraries obtained from control and overexpressing c-myc cells led to the identification of mRNA from the α-prothymosin gene (Eilers et al., 1991). This gene contains two E box motifs and its expression was found to be activated by Myc (Gaubatz et al., 1994). However, recently it has been reported that the Myc ER chimera contains within it a functional oestrogen-activated transcriptional activation domain which can falsify results (Solomon et al., 1995). In addition, α-prothymosin genes and reporter constructs driven by the α-prothymosin promoter do not display Myc dependent transcriptional activation properties, suggesting that in vivo this is not a true target of the c-myc gene (Mol et al., 1995).

E box motifs have also been found, and shown to bind Myc complexes, in a number of genes. These include human hsp70 (Taira et al., 1992), the dihydrofolate reductase gene (DHFR) (Mai & Jalava, 1994), p53 (Reisman et al., 1993), ECA39 (Benvenisty et al., 1992), ornithine decarboxylase (ODC) (Bello-Fernandez et al., 1993), cdc 25A (Galaktionov et al., 1996), the euukaryotic translation initiation factors eIF-4E and eIF-2α (Rosenwald et al., 1993b; Jones et al., 1996) and most recently, Myc-regulated DEAD box protein (MrDb) (Grandori et al., 1996).
Figure 1.7: Schematic diagram showing the regions of homology of Myc related proteins. Adapted from Ryan & Birnie, 1996.
Figure 1.8: Myc/Max/Mad transcription factor complexes and their effects on transcription upon binding to E-box (CACGTG) motifs. Arrows in bold indicate preferential complex formation.
At present ODC is the strongest contender as a direct physiological target of Myc. Its expression is indispensable for cellular proliferation as it catalyses the rate limiting step of polyamine biosynthesis (Pegg, 1986). In addition, ODC levels are regulated in a cell cycle dependent manner and are increased upon mitogenic stimulation and cellular transformation (Pegg, 1986). Two downstream CACGTG motifs transregulate the ODC promoter by binding Myc/Max dimers (Tobias et al., 1995). One upstream motif has also been implicated, particularly in transactivation succeeding long term quiescence (Pena et al., 1993; Pena et al., 1995; Tobias et al., 1995). ODC has also been identified as one mediator of c-myc induced apoptosis (Packham & Cleveland, 1994).

Another potentially interesting target for c-myc is cdc25A. This gene encodes a CDK-activating phosphatase expressed early in the G1 phase of the cell cycle (Jinno et al., 1994). Three possible E boxes are located within the first 2 introns, which are capable of binding Myc/Max heterodimers and can transactivate the promoter (Galaktionov et al., 1996). Furthermore, Myc induced apoptosis requires cdc25A (Galaktionov et al., 1996). The cellular targets of cdc25A are not well defined, although Cdk2 and Cdk4 are possible candidates (Terada et al., 1995). This may tie in with observations that c-myc causes the activation of cyclin E/Cdk2 which subsequently leads to the activation of cyclin A (Kim et al., 1994; Rudolph et al., 1996). This also correlates with the finding that overexpression of Myc leads to elevated levels of cyclin A and cyclin E mRNA (Jansen-Durr et al., 1993). Thus, c-myc may govern cell cycle progression by acting upstream of cyclin dependent kinases.

1.3.8 Repressor function of Myc

Myc can interact with the transcription factor TFII-I which activates core promoters through an initiator element (Inr) (Roy et al., 1993). This core sequence (CT CA(\(+1\))CTCTCT) is capable of binding other initiation factors including USF, ying-yang-1 (YY1) and E2F (Mai & Martensson, 1995). Binding of TFII-I and Myc to this element however represses transcription by preventing the formation of a complex between the TATA-binding protein (TBP), TFII-I and the promoter (Roy et al., 1993). Repression therefore requires the BR/HLH/LZ domain but also the Myc box II domain (Li et al., 1994). Several genes have been shown to contain Inr elements that are specifically repressed by c-myc. These include the \(\lambda\)5 promoter, a gene which is expressed only in pre B-cells, the terminal deoxynucleotidyl transferase (TdT) promoter (Mai & Martensson, 1995), and the promoters of the CAAT/Enhancer binding protein \(\alpha\) (C/EBP\(\alpha\)) and albumin genes (Li et al., 1994). Additionally, overexpression of Myc leads to a down regulation of cyclin D1 mRNA, due to the presence of an initiator element in the promoter region (Philipp et al., 1994; Mai & Martensson, 1995; Solomon et al., 1995).
1.3.9 Other proteins binding Myc

Within cells oncogenic proteins are often found complexed to tumour suppressor proteins (Ryan & Birnie, 1996). Myc is no exception to this, as the amino-transactivation domain in vivo was found to associate with the Rb-related protein p107, which is thought to mediate phosphorylation at a number of critical sites, thereby suppressing the transactivation potential of c-myc (Gu et al., 1994). In this regard, the NH₂ terminal domain has also been shown to interact with TBP, presumably allowing activation of transcription (Maheswaran et al., 1994).

Other factors binding Myc have also been identified. These include Nmi, a factor which also co-precipitates with N-myc (Bao & Zervos, 1996) and YY1 (Shrivastava et al., 1993). C/EBP transcription factors may also bind Myc due to the ability of the NH₂ terminal domain to downregulate C/EBP-dependent transactivation, by a mechanism distinct from inhibition via initiator elements (Mink et al., 1996).

1.3.10 Regulation of c-myc expression

It is clear therefore that c-myc is involved in many intricate control pathways, responding to numerous factors, culminating in its pivotal role in determining cell fate. As it plays such an important role it would be naive to believe c-myc expression could be controlled in a simple manner. Accordingly, regulation of c-myc expression is controlled in a variety of ways. Notably these include regulation at both the initiation and elongation stages of transcription, autoregulation, a number of different post-transcriptional control mechanisms and alternative translation initiation sites.

1.3.11 Transcriptional initiation

The c-myc gene contains 4 promoters; P1, P2, P3 and P0 which give rise to transcripts of 2.2 kb, 2.4 kb, 2.3 kb and 2.5-3.1 kb respectively (Battey et al., 1983; Bernard et al., 1983; Yang et al., 1985; Bentley & Groudine, 1986b). 75-90% of cellular transcripts originate from P2, with P1 producing only 10-25% (Battey et al., 1983; Bernard et al., 1983; Stewart et al., 1984; Taub et al., 1984; Yang et al., 1985; Bentley & Groudine, 1986b). The minor promoters, which do not contain TATA boxes, P3 and P0 contribute approximately 5% each towards total c-myc mRNA (Bentley & Groudine, 1986b; Ray et al., 1989) (Figure 1.6A).

Many DNase I hypersensitive sites, indicative of control regions, are found up to 4 kb upstream and 2 kb downstream of P1 and correspondingly many control sequence elements have been mapped. A myriad of binding factors to some of these regions have
been identified that co-dictate differential promoter usage and serve to regulate transcription initiation both positively and negatively (reviewed in Marcu et al., 1992).

There have also been reports of antisense transcripts bearing homology to the second intron of c-myc being detected in some cell lines upon depletion of polyamines (Celano et al., 1992). In this connection an antisense promoter has been mapped to 175 bp 5' of exon 3, within intron 2 of the murine c-myc gene (Spicer & Sonenshein, 1992). The function of such transcripts however is unknown although they presumably act as another regulatory mechanism to control gene expression (Celano et al., 1992).

1.3.12 c-myc autoregulation

It has been observed that during chromosomal translocations of c-myc, such as found in Burkitt's lymphoma and murine plasmacytoma, transcription predominates from the translocated allele, whereas the untranslocated allele is transcriptionally silent (ar-Rushdi et al., 1983; Nishikura et al., 1983; Siebenlist et al., 1984; Tachibana et al., 1993). This differential expression suggested that a negative autoregulatory loop may be functioning to down regulate the non-translocated allele (Nishikura et al., 1983). In addition, overexpression of exogenous c-myc has been shown to decrease endogenous c-myc levels, although this self-regulation is sometimes absent in transformed cell types (Grignani et al., 1990).

Autoregulation is exerted at the level of initiation of transcription and requires sequences in the C-terminal BR/HLH/LZ region for function (Penn et al., 1990). In this connection, a Myc binding site was identified in the human c-myc promoter region at a sequence also implicated as an origin of replication (Ariga et al., 1989). However, more recent studies suggest that there is a candidate transcriptional repressor element (Inr) around the P2 site (Kretzner et al., 1995). Other reports though support an indirect mechanism of c-myc autosupression perhaps by directly binding regulatory proteins known to influence its transcription (Buckle & Mechali, 1995).

1.3.13 Transcriptional elongation

In addition to controlling initiation of transcription a block to transcription elongation has also been identified for c-myc and a number of other proto-oncogenes (Spencer & Groudine, 1990). During differentiation of HL60 and MEL cells it was found that exon 1 and exon 2 transcript levels diverged indicative of discontinuous transcription (Bentley & Groudine, 1986a; Eick & Bornkamm, 1986; Mechti et al., 1986). Subsequently, the block to transcriptional elongation was mapped to a 95 bp region, 35 bp 5' of the exon 1/intron 1 boundary (Bentley & Groudine, 1988). This site contains a region of dyad symmetry
followed by a T rich tract, reminiscent of rho-independent terminators found in prokaryotes (Bentley & Groudine, 1988). However, attenuation using the above element appeared to be promoter dependent and more recently other sequences, found proximal to the P2 promoter, have been found to be necessary for premature termination (Krumm et al., 1992).

1.3.14 Post-transcriptional control mechanisms

Although transcriptional control plays a part in regulating \( c-myc \) expression the key component responsible for variations in steady-state levels of c-Myc is undoubtedly attributable to post-transcriptional mechanisms. The reason for this probably lies in the fact that both \( c-myc \) mRNA and protein have very short half lives of around 15 and 30 minutes respectively (Rabbitts et al., 1985).

mRNA stability

Loss of exon 1 by chromosomal translocations was found to increase \( c-myc \) mRNA stability, and correspondingly this region was originally believed to be required for transcript instability (Rabbitts et al., 1985). However, \( c-myc \) 5' UTR is not intrinsically unstable and AU rich sequences located in the 3' UTR were instead found necessary for the high rate of transcript turnover observed (Jones & Cole, 1987). In addition to this another destabilising element lying within the carboxy terminal coding region, the coding region determinant (CRD), has been identified (Wisdom & Lee, 1991). However, translation is required for maximal functioning of the element suggesting that a ribosome bound endonuclease may be involved. A 70 kD protein (coding region determinant-binding-protein: CRD-BP) has been purified from polysomes which appears to bind the CRD with high specificity and protects \( c-myc \) mRNA from endonucleolytic attack (Prokipcak et al., 1994).

mRNA targeting

Specific instability sequences (AUUUA) located in the 3' UTR instability determinant have also been identified as being involved in targeting \( c-myc \) mRNA to cytoskeletal bound polysomes in the perinuclear cytoplasm (Veyrune et al., 1996). Other nuclear protein transcripts also co-localise to this region (Hovland et al., 1995) which has led to the proposal that targeting of these messages facilitates the subsequent transport of these proteins into the nucleus (Hesketh et al., 1994).
mRNA induction

Post-transcriptional mechanisms are largely responsible for the induction of c-myc mRNA after inhibition of protein synthesis and during hepatic regeneration (Morello et al., 1990). Such in vivo transgenic studies have implicated both c-myc coding exons rather than the 5' and 3' UTRs as contributing to this control mechanism (Morello et al., 1993; Lavenu et al., 1995).

Translational control

A translational control mechanism residing in the first exon was originally postulated by Saito et al., arising from differential hypothetical secondary structures as a result of chromosomal translocations (Saito et al., 1983). Early in vivo studies examining translational efficiencies of c-myc mRNA in Burkitt's lymphoma cell lines though suggested that both truncated and full length transcripts were translated with equal efficiencies (Nilsen & Maroney, 1984; Butnick et al., 1985; Piechaczyk et al., 1985). In contrast, c-myc mRNAs lacking exon I were translated in vitro more efficiently when compared to full length transcripts (Darveau et al., 1985). Indeed, a 240 nt restrictive element within murine exon 1, was isolated and shown to inhibit translation of heterologous mRNAs, in some in vivo and in vitro systems (Parkin et al., 1988).

Similarly, differential translation of chloramphenical acetyl transferase (CAT) transcripts containing either Xenopus c-myc 5' UTR or mouse exon 1, has been observed in Xenopus oocytes and embryos (Lazarus et al., 1988; Parkin et al., 1988; Lazarus, 1992). Reporter constructs demonstrated that the oocyte specific translation inhibition was abrogated if sequences only at the 3' end of exon 1 were present, suggesting that promoter utilisation would influence the translational efficiency of transcripts (Lazarus, 1992). A more recent report indicated that this oocyte block to translation arose due to the inclusion of non-transcribed sequences (Fraser et al., 1996). When removed, constructs containing Xenopus c-myc 5'UTR showed no inhibition of translation in either oocytes or embryos. This was in direct contrast to human c-myc exon 1 where translation of reporters was prevented in both systems, possibly due to increased secondary structure (Fraser et al., 1996).

c-myc mRNA binding proteins

Two apparently unrelated proteins have also been discovered which can bind to c-myc mRNA and therefore could potentially be implicated in post-transcriptional control mechanisms. The first, a 55 kD protein of unknown function was discovered to bind a purine rich region located between P1 and P2 of the human c-myc transcript (Parkin &
Sonenberg, 1989). The second, thymidylate synthase (TS), a protein involved in maintenance of precursor deoxynucleotide levels, bound a 165 nt segment in the C-terminal coding region (Chu et al., 1995). Binding was inhibitory to in vitro translation of c-myc mRNA which coupled to the fact that TS is capable of binding its own transcript, thereby autoregulating its own expression, implicated that the association may be of functional significance (Chu et al., 1995).

1.3.15 Differential initiation of translation

c-Myc 1 (p67) and c-Myc 2 (p64) are both nuclear phosphoproteins with similar half lives of approximately 30 minutes (Hann & Eisenman, 1984). They differ only by 14 amino acids at the N-terminus which arises due to the differential usage of two translation start sites (Hann et al., 1988). The minor and heavier isoform, Myc 1, initiates from a non-AUG codon within exon 1 and has been found to be specifically activated at high cell densities due to methionine deprivation (Hann et al., 1992).

p67 expression is often lost in Burkitt’s lymphoma cell lines, due to loss or mutation of the exon 1 region (Hann & Eisenman, 1984; Hann et al., 1988). Similarly, during differentiation of murine erythroleukaemia (MEL) cells a change in the relative ratio of c-Myc 1: c-Myc 2 is observed (Spotts & Hann, 1990). Taken together these results suggest that the proteins may be functionally distinct. However, both p64 and p67, are independently able to transform rat fibroblasts in collaboration with bcr-abl, and are equally efficient in stimulating a Myc/Max responsive promoter (Blackwood et al., 1994). This suggests that the oncogenic potential of Myc simply lies in its deregulation rather than in an altered protein structure and the existence of a CUG codon could serve to allow basal level translation under growth limiting conditions (Blackwood et al., 1994). More recently it has been shown that the two proteins can differentially regulate transcription through a non-canonical DNA binding site (Hann et al., 1994). Specifically, c-Myc 1 can bind the CCAAT/Enhancer-binding protein (C/EBP) element and activate transcription whereas c-Myc 2 has no stimulatory effect on enhancer function. In contrast, both can transactivate E box myc sites (EMS) (Hann et al., 1994). Moreover, overexpression of c-Myc 1, in the absence of c-Myc 2, has been shown to inhibit cell growth (Hann et al., 1994).

It thus appears that the predominant isoform c-Myc 2 is responsible for growth and proliferation, whereas c-Myc 1 is required for inhibition of growth. It would be possible to envisage a situation whereby increasing the ratio of Myc 1: Myc 2 could activate/inactivate a different subset of genes involved in growth inhibition or differentiation. Correspondingly, if c-Myc 1 expression is lost, such as in Burkitt’s lymphoma, cells may lose the ability to be growth inhibited, which may contribute
towards neoplastic growth. Translational control may therefore be of primary importance in c-myc regulation.

1.4 Eukaryotic Translation

1.4.1 Global control of protein synthesis

Protein synthesis is the ultimate determinant governing the phenotypic expression of genetic information within a cell and therefore can be regarded as an integral component controlling gene expression. Indeed, translational processes consume a substantial quantity of metabolic energy generated within a cell and correspondingly any changes in growth rates are mirrored by alterations in protein synthesis rates by a variety of mechanisms. In some cases the exact manner in which this is achieved remains elusive although in other systems the mechanisms have been comprehensively delineated (reviewed in Redpath & Proud, 1994). Modulation of protein synthesis rates thus permits a rapid and universal regulation of many messages and additionally provides a controlling mechanism under circumstances where nuclear functions are absent (reviewed in Hentze, 1995).

1.4.2 General mechanism of protein synthesis

Protein synthesis can be divided into three stages 1) initiation 2) elongation and 3) termination. The first stage, initiation, involves the recruitment of the small (40S) and large (60S) ribosomal subunits to a mRNA, to form a translation competent 80S ribosome present at an initiation codon (AUG or CUG, GUG, ACG). The elongation phase then ensues which involves the sequential addition of individual amino acids to the C-terminus of the nascent polypeptide. This continues until the ribosome encounters a termination codon (UAA, UAG, UGA) which results in release of the completed polypeptide chain and the ribosomal subunits (reviewed in Hershey, 1991). Each stage of the process is catalysed by a large number of soluble protein factors which interact non-covalently with the translational apparatus (Table 1.5) These are divided into eukaryotic initiation factors (eIFs), eukaryotic elongation factors (eEFs) and eukaryotic release factors (eRFs) in accordance with the stage at which they act. The largest group is represented by the factors involved in initiation as this is the rate limiting step of the translational process (Hershey, 1991; Rhoads et al., 1994).

Most of the translation factors are abundantly expressed with the exception of the two initiation factors; eIF-4E and eIF-2B. However, many of the protein factors involved are phosphoproteins which supports the hypothesis that protein synthesis is primarily
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<th><strong>Factor</strong></th>
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<tr>
<td>eEF-1</td>
<td>51, 23, 49</td>
<td>binds aa-tRNA; GTPase; GTP:GDP exchange on eEF-1( \alpha ); GTP:GDP exchange with eEF-1( \beta )</td>
<td>( \alpha, \beta )</td>
<td>Y, Y</td>
</tr>
<tr>
<td>eEF-2</td>
<td>100</td>
<td>stimulates translocation; GTPase</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td><strong>Termination Factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eRF</td>
<td>54.0</td>
<td>recognizes stop codons, stimulates peptidyl-tRNA cleavage and release</td>
<td></td>
<td>N</td>
</tr>
</tbody>
</table>

**Table 1.5**: Soluble factors involved in eukaryotic translation. Adapted from Hershey, 1991.
controlled by the phosphorylation state and cellular concentrations of a few critical factors (reviewed in Rhoads, 1993; Hershey, 1994). Translation rates are also determined by elements intrinsic to a particular mRNA molecule (Kozak, 1989b), which can account for the approximately 100 fold range of translational efficiencies observed for cellular messages (Koch et al., 1980).

1.4.3 Initiation of protein synthesis

The process of initiation can be subdivided into four phases. 1) Ribosome dissociation into the 40S and 60S subunits. 2) Formation of the 43S pre-initiation complex, involving binding of Met-tRNA\textsubscript{i} to the 40S subunit. 3) Binding of this 43S complex to the mRNA and, in the case of most cellular mRNAs, scanning to the initiation codon. 4) Recruitment of the 60S subunit forming an elongation competent ribosome (Figure 1.9) (reviewed in Hershey, 1991). Furthermore, initiation can be qualitatively distinguished as either mobilisation, whereby the 43S pre-initiation complex interacts with a translationally inactive mRNA associated with proteins in the form of a messenger ribonucleoprotein particle (mRNP), or re-initiation which involves the association of the pre-initiation complex with mRNAs being actively translated on polysomes (Hershey, 1991).

1) Ribosome dissociation

Within a cell ribosomes are in equilibrium with their individual 40S and 60S subunits. However, reassociation is prevented and this equilibrium is shifted by initiation factors eIF-1A and eIF-3 which bind to the 40S subunit (reviewed in Hershey, 1991). In addition, eIF-3 contains an RNA recognition motif implying that it can bind both mRNA or ribosomal components. This is also consistent with its essential involvement in 43S pre-initiation complex binding to mRNA (Bommer et al., 1991).

2) Formation of the 43S pre-initiation complex

Initiator methionyl-tRNA\textsubscript{i} (Met-tRNA\textsubscript{i}) firstly associates with eIF-2.GTP to form a ternary complex, which is then competent to associate with the 40S ribosomal subunit, to form the 43S pre-initiation complex (Konieczny & Safer, 1983). However, because the binding of GDP to eIF-2 is 400 times tighter than the binding of GTP under physiological conditions, a guanine nucleotide-exchange factor, termed eIF-2B, is required for replacement of bound GDP by GTP (Rowlands et al., 1988). Furthermore, as Met-tRNA\textsubscript{i} dissociates from the ternary complex on hydrolysis of GTP, the aforementioned reaction (which is also highly dependent on phosphorylation state) dictates the recycling of the eIF-2 factor. Formation of the ternary Met-tRNA\textsubscript{i}.eIF-2.GTP complex, obligatory for 43S pre-initiation
Figure 1.9 : Schematic diagram of eukaryotic translational initiation.
complex formation, therefore constitutes a critical control point in the initiation of translation (reviewed in Hershey, 1991).

**eIF-2 and eIF-2B**

The eIF-2 polypeptide consists of three subunits, α, β and γ, all of which can be phosphorylated by a number of different kinases (reviewed in Redpath & Proud, 1994). However, phosphorylation of the α-subunit at Ser 51 is of principal importance as this leads to repression of protein synthesis by virtue of an increased binding affinity for eIF-2B but inhibition of eIF-2B exchange activity (Rowlands et al., 1988). Two mammalian eIF-2α kinases have been identified; haem controlled repressor (HCR) and dsRNA activated protein kinase (PKR). These kinases are activated under stress conditions, however, expression of inactive PKR in NIH3T3 cells was found to result in their malignant transformation implying a general requirement for this kinase under normal growth conditions (Koromilas et al., 1992b). In addition, as eIF-2B levels are normally limiting within a cell, phosphorylation of only 30% of the total eIF-2α present is sufficient to severely inhibit protein synthesis. Examination of eIF-2/eIF-2B ratios has indicated that this parameter varies considerably between cell types implying that different cell types may vary in their responsiveness to eIF-2α phosphorylation (Oldfield et al., 1994).

3) mRNA binding to the 40S ribosomal subunit

The activated 40S ribosomal subunit (43S pre-initiation complex) binding to mRNA principally involves three initiation factors; eIF-4F, eIF-4A and eIF-4B. For the majority of eukaryotic mRNAs these factors facilitate binding of the 40S subunit to the 5' -terminus and subsequent migration of the complex to the initiation codon (the scanning model) (Figure 1.10A). However a second model, originally identified for picornaviral transcripts but now also observed in a number of cellular mRNAs, involves the direct binding of the 40S subunit to an internal site within the mRNA either at, or slightly upstream of, the initiation codon (the internal entry model) (Figure 1.10B) (reviewed in Pain, 1996). In the scanning model subunits of the eIF-4F initiation factor are involved in recognition of the 5' end of the mRNA and recruitment of the 40S ribosomal subunit, however the detailed mechanics and sequential assembly of complex formation remain uncertain (Pain, 1996). Initiation factors eIF-4A and eIF-4B are then involved in movement of the complex in a 5'→3' direction and are most likely to simultaneously permit the unwinding of secondary structures within the 5' untranslated region (UTR) which impede the scanning ribosome (Kozak, 1989b). In this model, individual mRNAs which can differ significantly with respect to their 5' UTRs display considerable variations in translation rates as a direct consequence of dissimilar initiation rates (see Section 1.4.6). Other parameters are of importance in the internal mechanism of initiation (see Section
Figure 1.10: Diagram outlining the alternative models of translational initiation. (A) The conventional scanning model of initiation of translation. (B) Internal initiation of translation.
1.4.7) but all of the aforementioned initiation factors, or at least parts thereof, are required for initiation, indicating both the universal nature and the key regulatory role which mRNA: ribosome binding plays (reviewed in Jackson & Kaminski, 1995).

**eIF-4F**

The eIF-4F initiation factor complex is composed of three subunits. These are 1) eIF-4E: a 24 kD subunit, 2) eIF-4G: a 220 kD subunit and 3) eIF-4A: a 45 kD subunit (reviewed in Hershey, 1991). Both the eIF-4E and eIF-4A complexes can be isolated separately but eIF-4G has been only detected in eIF-4 complexes implying that it cannot bind mRNA in isolation (Rhoads et al., 1994). Furthermore, the property of each individual polypeptide appears to be enhanced when present in the eIF-4F initiation complex suggesting a degree of synergism between the components. In addition, the eIF-4E and eIF-4G subunits can be phosphorylated under certain physiological conditions which influences protein synthesis rates and emphasises the importance of these factors in initiation of translation (reviewed in Redpath & Proud, 1994).

**eIF-4E**

eIF-4E is the smallest subunit of eIF-4F and binds the 7-methyl guanosine cap (5'-m7G) characteristic of all eukaryotic cellular mRNAs (Shatkin, 1976). It has been implicated as a key component regulating translation primarily for two reasons. 1) eIF-4E is present in limiting quantities (0.01-0.2 molecules per ribosome) when compared to other initiation factors in cells (Duncan et al., 1987). 2) eIF-4E exists in both phosphorylated and non-phosphorylated forms (Hershey, 1989). Consistent with this essential role, overexpression of eIF-4E has been shown to cause deregulation of HeLa cell growth (De Benedetti & Rhoads, 1990) and induce malignant transformation of NIH3T3 and Rat 2 fibroblasts thereby classifying eIF-4E as an oncogene (De Benedetti & Rhoads, 1990). The mechanism by which this occurs however is unclear although eIF-4E overexpression has been demonstrated to facilitate the translation of mRNAs engineered to contain extensive secondary structure within their 5' UTR regions (Koromilas et al., 1992a). Such structural features are common amongst growth related proteins eg. oncogenes and growth factors and as a result are therefore thought to be normally inefficiently translated (Kozak, 1991). Further evidence to support this hypothesis has been provided from experiments which have demonstrated that the expression of cyclin DI (Rosenwald et al., 1993a; Rosenwald et al., 1995), ornithine decarboxylase (ODC) (Shantz & Pegg, 1994), fibroblast growth factor (FGF-2) (Kevil et al., 1995) and c-myc (De Benedetti et al., 1994) are increased in cell lines overexpressing eIF-4E.
Regulation of eIF-4E activity

The activity of the eIF-4E subunit, and hence eIF-4F complex formation, is controlled by two mechanisms; phosphorylation and by eIF-4E binding proteins. Phosphorylation of eIF-4E, recently redefined principally to Ser-209 (Joshi et al., 1995), correlates with an increase in the rate of protein synthesis and cell growth. Correspondingly treatment of cells with hormones, growth factors and mitogens stimulates phosphorylation of eIF-4E whereas during viral infection, mitosis and heat-shock, eIF-4E is hypophosphorylated (reviewed in Rhoads, 1993; Sonenberg, 1994; Pain, 1996). However, neither the physiological kinase nor the mechanism by which phosphorylation of eIF-4E enhances translation are clear (Morley et al., 1991).

Two eIF-4E binding proteins have been identified; eIF4E-BP1 and eIF4E-BP2 (Pause et al., 1994a), the former being the mammalian homologue of PHAS-I, a rat eIF-4E binding protein phosphorylated in response to insulin and other growth factors (Hu et al., 1994). Association of both these proteins with eIF-4E represses translation and is highly dependent on their phosphorylation states. Hence, in rat fat cells stimulated with insulin, PHAS-I becomes phosphorylated causing it to dissociate from eIF-4E and thereby relieves the translational repression (Pause et al., 1994a; Diggle et al., 1996). A conserved 12 amino acid sequence found within these binding proteins was found to be necessary for the interaction with eIF-4E. Since this motif was also present in eIF-4G it has been hypothesised that a functional competition between the eIF-4G and 4E binding proteins could regulate initiation complex formation (Haghighat et al., 1995; Mader et al., 1995).

eIF-4G

Until recently the function of eIF-4G was unknown but growing evidence now indicates that it acts as a bridge, bringing together the eIF-4E and eIF-4A components of the eIF-4F initiation complex (Pause et al., 1994a). This association appears to be enhanced, specifically activating translation initiation, by phosphorylation of eIF-4G by ribosomal protein S6 kinase (Morley et al., 1991; Morley & Pain, 1995). The mechanism by which this occurs however remains unclear.

During viral infection there is often a switch of translation from host encoded cellular mRNAs to viral transcripts. In some picornaviruses this is mediated by the production of a protease which cleaves the eIF-4G factor into an N-terminal and C-terminal domain serving to bifurcate the eIF-4E and eIF-4A binding functions (Lamphear et al., 1995). This C-terminal domain has been shown to be sufficient to support cap-independent translation, in the absence of eIF-4E, providing a rationale for the preferential translation of viral mRNAs due to their internal mechanism of ribosome entry (Ohlmann et al., 1996).
eIF-4A

eIF-4A was originally identified as the archetypal member of the DEAD box family of proteins which all display RNA helicase activity (Linder et al., 1989). It is capable of binding ATP and exhibits RNA-dependent ATPase activity in addition to ATP-dependent RNA helicase activity in conjunction with eIF-4B (Grifo et al., 1984; Rozen et al., 1990). It was therefore postulated that eIF-4A functioned to unwind secondary structure within the 5'UTR (Rozen et al., 1990). Primarily eIF-4A functions as a subunit of eIF-4F, but free eIF-4A is additionally required to recycle into the eIF-4F complex during translation (Pause et al., 1994b).

4) Formation of 80S initiation complex

Joining of the 60S large ribosomal subunit occurs when the pre-initiation 40S complex is correctly positioned at the initiation codon. The process requires a further initiation factor, eIF-5 which is thought to specifically interact with eIF-2 (Chaudhuri et al., 1994). GTP hydrolysis ensues resulting in release of eIF-2.GDP, eIF-1A and eIF-3 (Raychaudhuri et al., 1985). Little else is known of what governs and regulates 80S assembly but as eIF-5 can be phosphorylated and association of the 60S subunit is slow relative to 48S complex formation, this step may confer an additional control point in initiation of translation (Anthony & Merrick, 1992; Chevesich et al., 1993). At this stage the 80S ribosome is competent to begin the elongation phase of protein synthesis.

1.4.4 Elongation of protein synthesis

The elongation phase of protein synthesis occurs via a cyclic pathway permitting the genetic information within each triplet codon to be translated into amino acids which are then covalently joined to one another resulting in a polypeptide chain (Figure 1.11). Four steps are critical in this pathway. 1) The binding of amino acyl tRNA to the A site of the ribosome which is catalysed by eEF-1α. 2) GTP hydrolysis and the release of eEF-1α.GDP. 3) Peptide bond formation catalysed by the 60S ribosomal based peptidyl transferase activity. 4) Translocation of the peptidyl-tRNA from the A to the P site of the ribosome, movement of the ribosome by three bases and translocation of the deacylated tRNA from the P to the E site. These reactions are mediated by eEF-2 which possesses ribosome-dependent GTPase activity (Nygard & Nilsson, 1990). On average 6-8 amino acids are incorporated per second with a high fidelity rate (Hershey, 1991). Both eEF-1 and eEF-2 can be phosphorylated, however, the role of this with regard to controlling protein synthesis remains relatively ill-defined (Redpath & Proud, 1994). Similarly, although eight amino acyl-tRNA synthetases have been identified as phosphoproteins the functional significance of these modifications remains unclear (Hershey, 1989).
Figure 1.11: Elongation and termination of protein synthesis.
1.4.5 Termination of protein synthesis

The termination of protein synthesis in eukaryotes is relatively poorly understood and is thought to require only the presence of a stop codon (UAA, UGA or UAG) and a single release factor (eRF) (reviewed in Hershey, 1991). This factor is hypothesised to recognise a stop codon, bind to the ribosome and subsequently cleave the bond between the last peptidyl-tRNA and the polypeptide chain (reviewed in Tuite & Stansfield, 1994). Three release factors have been identified in prokaryotes to catalyse different steps in this process; RF-1, RF-2 and RF-3 and consequently eukaryotic homologues would be predicted. In this connection, mutants of a S. cerevisiae gene, Sup45p, have been shown to display a decreased rate of polysomal ribosome release (Tuite & Stansfield, 1994). However, further knowledge into eukaryotic termination will undoubtedly be forthcoming from unequivocal identification of the factors involved.

1.4.6 Determinants of protein synthesis rates

In addition to regulation of protein synthesis rates by altering the translational components, primarily by phosphorylation/dephosphorylation reactions, five inherent features of a mRNA govern its "translatability" (Kozak, 1989b). These include: 1) the m^7G cap 2) context of AUG/initiation codon 3) position of AUG codon 4) leader length and 5) secondary structure.

1) m^7G cap

All eukaryotic cellular mRNAs, except those of organellar origin, are post-transcriptionally modified by the addition of a methylated guanosine residue at the 5' end. This serves a number of purposes including: a) nucleocytoplasmic transport b) splicing c) 3' end processing d) protection against 5' exonucleolytic degradation and e) initiation of translation (Hershey, 1991). As previously mentioned, eIF-4E interacts with the cap structure and therefore inaccessibility of the cap due to higher-order secondary and tertiary structures correlates with a reduction in the translational efficiency (Lawson et al., 1988). Similarly, uncapped messages, both in vivo and in vitro, generally tend to be poorly translated.

2) Context of AUG/initiation codon

Examination of 699 vertebrate mRNA sequences revealed the presence of a consensus sequence around the AUG initiation codon: GCCA/GCCAUGG (Kozak, 1987a). This sequence was also shown experimentally, by the systematic mutation of nucleotides surrounding the AUG initiator codon, to be the optimal context for translational initiation.
(Kozak, 1986; Kozak, 1987b). However, the purine at position -3 (where +1 is the A of the initiation codon) and a G at position +4 were the strongest contributors and were found to be the most highly conserved (Kozak, 1987a; Kozak, 1987b). Indeed, mRNAs of unfavourable context were found to encode growth factors and cytokines suggesting that poor translation is a prerequisite in preventing over production of deleterious products (Kozak, 1994a).

Consistent with this, the initiation of protein synthesis from non-AUG initiation codons (CUG, GUG or ACG), as often observed in growth related mRNAs, is more inefficient than initiation of translation from a standard AUG codon (Kozak, 1990). In this case positions +5 and +6 are also major determinants of initiation efficiency (Boeck & Kolakofsky, 1994). It has also been reported that initiation codons within a poor context can be enhanced by increasing the secondary structure approximately 14 bases upstream (Kozak, 1990). This is postulated to retard the scanning 40S ribosome thereby extending the time available for recognition of the initiation codon (Kozak, 1990).

3) Position of the AUG codon

Eukaryotic ribosomes almost exclusively initiate translation at the 5' proximal AUG codon, a finding consistent with the scanning model of translational initiation and concurrent with the observation that fewer than 10% of vertebrate mRNAs examined contained AUG codons proceeding the major open reading frame (ORF) (Kozak, 1987a). Moreover, this minor population were found to encode proto-oncogenes, growth factors and cell-surface receptors implying that these upstream AUG codons are of functional significance (Kozak, 1987a). Indeed, insertion of AUG codons upstream of the major ORF are known to be inhibitory on translation presumably by "stalling" scanning ribosomes (Kozak, 1986). Three cellular mechanisms appear to exist to circumvent this potential problem posed by upstream initiation codons; a) alteration of mRNA structure, b) leaky scanning and c) reinitiation if the upstream AUG encodes a short upstream open reading frame (uORF) (Kozak, 1991).

a) Alteration of mRNA structure

In a number of genes upstream initiation codons are removed by structural alterations to the mRNA such as alternative splicing or differential promoter usage thereby generating two different mRNAs and functionally distinct proteins which initiate at the first initiator codon (Kozak, 1991).

b) Leaky scanning

This model postulates that if the first initiation codon occurs in a sub-optimal context a fraction of scanning ribosomes will not recognise this initiation codon and bypass it,
initiating instead at a downstream site (Kozak, 1991). It is therefore possible to produce two different proteins from one mRNA (reviewed in Kozak, 1991). This mechanism may be widespread amongst proto-oncogenes where less efficient CUG or other non-AUG initiation codons are prevalent upstream of the major ORF (reviewed in Hann, 1994). In synthetic constructs however, context dependent leaky scanning fails if a second AUG codon is placed some distance from the first (70 nt) suggesting that elongating 80S ribosomes may mask potential downstream start sites even when they are in a better context (Kozak, 1995).

c) Reinitiation

If a downstream AUG is closely followed by a termination codon thereby forming a short uORF reinitiation at an upstream AUG is possible (reviewed in Geballe & Morris, 1994). In general this phenomenon is favoured by short uORFs and long intercistronic spaces (Kozak, 1991). These facts have been accommodated in the following hypothesis whereby some essential initiation factor is retained for a short period during translational elongation but is lost if the minicistron is extended. Also, by increasing the intercistronic distance the time available for a scanning ribosome to reacquire a Met-tRNA\textsubscript{i} eIF2.GTP ternary complex, essential for reinitiation, is lengthened (Kozak, 1992). In some cases the surrounding sequences or the peptide sequence generated by the uORF can also influence the frequency of reinitiation (Geballe & Morris, 1994). The presence of an uORF normally serves to suppress translation from the main downstream cistron thereby controlling expression by a translational mechanism. The paradigm for regulation in this manner is the \textit{S. cerevisiae} gene GCN4 (reviewed in Hinnebush, 1993).

4) Leader length

The 5'UTR of most vertebrate mRNAs is between 20-100 nucleotides in length, with the notable exception of proto-oncogenes which are invariably longer than 100 nucleotides (Kozak, 1987a). A minimal length of 10-20 nts is required for efficient initiation and correct recognition of the initiator AUG codon but the efficiency of translation can be increased if the leader length is increased (up to 60 nts), providing it is devoid of secondary structure (Kozak, 1994a). This increased translational efficiency is also independent of the primary nucleotide sequence inserted, suggesting that the increased leader length merely functions to permit the binding of additional 40S ribosomal subunits (Kozak, 1994b). Within a cellular context however, the advantage of an extended leader sequence is often abrogated by an accompanying increase in the secondary structure and leader length \textit{per se} is therefore not an adequate indicator of translational efficiency.
5) **Secondary structure**

Higher order structures within the 5' UTR are probably the most universal feature that determines the translational efficiency of a particular mRNA. In general, any secondary structure serves to repress translation by thwarting the progression of the scanning ribosome. However, both the stability and position of such secondary structures influences the overall effect on protein synthesis (Kozak, 1989a). This was discovered from *in vitro* translation experiments whereby the introduction of a moderately stable stem loop (-30 kcal/mol) into a reporter construct decreased translation if inserted at the 5' end near the cap structure but had no effect if placed further downstream (52 nts) (Kozak, 1989a). Small amounts of secondary structure (approximately -6.0 kcal/mol) lying close to the 5' terminus have also been reported to restrict translation (Kozak, 1994b). Thus the initial binding of the 40S ribosomal subunit and initiation factors requires a region devoid of secondary structure. Once initiated however, the scanning ribosome can melt secondary structures within its path (Kozak, 1994a). This statement is only partially correct as the introduction of more stable structures (-61 kcal/mol) 71 nucleotides from the cap were found to completely inhibit translation (Kozak, 1989a). It was postulated that this block to protein synthesis arose due to the "stalling" of scanning ribosomes (Kozak, 1989a). Similar results have been reported for yeast systems, although the translational apparatus appears to be more sensitive to secondary structure and, unlike higher eukaryotes, secondary structures located near the 5'-cap do not seem to impede 40S ribosomal binding (Sagliocco *et al.*, 1993).

It follows from these results therefore, that it may not be coincidental that genes which display a high GC content within the 5' UTR (>70% GC), and accordingly are predicted to be highly structured, encode oncogenes, growth factors and components of signal transduction pathways (Kozak, 1991). Indeed, many of these 5'UTRs when placed upstream of reporter constructs substantially reduce the translational efficiency both *in vivo* and *in vitro* (Kozak, 1991). This is also consistent with the fact that loss of these inhibitory regions is often associated with a transformed phenotype, suggesting that a low translational efficiency, arising from the excessive secondary structure within the 5'UTR, is essential for regulated gene expression (Kozak, 1991; Rosenwald, 1996). Such mRNAs presumably are therefore only efficiently translated under specific circumstances, eg. growth stimulation, but the mechanisms by which this is achieved are not fully established.

One possibility is that changes in the general components of the translational apparatus, eg. by phosphorylation, could selectively enhance translation of these transcripts (Hershey, 1989). Indeed, as previously discussed, overexpression of eIF-4E specifically promotes the translation of some growth-related transcripts. However, specific cellular
factors may also be required as exemplified by the variable translation of *c-myc* mRNA between different *in vivo* and *in vitro* systems (see Section 1.3.14). Such factors, either in the form of a protein or an RNA component, may be required to specifically destabilise structures within the 5'UTR or adapt the translational machinery to permit translation. Alternatively these factors could act by sequestering translational repressor proteins that normally bind to the structured 5'UTR regions or act to promote internal initiation of translation. In the case of growth related proteins, which posses characteristics detrimental to translational efficiency in almost every one of the five categories of translation determinants, the scanning model would impose severe restrictions on translation. However, an internal mechanism of ribosome entry could bypass these restraints.

### 1.4.7 Internal initiation of translation

During picornaviral infection of mammalian cells it was noted that concurrent with a down-regulation of translation of host-encoded transcripts there was efficient translation of virion RNAs. However, this finding was inconsistent with several features of picornaviral RNAs which would be predicted by the scanning model to confer very low translational efficiency. Firstly, the 5'UTRs of picornavirus RNAs are between 610-1400 nucleotides long and highly structured. Secondly, although the 5' end of the virion RNA possesses a covalently linked virally encoded protein this is rapidly removed following uncoating of the virus generating an RNA that is effectively uncapped. Thirdly, many AUG codons (up to a maximum of 15) are found within the 5'UTR and as the majority of these are not conserved between species are unlikely to function as genuine translation initiation sites but would severely impede progression of the scanning ribosome (Jackson *et al.*, 1994). A mechanism of internal initiation of translation was therefore postulated and demonstrated using a dicistronic construct whereby translation of the downstream cistron was achieved by insertion of the picornavirus 5'UTR between the two cistrons, even under conditions that prevented cap-dependent translation of the upstream cistron (Pelletier & Sonenberg, 1988). Direct internal entry of ribosomes was also confirmed by showing that artificial circular mRNAs containing the picornaviral 5'UTR could be translated and were associated with the ribosomes (Chen & Sarnow, 1995).

The use of such dicistronic constructs has become standard to assess the ability of a mRNA to direct internal initiation, but careful interpretation of the results is sometimes required (Jackson *et al.*, 1995). This system was also utilised to determine the minimal region required for internal initiation by assaying the effects of progressive deletions of different picornavirus 5' UTRs (Borman & Jackson, 1992; Jackson *et al.*, 1994). From comparative studies this minimal element defined as the internal ribosome entry segment (IRES), is approximately 450 nucleotides in length for picornaviruses, however there is no
general consensus IRES sequence (Jackson et al., 1994). Instead based on their sequence and secondary structure IRESes can be divided into two large groups and one minor group (Table 1.6) (Jackson & Kaminski, 1995). Within each group there is a strong conservation of deduced secondary structure and a degree of primary sequence homology but there is very little conservation of either between the groups, except for a pyrimidine rich tract lying about 25 nucleotides 5' to the 3' end of the IRES (Jackson et al., 1994).

In addition to classification of IRESes on the aforementioned basis there is a distinct functional difference between the groups with regard to the initiation start site. In all cases the ribosome binding site occurs at an AUG codon located at the 3' end of the IRES located about 25 nts downstream of the conserved polypyrimidine tract. However, although cardioviruses and hepatoviruses use this as a functional initiation site aphthoviruses tend to use the next downstream initiation codon. Moreover, entero-/rhinoviruses appear to utilise the IRES as a "ribosome landing pad" and then scan to the authentic AUG codon some 40-160 nts downstream (reviewed in Jackson et al., 1994). A further difference between the groups is that the requirements for optimal internal initiation of translation vary greatly in vitro and their translational efficiencies vary depending on the particular in vitro translation system used (Borman et al., 1995; Jackson & Kaminski, 1995).

Although the IRES groups appear diverse it is thought that three key features are universally required for IRES functioning which have been incorporated into a general model of internal initiation (Figure 1.12) (Jackson et al., 1994). 1) The secondary/tertiary structure which the IRES forms is critical for internal initiation by probably allowing essential short primary sequence motifs, often found in unpaired segments, to be brought together and create binding sites for ribosomes, or other factors, that may facilitate initiation. 2) The ribosome binding site occurs at a conserved AUG triplet at the 3' end of the IRES. 3) The selection of the ribosome entry site is partly determined by its distance from motifs probably found upstream within the IRES (Jackson et al., 1994). The most likely motif to fulfil this criterion is the polypyrimidine tract. This is consistent with the observation that both this region and the AUG ribosome entry site are capable of base pairing to the 18S rRNA in a manner akin to the Shine Dalgarno sequence found within prokaryotic mRNA sequences (Scheper et al., 1994).

**Factors required for internal initiation**

Internal initiation appears to require all of the canonical initiation factors including components of eIF-4F complex (reviewed in Jackson & Kaminski, 1995). This apparent paradox was reconciled by the finding that picornaviral proteases cleave eIF-4G thereby separating the eIF-4E and eIF-4A/eIF-3 binding domains, the former of which is
<table>
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<th>Abbrev.</th>
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<tr>
<td>Coxsackie B group</td>
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<tr>
<td>Aphthoviruses</td>
<td>Foot and mouth disease virus</td>
<td>FMDV</td>
</tr>
<tr>
<td>III Hepatoviruses</td>
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<td>HAV</td>
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</table>

Table 1.6: Classification of picornaviruses. (Taken from Jackson et al., 1990).
Figure 1.12: Schematic general model for internal initiation of picornavirus RNA translation. The IRES is proposed to be a complex structure with many secondary and tertiary interactions which serve to present a number of short primary sequence motifs (thickened lines) thought to permit binding of specific RNA-binding proteins (X and Y) allowing direct recruitment of the ribosome (shaded oval). The AUG at the 3' end of the IRES, lying approximately 25 nucleotides downstream of a conserved polypurine tract, acts as the actual ribosome entry site. However, only cardioviruses initiate translation at this site. Aphthoviruses, enteroviruses and rhinoviruses instead scan to the next AUG downstream. Taken from Jackson & Kaminski, 1995.
unnecessary for internal initiation (Mader et al., 1995). UV cross-linking studies have recently demonstrated the association of eIF-4B with the IRES from foot and mouth disease virus (Meyer et al., 1995). This interaction however was indirect implying that additional factors were required for binding (Meyer et al., 1995).

Further evidence to support the requirement of trans-acting factors has come from in vitro translation studies using different cell-free systems. It was found that no picornaviral IRES could function in the wheat germ system and rabbit reticulocyte lysates could only support IRES function from the cardio-/aphthovirus group unless supplemented with HeLa cell extracts, which then allowed the translation of entero-/rhinoviruses, or with liver cytoplasmic cell extracts to permit Hepatitis A virus IRES functioning (Borman et al., 1993; Glass & Summers, 1993). In all cases it was presumed that addition of cell extracts provided the appropriate factors in sufficient quantities to enable internal initiation of translation.

Much effort has therefore been expended in trying to identify such factors principally using a UV cross-linking approach. However, although this has identified a large number of candidate proteins it is uncertain if they are actually required for IRES activity. Similarly, many proteins appear to be IRES specific. In general, a larger number of proteins appear to bind the IRESes of entero- and rhinoviruses (Borman et al., 1993). Two proteins that have been identified to bind picornaviral IRESes and which have been shown to be of functional significance are the poly-pyrimidine tract binding protein (PTB) and the autoimmune antigen La.

**PTB**

Poly-pyrimidine tract binding protein (PTB) or p57 has been observed to bind both entero-/rhinovirus and cardio-/aphthovirus IRESes although cross-linking to the latter group is stronger (Borman et al., 1993; Witherell et al., 1993; Hellen et al., 1994). The strongest affinity binding site maps to a stem loop at the 5' end of the EMCV IRES and mutations which disrupted PTB binding correlated with decreased cap-independent translation implying a functional role for PTB (Jang & Wimmer, 1990; Kaminski et al., 1995). Similarly, antibodies to PTB were found to disrupt cap-independent translation although addition of recombinant PTB did restore EMCV IRES function (Hellen et al., 1993).

In another study EMCV IRES activity was found to be restored on addition of recombinant PTB in cell extracts partially depleted of PTB, with PTB specifically promoting 48S pre-initiation complex formation (Borovjagin et al., 1994). Other results also support this requirement for PTB in EMCV IRES function but PTB does not appear to be a universal factor for internal initiation as rabbit reticulocyte lysates depleted of PTB
were still functional in internal initiation of TMEV and Hepatitis C virus (Kaminski et al., 1995). PTB has therefore been postulated to act as a chaperone providing the correct IRES conformation facilitating the binding of other factors (Kaminski et al., 1995).

La

The autoimmune antigen La (p52) has been reported to stimulate poliovirus translation in rabbit reticulocyte lysates and suppress the production of aberrant proteins in a manner reminiscent of HeLa cell extra supplementation. (Meerovitch et al., 1993; Svitkin et al., 1994). However, the concentrations of recombinant La required for the augmented IRES activity are very high and the physiological significance of these results has been questioned (Jackson & Kaminski, 1995). Immunodepletion of La from HeLa cell extracts was sufficient to inhibit poliovirus translation suggesting that La may play a functional role in internal initiation in this system (Belsham et al., 1995). As La has been shown to be capable of binding and unwinding dsRNA \textit{in vitro} (Xiao et al., 1994) this may not be implausible. Furthermore, a small yeast RNA which inhibits internal initiation of poliovirus RNA has been found to bind La protein (Das et al., 1996). The suppressive effect of this inhibitory RNA could also be removed by addition of exogenous La, again implicating that La protein is involved in internal initiation (Das et al., 1996).

Other viral and cellular IRESes

In addition to picornaviruses, IRESes have been found in pestiviruses and hepatitis C virus (Jackson & Kaminski, 1995). In both cases the IRES extends into the coding sequence and lacks the conserved oligopyrimidine tract (Jackson & Kaminski, 1995). A small number of cellular mRNAs have also been identified, by virtue of the dicistronic assay, to contain IRESes. These include mammalian immunoglobulin heavy-chain binding protein (BiP) (Macejak & Sarnow, 1991), \textit{Drosophila melanogaster} antennapedia mRNA (Oh et al., 1992), human fibroblast growth factor 2 (FGF-2) mRNA (Vagner et al., 1995), eIF-4G (Gan & Rhoads, 1996) and two yeast transcription factors TFIID and HAP4 (Iizuka et al., 1994). These diverse members are not known to contain conserved nucleotide sequences but all possess long 5'UTRs which are predicted to be highly structured due to their high GC content. Indeed, the fact that some of these 5'UTRs were inhibitory to translation in certain systems implicated trans-acting factors were required for their translation (Prats et al., 1992).

The trans-acting factors required for internal initiation have not yet been elucidated for any of the aforementioned cellular IRESes although preliminary data for the BiP IRES have identified two proteins, p95 and p65, as possible candidates (Iizuka et al., 1995) Regulation of specific trans-acting factors could allow the internal initiation of translation.
to be controlled, permitting expression of the protein, or a particular isoform of the protein, only in certain circumstances. Such a mechanism could modulate the expression of a number of genes and provide a more selective control than altering general initiation components but a less specific control mechanism than that conferred by translational repressors.

1.4.8 Translational regulation by specific protein/mRNA interactions

Translational regulation of specific mRNAs by specific proteins could be envisaged to occur in a manner akin to activation and repression of transcription by transcription factors, with translational repressor and activator proteins binding in the 5'UTR to influence the initiation of translation (Kozak, 1992). To date however, with the possible exception of internal initiation factors, no translational activator proteins are known and many translational repressors bind within the 3'UTR (reviewed in Standart & Jackson, 1994). Many of these proteins are poorly characterised but in general proteins binding to RNA recognise particular structural motifs rather than primary sequence determinants alone (Frankel et al., 1991; McCarthy & Kollmus, 1995). The best characterised and the paradigm for translational control by a binding protein is undoubtedly ferritin mRNA whose translation is regulated in response to iron concentrations by virtue of an iron-responsive element (IRE) which is capable of binding an iron regulatory protein (IRP) (reviewed in Klausner et al., 1993).

1.4.9 Regulation by 3'UTR motifs

Regulation of protein synthesis by sequences in the 3'UTR would at first appear paradoxical but there is growing evidence that the 5' and 3' ends of mRNAs can interact (reviewed in Decker & Parker, 1995). Translational control in this manner is particularly well documented for the spatial and temporal control of mRNAs during early development where nuclear functions are absent. In some cases specific sequence elements have been identified and while the mechanisms of translational repression remain to be elucidated two possible scenarios have been postulated; 1) These elements promote poly(A) tail shortening or 2) they act independently of the poly(A) tail (Standart & Jackson, 1994; Decker & Parker, 1995).

The main premise for these statements comes from the fact that polyadenylation, while not an absolute requirement, enhances translation (Jacobson & Favreau, 1983). This became evident from microinjection studies but was in correlative agreement with observations that translational activation of mRNAs during maturation or early embryogenesis is associated with poly(A) tail lengthening whereas translational inactivation is accompanied by poly(A) tail shortening (reviewed in Bachvarova, 1992).
Translational activation via the poly(A) tail length may, in part, be mediated by poly(A) binding protein (PABP). Evidence for a direct involvement in translation came from studies on yeast mutants of PABP which were found to be suppressed by mutations in a structural component of the 60S ribosomal subunit (Sachs & Davis, 1989). This is also consistent with the finding that the less efficient recruitment of poly(A)$^-$ mRNA into polysomes, compared to poly(A)$^+$ mRNA, was probably attributable to a deficiency in 60S ribosomal subunit joining (Munroe & Jacobson, 1990). Addition of exogenous poly(A) was also found to inhibit protein synthesis in vitro, presumably by sequestration of PABP, thus implying that it is required for translation (Jackson & Standart, 1990). Other studies have identified eIF-4 initiation factors as candidates that interact with poly(A) tails thereby providing collateral evidence for 5' and 3' end interaction (Gallie & Tanguay, 1994).

Translational regulation can therefore be mediated at a number of different levels providing controlled gene expression particularly in situations where nuclear functions are absent and permitting rapid cellular responses to external cues. However, in addition to all the aforementioned determinants that govern translation per se quantitative changes in mRNA levels within a cellular context effectively alter protein synthesis. Thus parameters encompassing transcriptional rates, mRNA stabilities, nuclear transport and subcellular localisation of mRNAs become relevant factors influencing translation.
1.5 Project Background and Aims

It was observed that in cell lines derived from multiple myeloma patients there was a 10-25 fold increase in c-myc protein levels (Figure 1.13). This was not accompanied by a concomitant increase in c-myc mRNA levels and was not attributable to a prolonged protein half life in these cells (Figure 1.13). It thus appeared that enhanced translation could be responsible for the augmented c-myc protein levels in the MM cell lines.

The aim of this project was to try to rationalise these observations and delineate the putative translational control mechanism functioning for c-myc within these MM cell lines. As c-myc exon 1 had previously been implicated in translational regulation it was decided to focus attention on this region. The main aims of the project were therefore threefold:

1) To determine if primary sequence alterations of c-myc exon 1 could be responsible for the elevated c-myc protein levels.

2) To search for RNA-binding proteins that were capable of binding the c-myc 5'UTR and thus potentially modulate translation.

3) To examine the translational efficiencies of reporter constructs containing c-myc exon 1 in in vitro translation systems.

It was hoped that investigation of all the aforementioned parameters would give some indication of the underlying cause responsible for the aberrant translational control of c-myc displayed in the MM cell lines. An additional aim of the project was to characterise the MM cell lines which have been in culture for many years and to try to isolate new MM cell lines to determine if translational deregulation of c-myc is a widespread phenomenon in MM that may contribute towards the pathogenic disease state.
Figure 1.13: (A) \( c\text{-}myc \) protein levels calculated from an ELISA system. This method utilises two anti-peptide antibodies which recognise different epitopes of the human \( c\text{-}myc \) protein. The results plotted are average values obtained from three independent experiments. (B) \( c\text{-}myc \) mRNA levels calculated from Northern blot analysis. \( c\text{-}myc \) mRNA levels were calculated from phosphorimager analysis and normalised to levels of the control mRNA glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Control cell lines; GM0892A, GM1953, Colo320. MM cell lines; GM2132, GM1500, GM1311, GM6923.
CHAPTER 2

Materials and Methods
Materials and methods

2.1 General Reagents

2.1.1 General Reagents
Unless otherwise stated all chemical reagents were of analytical grade and were obtained from BDH laboratory supplies (Lutterworth, Leicestershire, UK), Fisons (Loughborough, Leicestershire, UK), ICN Flow Ltd (Thame, Oxfordshire, UK) or Sigma Chemical company Ltd (Poole, Dorset, UK). Products for molecular biological techniques were routinely purchased from Anachem (Luton, Bedfordshire, UK), Boehringer Mannheim UK Ltd (Lewes, East Sussex, UK), Gibco-BRL (Paisley, Scotland), Stratagene Ltd (Cambridge, UK), New England Biolabs (NEB) (c/o CP Labs, Bishops Stortford, Hertfordshire, UK) and Pharmacia Biotech (Milton Keynes, Buckinghamshire, UK). Agarose of molecular biology grade for use in all standard procedures was purchased from Immunogen International Ltd (Sunderland, Tyne & Wear, UK). Reagents for bacterial cell culture were obtained from Oxoid (Unipath, Basingstoke, Hampshire, UK). Foetal calf serum (FCS) for mammalian tissue culture was obtained from Advanced Protein Products (APP) (Brierly Hill, UK) and human serum and human plasma were obtained from the Trent blood transfusion service (Sheffield, UK). All tissue culture plasticware was supplied by Nunc products (Gibco-BRL). Nitrocellulose membranes for blotting procedures were obtained from Schleicher and Schuell (c/o Anderman & Company, Surrey, UK), Bio-Rad laboratories (Hemel Hempstead, Hertfordshire, UK) and Gelman Sciences Ltd (Northampton, UK). Radiolabelled chemicals were obtained from Amersham International Plc (Little Chalfont, Buckinghamshire, UK).

2.1.2 Antibodies
The Myc monoclonal antibody 9E10, as originally described by Evan et al, 1985, was obtained from super concentrated tissue culture supernatant generated from the hybridoma cell line Myc9E10F5, in collaboration with Dr Tim Harrison (Biochemistry Department, Leicester University, Leicester, UK). This antibody was normally diluted 1:400 - 1:600 for use in immunoblots. Monoclonal La antibody was routinely diluted 1:60 for use in Western blot analysis and was a kind gift from Prof Mike Clemens (St George's Hospital Medical School, London, UK). Polyclonal serum raised against polypyrimidine tract binding protein (PTB) and recombinant GST-PTB fusion protein, for use as a positive control, were generously provided by Dr Richard Jackson (Biochemistry Department, Cambridge University, Cambridge, UK). This antibody was normally diluted 1:5000 for immunoblot analysis. Secondary antibodies raised against mouse and rabbit IgG and conjugated to horseradish peroxidase were obtained from Sigma and routinely diluted 1:2000. Cell surface antigen antibodies CD38 and CD45 were purchased from Cymbus Bioscience Ltd (Southampton, UK).
2.2 Tissue Culture Techniques

2.2.1 Tissue culture media and supplements

**RPMI 1640 medium**: Roswell Park Memorial Institute 1640 medium, with L-glutamine (Gibco-BRL) was supplemented with 15% foetal calf serum (FCS) (Advanced Protein Products) and gentamicin (10 µg/ml) (Sigma).

**IMDM medium**: Iscove's modified Dulbecco's medium (Sigma) was supplemented with 1% methylcellulose, 5% FCS, 30% heat inactivated human plasma, β-mercaptoethanol (10 µM), EDTA (50 µM) and gentamicin (10 µg/ml).

**DMEM medium**: Dulbecco's modified Eagle medium, without sodium pyruvate (Gibco-BRL) was supplemented with 10% FCS and gentamicin (10 µg/ml).

**McCoy's medium**: McCoy's 5A modified medium, with L-glutamine (Sigma), was supplemented with EDTA (50 µM).

2.2.2 Cell Lines

<table>
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<tr>
<th>Cell Line Name</th>
<th>Cell Line Type</th>
<th>Growth medium</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>Colo320 DM</td>
<td>Colon adenocarcinoma</td>
<td>RPMI (10 % FCS)</td>
<td>ATCC</td>
</tr>
<tr>
<td>HL60</td>
<td>Promyelocytic leukaemia</td>
<td>RPMI (10 % FCS)</td>
<td>ATCC</td>
</tr>
<tr>
<td>GM03201</td>
<td>EBV immortalised lymphoblastoid cell line (LCL)</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>GM0892A</td>
<td>EBV immortalised lymphoblastoid cell line (LCL)</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>GM1953</td>
<td>EBV immortalised lymphoblastoid cell line (LCL)</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>U266</td>
<td>Multiple myeloma</td>
<td>RPMI (15 % FCS)</td>
<td>ATCC</td>
</tr>
<tr>
<td>GM2132</td>
<td>Multiple myeloma</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>GM1311</td>
<td>Multiple myeloma</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>GM6923</td>
<td>Multiple myeloma</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>GM1500</td>
<td>Multiple myeloma</td>
<td>RPMI (15 % FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
<td>CRL7541</td>
<td>Human stromal BM multiple myeloma</td>
<td>DMEM (10 % FCS)</td>
<td>ATCC</td>
</tr>
<tr>
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<td>Human stromal BM multiple myeloma</td>
<td>DMEM (10 % FCS)</td>
<td>ATCC</td>
</tr>
<tr>
<td>CRL7542</td>
<td>Human stromal BM normal</td>
<td>DMEM (10 % FCS)</td>
<td>ATCC</td>
</tr>
</tbody>
</table>

Table 2.1: Cell lines utilised with details of their origin and in vitro growth requirements. All lines were purchased from either the American Type Culture Collection (ATCC) or the National Institutes of General Medical Sciences Human Genetic Mutant
Cell Repository (NIGMS). (BM; bone marrow).

2.2.3 Maintenance of cell lines
All of the cell lines listed in Table 2.1 were cultured in the media indicated in sterile flasks (Nunclon, Gibco-BRL) and maintained at concentrations between $5 \times 10^5$-1x$10^6$ cells/ml. The adherent cell lines CRL7541, CRL7544 and CRL7542 were grown to confluence and treated with trypsin-EDTA solution (Gibco-BRL) and then split 1:4. Cells were routinely grown at 37°C in a humidified atmosphere containing 5% CO₂. Cell manipulations were carried out in a class II microbial safety cabinet.

2.2.4 Mononuclear cell preparation
5 ml of either blood or bone marrow (aspirate) was removed from the patient and immediately added to 5 ml McCoy's medium (50 μM EDTA) to prevent clotting. The sample was then applied to 10 ml Ficoll Paque (Pharmacia) pre-warmed to 37°C, and centrifuged at 1,800 rpm for 20 mins at room temperature (Sorvall RT600B). The mononuclear cells banding at the interface were carefully removed and added to approximately 8 ml McCoy's medium and then layered onto a second Ficoll Paque gradient (8 ml) and subjected to a further centrifugation. The mononuclear cells at the interface were removed, counted and diluted into 2 ml McCoy’s medium prior to in vitro culture.

2.2.5 In vitro growth of mononuclear cell preparations
Mononuclear cell preparations were cultured in vitro using a method adapted from Takahashi et al, 1985. Briefly; 1 ml, 0.75 ml, 0.5 ml and eight serial dilutions of 0.5 ml of the 2 ml mononuclear cell preparation in McCoy’s medium (original concentration; approximately 3.0x$10^5$ cells/ml) were added to 1.5 ml semi-solid Iscove's modified Dulbecco's medium (IMDM) in 24 well plates (Nunclon, Gibco BRL). This medium, as indicated above (Section 2.2.1), was supplemented with methylcellulose (1%), 5% FCS, β-mercaptoethanol (10 μM), EDTA (50 μM), gentamicin (10 μg/ml) and heat inactivated human plasma (30%) to favour the growth of myeloma cells. Cultures were incubated under standard conditions (Section 2.2.3) and examined every 4-8 days. At approximately 20 day intervals 0.5-1 ml quantities of IMDM lacking methylcellulose were added to each of the samples to replenish nutrients and loss of media by evaporation.

2.2.6 Subculturing of myeloma cells
At approximately 20 day intervals myeloma cells were picked using a 5 μl glass pipette and subcultured in liquid RPMI 1640 medium with or without recombinant interleukin-6 (IL-6) (1 ng/ml). As the plasma cells were tightly associated with the stromal cells (bone marrow samples), it was often necessary to dislodge the stromal cells from the substratum with the pipette.
2.3 Cytochemical Techniques

2.3.1 Cytochemical solutions
PBS (Phosphate buffered saline); 150 mM NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.2. 10 tablets of PBS (ICN Flow Laboratories) were dissolved in 1 l of sterile water. Aliquots were then sterilised by autoclaving at 120°C for 20 mins at 15 psi.

Giemsa solution; Giemsa solution (BDH) was diluted 1:9 in phosphate buffer pH 6.8.
May Grunwald solution; May & Grunwald staining solution (BDH) was diluted 1:1 in phosphate buffer pH 7.2.

2.3.2 Slide preparation
Cells were harvested by centrifugation at 1,000 rpm and washed three times in phosphate buffered saline (PBS). Samples obtained from bone marrow cultures were passed through a 25 gauge needle, prior to centrifugation, to disperse cell clumps. Cells were then counted and diluted to a density of approximately 5×10⁴ cells/ml in PBS containing 0.1% sodium azide, 5% FCS. 500 µl samples were transferred to cytospin chambers (Shandon) and centrifuged at 500 rpm for 8 mins (low acceleration). Slides were fixed in absolute methanol for 10 mins at -20°C and then either directly stained or air dried prior to storage. 5 µl samples obtained from MM patients were directly smeared on to slides, allowed to dry and then fixed in absolute methanol as described above.

2.3.3 Giemsa staining
Fixed samples were stained in Giemsa solution for 1 hour at room temperature. After rinsing three times in distilled water, samples were dehydrated in isopropyl alcohol for 1 min and then stored dry in the dark. Slides were examined under a Olympus BH-2 light microscope and photographs were taken using an Olympus C-35 AD-2 camera on Fuji-RDP film (ASA 400) uprated to ASA 1000 during development.

2.3.4 May Grunwald Giemsa (MGG) staining
Slides were immersed in May Grunwald solution for between 5-15 mins and immediately transferred to Giemsa solution (see 2.3.2) for a further 20-30 mins. Excess stain was then removed by rinsing the samples in distilled water for a minimum of 15 mins. Slides were stored dry in the dark.
2.4 Immunofluorescence Techniques

2.4.1 Immunofluorescence solutions
PBS (Phosphate buffered saline); 150 mM NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.2.
10% NGS/PBS; 10% heat inactivated normal goat serum (HI NGS), 0.1% sodium azide in PBS.
5% ABS/PBS; 5% heat inactivated human AB serum (HI ABS), 0.1% sodium azide in PBS.
DABCO mounting solution; 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO), 10% glycerol in PBS.

2.4.2 Fluorescent activated cell sorting (FACS)
Cells were diluted to a density of 1x10⁵ cells/ml and then harvested after 16 hours by centrifugation at 1,000 rpm for 5 mins. 1x10⁶ cells were resuspended in 40 µl 10% NGS/PBS and incubated on ice with an equal volume of antibody diluted in 5% ABS/PBS for 30-60 minutes. Fluorescein isothiocyanate (FITC) conjugated CD38 antibody (Cymbus Bioscience Ltd) was diluted 1:4 and phycoerythrin (PE) conjugated CD45 antibody was diluted 1:10 (Cymbus Bioscience Ltd). Mouse IgG FITC and PE conjugated antibodies (Becton Dickinson), used as non-specific negative controls, were diluted 1:50 and 1:20 respectively. Cells were then washed three times in PBS and finally resuspended in 1 ml PBS prior to analysis on a FACScan flow cytometer (Becton Dickinson). Data acquisition and subsequent analysis was performed using Becton Dickinson Lysis II software.

2.4.3 CD38 enrichment by FACS
Peripheral blood samples were treated with CD38 antibody, at an appropriate dilution, as described above (Section 2.4.2) but sodium azide was omitted from all solutions. Cells were then sorted into CD38 positive and negative populations using a Vantage flow cytometer (Becton Dickinson) and collected in a PBS solution. Cells were cultured in vitro at concentrations of approximately 1x10⁵ cells/ml as described in section 2.2.5.

2.4.4 CD38/CD45 immunofluorescence
Cells were treated with antibody as described for FACS analysis (Section 2.4.2) but resuspended at a concentration of 5x10⁴ cells/ml in PBS containing 0.1% sodium azide, 5% FCS. 500 µl samples were transferred to cytospin chambers (Shandon) and centrifuged at 500 rpm for 2 mins (low acceleration). Slides were then air dried for approximately 30 mins and mounted with coverslips in DABCO solution to prevent fading, and subsequently stored in the dark at 4°C. Samples were examined using an
Olympus BH-2 immunofluorescence microscope and photographs were taken using an Olympus C-35AD-2 camera on Fuji-RDP film (ASA 400) uprated to ASA 1000 during development.

2.4.5 κ/λ immunoglobulin light chain immunofluorescence: Cell aggregates were disrupted by passing the samples through a 25 gauge needle and harvested by centrifugation at 1,000 rpm. After three washes with PBS cells were resuspended at a concentration of 5x10⁴ cells/ml in PBS containing 0.1% sodium azide, 5% FCS and centrifuged on to slides as described above (Section 2.4.2). Samples were then air dried for at least 30 mins and desiccated overnight at 4°C. The slides were then fixed for 15 mins in methanol at 4°C and immediately washed in PBS for a further 15 mins. κ and λ immunoglobulin light chain FITC conjugated antibodies (Atlantic Antibodies) were diluted 1:10 in 0.005% Evans blue in PBS and incubated with the samples in a moist chamber for 30 mins. Excess antibody was removed by washing the slides in PBS for 15 mins. Coverslips were mounted in DABCO solution, stored and examined as described above (Section 2.4.4).

2.5 Bacterial Methods

2.5.1 Bacterial media and supplements

Agar plates; 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl dissolved in 1 l distilled water and 15 g agar. Aliquots were sterilised by autoclaving at 120°C for 20 mins at 15 psi.

Ampicillin; 50 mg/ml ampicillin dissolved in sterile, distilled water and filter sterilised (1000x stock).

IPTG (Isopropyl-β-D-thiogalactopyranoside); 25 mg/ml solution dissolved in sterile, distilled water.

LB (L-Broth); 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl dissolved in 1 l distilled water. Aliquots were sterilised by autoclaving at 120°C for 20 mins at 15 psi.

M9 Salts; 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl dissolved in 1 l distilled water. Aliquots were sterilised by autoclaving at 120°C for 20 mins at 15 psi.

Minimal media; 20% M9 salts, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.2% glucose, 0.01 mg thiamine.

Top agar (0.8%); 8 g Bacto-agar dissolved in 1 l distilled water. Aliquots were sterilised by autoclaving at 120°C for 20 mins at 15 psi.

X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside); 2% solution in dimethylformamide.
2.5.2 Bacterial strains
The *E. coli* strain JM109 was used in all bacterial manipulations. JM109; e14~(mcrA)rec A1, endA1, gyr A96, thi-1, hsdR17, supE44, relA1, Δ(lac-proAB) F'\*traD36, proAB, lacZΔM15.

2.5.3 Preparation of competent cells
A single *E. coli* JM109 colony was picked from an agar plate and grown overnight in 5 ml L-Broth at 37°C with aeration. 100 µl was then used to inoculate 5 ml minimal media and grown for a further 3 hours at 37°C to ensure retention of the F plasmid. The culture was then transferred to a flask containing 250 ml LB (20 mM MgSO₄) and grown at 37°C until an optical density of 0.48-0.50 (A600nm) was achieved. The cells were chilled on ice for 5 mins and pelleted at 5,000g for 5 mins at 4°C, resuspended in 100 ml ice cold TfbI (30 mM KAc, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂, 15%(v/v)glycerol; pH 5.8, filter sterilised) and re-pelleted. Subsequently the pellet was resuspended in 10 ml TfbII (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂, 15%(v/v)glycerol; pH 6.5, filter sterilised) and incubated on ice for 15 mins - 16 hours. The competent cells were flash frozen, on dry ice, in 200 µl aliquots and stored at -70°C until required.

2.5.4 Transformation of bacterial cells
Ligation products (Section 2.6.14) or plasmid DNA (10 ng) were diluted in sterile, distilled water to a final volume of 25 µl. 35 µl of competent JM109 bacterial cells were added and then incubated on ice for a minimum period of 20 mins. Samples were heated at 37°C for 5 mins, 100 µl of L-broth was added and they were then grown at 37°C with aeration for approximately 20-40 mins. Samples were spread on to L-agar plates containing the appropriate supplements. Plates were incubated at 37°C for 16-20 hours.

2.5.5 Transfection of bacterial cells
Ligation products (Section 2.6.14) or plasmid DNA (10 ng) were diluted in sterile, distilled water to a final volume of 25 µl to which 35 µl of competent JM109 bacterial cells were added. The mixture was incubated on ice for a minimum of 20 mins and then heated at 37°C for 5 mins. Cells were immediately placed back on ice. 140 µl of JM109 lawn cells, from a growing culture, were added to the heat shocked cells prior to the addition of 3 ml of top agar (0.8%) containing 0.02% X-GAL, 0.25 mg/ml IPTG at 55°C. The top agar cell mixture was then poured on to an L-agar plate and incubated at 37°C for 16-20 hours.

2.6 Nucleic Acid Methods

2.6.1 Buffers/Stock solutions
Acrylamide gel mix (sequencing-6%); 84 g urea, 20 ml 10xTBE, 30 ml 40% acrylamide made up to 200 ml with distilled water. Store at 4°C. 50 µl TEMED and 50 µl APS
(25%) were used to induce polymerisation.

20xSSC (Sodium chloride, sodium citrate); 3 M NaCl, 3.5 M NaCitrate, pH 7.0.
TAE (Tris-Acetate-EDTA); 0.04 M Tris-acetate pH 8.0, 1 mM EDTA.
TBE (Tris-Borate-EDTA); 89 mM Tris-HCl pH 8.0, 89 mM boric acid, 2.5 mM EDTA.
10xTBE agarose gel loading dye; 0.25% bromophenol blue, 0.25% xylene blue, 15% ficoll in 10xTBE.
TE (Tris EDTA); 10 mM Tris pH 8.0, 1 mM EDTA.
TNE (Tris, Sodium chloride, EDTA); 50 mM Tris, 100 mM NaCl, 5 mM EDTA.

2.6.2 Plasmids

<table>
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<tr>
<th>Plasmid</th>
<th>Vector purpose</th>
<th>Source/Reference</th>
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<td>Sequencing</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pMC41</td>
<td>c-myc positive control</td>
<td>Watt et al., 1983b</td>
</tr>
<tr>
<td>pBluescript SK</td>
<td>In vitro transcription</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pSK GAPDH</td>
<td>GAPDH transcript</td>
<td>Dugaiczyk et al., 1983</td>
</tr>
<tr>
<td>pJHRV(10-605)</td>
<td>IRES transcript</td>
<td>Borman &amp; Jackson, 1992</td>
</tr>
<tr>
<td>pGL3 control vector</td>
<td>Luciferase reporter</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-luc</td>
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<td>Promega</td>
</tr>
<tr>
<td>pXLJ'0</td>
<td>Dicistronic reporter construct</td>
<td>Borman &amp; Jackson, 1992</td>
</tr>
<tr>
<td>pXLJ0DA1099</td>
<td>Dicistronic TMEV IRES positive control</td>
<td>Hunt et al., 1993</td>
</tr>
</tbody>
</table>

Table 2.2: Plasmids used during the course of this study. The plasmid pSK GAPDH containing the chicken glyceraldehyde 3 phosphate dehydrogenase gene was a generous gift from Prof David Critchley (Biochemistry Department, Leicester University, Leicester, UK). The constructs pJHRV(10-605), pXLJ'0 and pXLJ0DA1099, as detailed in Chapter 6, were all kindly provided by Dr Richard Jackson (Biochemistry Department, Cambridge University, Cambridge, UK).

2.6.3 Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems model 394 machine (Protein and Nucleic Acid Sequencing Laboratory, Leicester University) at a 0.2 μM scale. Oligonucleotides were purified from the by-products of synthesis by precipitation with 0.1 vol. 3M sodium acetate (pH 5.2) and 3 vols. ethanol. Samples were incubated at -20°C for at least 30 mins and the precipitated DNA pelleted by centrifugation at 13,000 rpm for 20 mins. After washing with 70% ethanol, samples were briefly dried and then resuspended in 100-200 μl TE.
2.6.4 Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>FP1</td>
<td>5' GCCGGATCCGGCCTTTATAATGCGAG 3'</td>
</tr>
<tr>
<td>NS5350</td>
<td>5' TGGCTCCCCTCCTGC 3'</td>
</tr>
<tr>
<td>FP2431</td>
<td>5' TTCCCGCCAAGCCTCTGAGA 3'</td>
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<tr>
<td>FP2501</td>
<td>5' TAATTCCAGCGAGGGCAGA 3'</td>
</tr>
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<td>FP2610</td>
<td>5' TATTCGCTCCGGATCTCCCT 3'</td>
</tr>
<tr>
<td>FP2670</td>
<td>5' TGCCGCATCCACGAAACTTT 3'</td>
</tr>
<tr>
<td>FP2731</td>
<td>5' TTGTAAGTTCACGTGCAAAG 3'</td>
</tr>
<tr>
<td>FP2</td>
<td>5' GTGGAATTCTGGTTTTCCACTACCGAAA 3'</td>
</tr>
</tbody>
</table>

Table 2.3: Nucleotide sequence of oligonucleotide primers used for PCR amplification and sequencing of c-myc exon 1.

2.6.5 Determination of nucleic acid concentrations

DNA or RNA concentrations were determined spectrophotometrically by monitoring the absorbance at 260nm. 50 µg/ml dsDNA, 40 µg/ml RNA and 20 µg/ml oligonucleotide were taken to be equivalent to 1 OD unit.

2.6.6 Phenol/chloroform extraction

Contaminating proteins were removed from nucleic acid solutions by the addition of 1 volume of 50% equilibrated phenol, 49% chloroform, 1% isoamyl alcohol (IAA). After vigorous vortexing/shaking to mix the layers the phases were separated by centrifugation at 13,000 rpm for 5 mins and the upper inorganic phase was removed to a fresh tube. DNA was then ethanol precipitated as described below (Section 2.6.7).

2.6.7 Ethanol precipitation of DNA

DNA was precipitated by the addition of 0.1 vol. 3M sodium acetate (pH 5.2) and 3 vols. ethanol and then pelleted by centrifugation at 13,000 rpm for 10 mins. The pellet was washed in 70% ethanol to remove any excess salt, dried briefly and then resuspended in the appropriate buffer. This was normally TE or sterile distilled water.

2.6.8 Agarose gel electrophoresis

DNA fragments were separated by electrophoresis through agarose gels (normally 0.8%, 1.0%, or 2% w/v) in 1xTBE buffer. Gels were prepared by melting agarose in 1xTBE buffer. Once cooled and cast, they were run submerged in 1xTBE buffer in horizontal electrophoresis tanks at up to 8 V/cm. Samples were mixed with 1/10 vol. TBE loading buffer prior to electrophoresis. After electrophoresis was complete gels were stained in ethidium bromide (1.3 mg/l in 1xTBE) for 15-20 mins. The DNA was visualised and

49
photographed (Polaroid film) on a UV transilluminator.

2.6.9 Preparation of genomic DNA
1 ml of cells (approx. 5x10^5 cells) was removed from each of the cell lines. Cells were pelleted by centrifugation at 6,500 rpm for 10 mins and resuspended in 1 ml sterile PBS. To this, 3 ml stock proteasing solution (1xTNE diluted 10:1 in Tris-HCl pH 8.0), 80 µl 25% SDS and 20 µl proteinase K (20 mg/ml) was added. The mixture was then gently rocked at 37°C for 4 hours. Contaminating proteins were extracted with phenol and chloroform, and the resulting genomic DNA precipitated with 2.5 vols. ethanol. After centrifugation for 15 mins at 8,000 rpm at 4°C the supernatant was removed and the pellet dried and resuspended in 100 µl TE.

2.6.10 Polymerase chain reaction
Primers FP1 and FP2 were used to amplify c-myc exon 1 (2289-2881). PCR reactions contained 10 µl 10x PCR Buffer (Advanced Biosystems), 10 µl MgCl2 (25 mM), 1 µl dNTPs (10 mM of each of dATP, dCTP, dTTP, dGTP), 0.2 µl Taq DNA polymerase (Advanced Biosystems), 0.7 µg DNA or 2 µl of cDNA obtained after reverse transcription (Section 2.7.3), 2 µl of each oligonucleotide (20 µM) and sterile, distilled water to 100 µl. Samples were overlaid with 50 µl of paraffin oil to minimise evaporation. The PCR reactions were carried out in a Perkin Elmer Cetus DNA Thermal Cycler at 94°C for 3 mins followed by 37 cycles of [94°C for 2 mins, 63°C for 3 mins, 72°C for 2 mins] and 72°C for 10 mins. After the amplification was complete a 12 µl sample was removed and run on a 1.0% agarose gel. PCR amplification using the FP2/FP2501 primer pair was carried out identically except that the annealing temperature of the PCR was reduced from 63°C to 57°C.

2.6.11 Restriction enzyme digestion
Approximately 1 µg DNA was digested with restriction enzymes in a total volume of between 10-40 µl under conditions recommended by the suppliers. Reactions were incubated at the appropriate temperature for 1-2 hours.

2.6.12 End filling
The Klenow fragment of E.coli DNA polymerase I was used to fill in the 5' overhangs of vector and/or fragments created by digestion with certain restriction enzymes to form flush ends permitting cloning into blunt end restriction sites. 1 unit of Klenow was added for each µg of DNA present and incubated at room temperature for 15 mins in the presence of the appropriate deoxynucleotide triphosphates. The enzyme was then inactivated by heating to 75°C for 10 mins.
2.6.13 Alkaline phosphatase treatment of DNA
To prevent vector re-ligation following restriction enzyme digestion the vector ends were dephosphorylated using calf intestinal alkaline phosphatase (CIP). After restriction digestion CIP buffer, appropriately diluted, and 2 units of CIP enzyme were added to give a final volume of 200 μl. Samples were then incubated at 37°C for 1 hr, phenol extracted and the DNA precipitated with ethanol. Samples were then resuspended in 10 μl sterile distilled water.

2.6.14 Ligation of DNA
Ligations were performed in a total volume of 10 μl. Vector DNA (0.1 μg) was mixed in a 1:3 molar ratio with insert DNA in the presence of 1 unit T4 DNA ligase in the ligase buffer supplied. The reaction mix was incubated at room temperature for 3-4 hours or at 16°C overnight and 5 μl was then used in transfections or transformations (Sections 2.5.4 & 2.5.5).

2.6.15 Small scale preparation of plasmid DNA
A single plaque or colony was picked and grown overnight in 3 ml of LB. 1.6 ml was then removed and centrifuged at 13,000 rpm for 30s. If necessary the supernatant was carefully removed into a fresh tube for preparation of single strand DNA (Section 2.6.20). Plasmid DNA was prepared using a method adapted from Morelle, 1989. Briefly, the pellet was resuspended in 100 μl solution 1 (25 mM Tris HCl pH 8.0, 10 mM EDTA, 50 mM Glucose) and after a 5 min incubation on ice 200 μl solution 2 (1% SDS, 0.2M NaOH) was added. The mixture was then incubated on ice for a further 5 mins prior to the addition of 150 μl of 7.5M NH₄Ac (pH 7.8). The solution was gently mixed and incubated on ice for a final 5 mins. The precipitated matter was removed by centrifugation (13,000 rpm) for 10 mins and the supernatant removed and ethanol precipitated. The final pellets were normally resuspended in 30 μl TE and 6 μl of this was used in restriction digests.

2.6.16 Large scale preparation of plasmid DNA
Large scale preparations of plasmid DNA were carried out as described in Sambrook et al, 1989. A 200 ml flask of L-Broth, containing the appropriate antibiotic supplements was inoculated with a 3 ml inoculum and then grown shaking at 37°C for 12-16 hours. The cells were then pelleted by centrifugation at 5,000 rpm in a GS3 rotor (Sorvall RC-5B, DuPont Instruments) for 10 mins at 4°C. Pellets were resuspended in 7.7 ml ice cold Solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA), transferred to SS34 tubes and incubated for 5 mins at room temperature. The cells were then lysed in 15.3 ml of Solution 2 (0.2M NaOH, 1% SDS), mixed, and incubated on ice for 10 mins, prior to the addition of 11.5 ml of 3M KOAc pH 4.8 and a further 10 min incubation on ice. Precipitated matter was removed by centrifugation at 15,000 rpm for 30-60 mins at 4°C and DNA in the supernatant precipitated by the addition of 0.6 vol. isopropanol. DNA was
then pelleted by centrifugation at 9,500 rpm for 20 mins at 20°C, dried and resuspended in 8 ml of TE with 8g CsCl and 500 μl ethidium bromide (10 mg/ml). CsCl gradients were then centrifuged at 45,000 rpm for 20-40 hours and plasmid DNA was removed with a syringe and needle. The ethidium bromide was extracted using ITC solution (20% w/v CsCl, 10 ml 50 mM Tris-HCl, 5 mM NaCl, 50 mM EDTA, 80% v/v isopropanol). 150 μl of clarified solution was then mixed with 600 μl of sterile, distilled water, 75 μl 3M NaOAc and 600 μl of isopropanol and centrifuged for 10 mins. The pellet was then resuspended in 100 μl of sterile, distilled water and precipitated with 1/10 vol. 3M NaOAc and 2.5 vols. ethanol. After centrifugation for 10 mins at 13,000 rpm samples were finally resuspended in 100-200 μl of sterile, distilled water.

2.6.17 Purification of DNA by NaI and glassmilk

Solutions containing DNA (greater than 200 bp) were incubated with 3 volumes of NaI solution and 5 μl glassmilk for 5 mins at room temperature. The glassmilk was pelleted and then washed three times in "NEW WASH" and the DNA was eluted by resuspending the pellet in 6-8 μl sterile, distilled water and incubating at 50°C for 3 mins. After repelleting the glassmilk, the supernatant was removed to a fresh tube and the elution process was repeated. (Manufacturer's instructions-Geneclean II, BIO 101; supplied by Anachem).

2.6.18 Gel isolation of DNA

DNA fragments were separated by agarose gel electrophoresis as described (Section 2.6.8), except low melting point agarose (Gibco-BRL) and lxTAE buffer were used. DNA was visualised with ethidium bromide (1.3 mg/l) staining using a low intensity UV source and fragments of the required size were excised from gels with minimal residual agarose. Gel pieces were weighed and 3 volumes of NaI solution were added. The agarose was then melted by incubating the samples at 50°C for 20 mins. 5 μl of glassmilk was added and the samples were treated as described in Section 2.6.17.

2.6.19 Southern blotting procedure for DNA

DNA fragments were separated by agarose gel electrophoresis and examined after ethidium bromide staining on a UV transilluminator. Gels were then placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 1 hour. Gels were then neutralised in 1M Tris-HCl pH 8.0, 1.5 M NaCl for 1 hour and blotted on to Zeta probe membrane (BioRad) pre-soaked in 2xSSC. Filter wicks were soaked in 20xSSC and DNA was blotted on to the membranes for 1.5-2 hours using Quickdraw™ blotting paper (Sigma). Membranes were then briefly soaked in 6xSSC and the DNA immobilised on the membranes by baking at 80°C for 30 mins. Prior to hybridisation membranes were soaked in 2xSSC and then pre-hybridised in 10-15 ml of Church-Gilbert buffer (0.25 M Na₂HPO₄ pH 7.2, 7% SDS, 1
mM EDTA) in the presence of 1 ml boiled, alkali denatured salmon sperm DNA (10 mg/ml) in a hybridisation chamber at the appropriate temperature for 1.5-2 hours. Radiolabelled DNA probes were then added and hybridised overnight, again at the appropriate temperature. (For the FP2610 oligonucleotide this was calculated from the melting temperature; $T_m=2(A+T)-4(G+C)-7$ to be 55°C). Membranes were then washed in a solution of 2xSSC containing 0.1% SDS and exposed directly to Kodak XAR-5 film at -70°C for 16-80 hours.

### 2.6.20 End labelling of DNA fragments

DNA fragments or oligonucleotides were end labelled with $^{32}$Pγ-ATP using T4 polynucleotide kinase (PNK). 150 ng of DNA was incubated with 1 unit of T4 PNK in the presence of PNK buffer, appropriately diluted, 3 mM dithiothreitol (DTT), 2.5 µl $^{32}$Pγ-ATP (300 Ci/mmol) in a total volume of 30 µl at 37°C for 60 mins. Unincorporated radionucleotide was then removed by centrifugation through a 1 ml Sephadex G-50 column.

### 2.6.21 Preparation of single stranded DNA

Cells from overnight cultures were pelleted as detailed in Section 2.6.15 and the supernatants carefully removed into fresh tubes. 300 µl 20% PEG (polyethylene glycol) in 2.5M NaCl was added to the supernatants to precipitate the M13 bacteriophage. Samples were vortexed and incubated at room temperature for 20 mins or at 4°C overnight. Phage particles were recovered by centrifugation at 13,000 rpm for 10 mins and the resulting pellets resuspended in 200 µl TE. A 20 µl aliquot was removed and stored at -20°C as infectious phage stock. To the remainder of the sample an equal volume of phenol was added. After vigorous vortexing and centrifugation the aqueous layer was chloroform extracted and single stranded DNA precipitated with 0.1 vol. 3M NaOAc (pH 5.2) and 3 vols. ethanol. Pellets were finally resuspended in 30 µl TE.

### 2.6.22 Sequencing

Single stranded DNA was prepared as above (Section 2.6.20) and 5 µl was used in sequencing reactions. 5 µl sterile, distilled water, 2 µl M13 Universal primer (4.44 ng/ml) or synthesised primer (20 ng/ml) and 2 µl of annealing buffer (Pharmacia) were added to the DNA before incubation at 70°C for 2 mins, followed by incubation at 37°C for 15 mins. Samples were labelled for 5 mins at room temperature using 0.5 µl $^{35}$S α-dATP (1270 Ci/mmol), 3 µl labelling mix A (Pharmacia) in the presence of 1 unit T7 DNA polymerase. Chain elongation was terminated by addition to 2.5 µl of each termination mix (G, A, T or C) and the reaction stopped after 5 mins at 37°C by the addition of 5 µl stop solution (Pharmacia). 2 µl of each sample was then run on an 6% polyacrylamide gel at 38W (2500V, 20A). Gels were dried for 1 hour at 80°C and exposed to Kodak XAR-5 film for 16-80 hours at room temperature.
2.7 RNA Techniques

2.7.1 RNA solutions

**DEPC treated water;** distilled water was treated with diethylpyrocarbonate (DEPC) (0.1%) for 2-16 hours at 37°C and then autoclaved at 120°C for 20 mins at 15 psi.

**D-Base;** 100 mM KCl, 0.2 mM K-EDTA pH 8.0.

**GITC;** 4 M guanidinium isothiocyanate, 0.5% sarcosyl, 25 mM sodium citrate in DEPC treated water.

2.7.2 Preparation of total cellular RNA

Total cellular RNA was prepared according to the method of Chomczynski & Sacchi, 1987. Cells were counted, harvested and washed in PBS and then resuspended in Solution D (72 µl β-mercaptoethanol added to 10 ml GITC solution) (1 ml per 10 x 10^6 cells). Samples were vortexed vigorously to shear the DNA and then vortexed further after the addition of 100 µl 2M sodium acetate pH 4.0, 1 ml water saturated phenol (Sigma) and 200 µl chloroform/isoamyl alcohol (49:1). The aqueous and organic phases were separated by centrifugation at 13,000 rpm for 15 mins at 4°C and the aqueous layer containing RNA complexed with guanidinium was transferred to a fresh tube. RNA was precipitated for 20-30 mins at -20°C by the addition of an equal volume of isopropanol and pelleted by centrifugation at 13,000 rpm for 15 mins at 4°C. RNA pellets were carefully washed with 75% ethanol, dried and then resuspended in 20-30 µl of 0.1% DEPC treated sterile, distilled water. RNA concentrations were then calculated from A_{260nm} values and its purity assessed from the A_{260nm}/A_{280nm} ratio.

2.7.3 Reverse transcription of RNA

Contaminating DNA was removed from RNA samples by pre-treatment of RNA samples (approx. 1 µg) with 1 unit of DNase I for 30 mins at 37°C. Reaction mixtures normally contained 0.5 mM dNTPs, 10 mM DTT, 1 µl random hexamer primers (100 pmoles/µl), 1 unit RNasin (Promega) and reverse transcription buffer appropriately diluted in DEPC treated water in a total volume of 20 µl. The DNase I enzyme was then heat inactivated at 95°C for 10 mins and 200 units of Moloney Murine Leukaemia virus (Mo-MuLV) reverse transcriptase was added after cooling briefly on ice. Samples were incubated at room temperature for 5-15 mins and subsequently incubated at 37°C for 1 hour. The enzyme was heat inactivated at 95°C for 10 mins. 2 µl samples were routinely used in reverse transcription polymerase chain reactions (RT-PCR) (Section 2.6.10).

2.7.4 In vitro transcription

Vectors were linearised downstream of inserts, phenol and chloroform extracted, and then ethanol precipitated. 1 µg of restricted template was incubated with 1 µl of each rNTP (10
mM)(rATP, rGTP, rCTP, rUTP), 1 μl 0.75M DTT, 1 μl RNasin ribonuclease inhibitor (Promega), 5 μl 5x transcription buffer (Stratagene) and 10 units of T3, T7 or S6 RNA polymerase at 37°C for 1 hour. Transcripts were purified by addition of 10 units RNase free DNase for 20 mins at 37°C and after phenol and chloroform extractions they were precipitated using 0.1 vol. 3M NaOAc (pH 5.2) and 2.5 vols. ethanol. Radiolabelled transcripts were synthesised as above but 1 μl of either [α^32P] rUTP (800 Ci/mmol), [α^32P] rGTP (800 Ci/mmol) or [α^32P] rCTP (3000 Ci/mmol) was added in place of the appropriate unlabelled nucleotide. Unincorporated nucleotides were removed by centrifugation through a 1 ml Sephadex G-50 column. Transcript concentrations were determined by either Cerenkov scintillation counting or A_{260nm} values.

### 2.8 Protein Techniques

#### 2.8.1 Stock solutions/buffers

**Coomassie stain:** 0.1% Coomassie brilliant blue R-250 dissolved in 5:1:5 methanol:acetic acid:water.

**1x SDS sample buffer:** 50 mM Tris pH 6.8, 10% glycerol, 4% SDS, 0.1% bromophenol blue, 10% β-mercaptoethanol, 1 mM EDTA.

**SDS-PAGE resolving buffer:** 1.5 M Tris, 0.24% TEMED, 1% SDS pH 8.8.

**SDS-PAGE stacking buffer:** 0.25 M Tris, 0.12% TEMED, 0.2% SDS pH 6.8.

**SDS running buffer:** 25 mM Tris, 192 mM glycine, 0.1% SDS pH 8.3.

**TBST (Tris buffered saline, tween):** 10 mM Tris pH 8.0, 0.9% NaCl, 0.1% Tween.

#### 2.8.2 Nuclear free cell extract preparation

60-90x10^6 cells were harvested by centrifugation at 1,000 rpm for 5 mins and then washed in PBS. Cell pellets were resuspended in 600 μl polysome buffer (300 mM KCl, 5 mM MgCl₂, 10 mM HEPES pH 7.4) containing 0.5% NP40 to induce cell lysis. Nuclei were removed by centrifugation at 13,000 rpm for 10 mins.

#### 2.8.3 Preparation of HeLa cell extracts

HeLa cell extracts were a kind gift from Richard Jackson (Biochemistry Department, Cambridge, UK) and were made as described in Borman & Jackson, 1992.

#### 2.8.4 Determination of protein concentration - Bradford assay

Cell extracts were typically diluted 1:10 and 1:20 in sterile, distilled water. Stock BSA (2 mg/ml) was similarly diluted to concentrations of between 0.1-1.5 mg/ml. Bradford reagent was added according to the manufacturer's instructions (Pierce and Warriner) and absorbance at 630nm was monitored using a microtitre plate reader (Bio-tek instruments). Concentrations were then determined from a standard curve.
2.8.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli, 1970. Briefly, gels were cast and run in a Bio-Rad Protean II system and were prepared as detailed in Table 2.4 using a 30%:0.8% acrylamide:bisacrylamide stock solution. Gels were polymerised by the addition of ammonium persulphate solution. Samples were either resuspended in 1xSDS sample buffer or added to an equal volume of 2xSDS sample buffer prior to boiling and loading. Gels were then run in SDS running buffer for 6-20 hours at between 8-40 mA depending on the resolution required.

<table>
<thead>
<tr>
<th>%</th>
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<th>Stacking Gel</th>
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<td>10.0%</td>
</tr>
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<td>135 µl</td>
</tr>
<tr>
<td>Total</td>
<td>35 ml</td>
<td>35 ml</td>
</tr>
</tbody>
</table>

Table 2.4: Preparation of polyacrylamide gels. (* denotes stacking buffer).

2.8.6 Coomassie staining
Gels were stained in a Coomassie solution at room temperature for approximately 1 hour and then destained in (5:1:5) methanol:acetic acid:water for 1-5 hours. During this period the destaining solution was changed frequently. Gels were then stored in water or dried for 2 hours at 80°C to permit prolonged storage.

2.8.7 Silver staining
Gels were fixed in 50% methanol, 5% acetic acid for 30 mins, 7% methanol, 5% acetic acid for 30 mins and 10% glutaraldehyde for 30 a further mins. After a brief rinse in distilled water, gels were washed for a period of 80 mins with four changes of distilled water. Gels were then incubated for 30 mins in 1 mg DTT dissolved in 200 ml distilled water followed by 30 mins in 0.1% AgNO₃ solution. Excess Ag⁺ was removed by rinsing in distilled water and gels developed in 385 mM Na₂CO₃ dissolved in 200 ml water with 0.03% formaldehyde. The reaction was stopped by the addition of 50% citric acid solution and gels were subsequently rinsed in water. If prolonged storage was required, gels were then dried at 80°C for 2 hours.

2.8.8 Transfer of proteins on to nitrocellulose membranes
Cell extracts were separated by SDS-PAGE and then transferred on to nitrocellulose (Schleicher and Schuell) by electroblotting in transfer buffer (50 mM Tris, 192 mM glycine, 20% methanol) for 2.5 hours at 85 V as originally described by Towbin et al, 1979. Protein transfer was visualised temporarily by staining with Ponceau-S solution.
(0.5% w/v in 5% w/v trichloroacetic acid[TCA]).

2.8.9 North western blotting
Proteins immobilised on nitrocellulose were allowed to renature by incubating the membranes in 5% BSA dissolved in 10-15 ml D67NP40 solution (filter sterilised 65% v/v D-Base, 6.7 mM triethanolamine (TEA) pH 7.9, 170 mM DTT, 0.05% NP40) containing 1 mg/ml alkali denatured salmon sperm DNA and 0.02 mg/ml yeast tRNA to block non-specific protein and nucleic acid binding sites. After at least 1 hour at room temperature 32P labelled RNA transcripts were added and then incubated for a further hour at 30°C. Membranes were washed in D67NP40 solution and exposed directly to Fuji-RX X-ray film for 1-40 hours at room temperature.

2.8.10 Western blotting/immunodetection
Proteins immobilised on to nitrocellulose after SDS-PAGE (2.8.8) were detected immunologically using antibodies to either c-myc, actin, La or polypyrimidine tract binding protein (PTB). Non-specific binding sites were first blocked by incubating the nitrocellulose membranes in a 5% dried milk solution in TBST for 1-4 hours at room temperature. Membranes were then incubated in 5-10 ml of appropriately diluted primary antibody (Section 2.1.2) in 5% milk TBST for a period of 1-2 hours with constant agitation. Excess antibody was removed by rinsing for three 10 min periods in TBST solution. Mouse (c-myc, actin, La) or rabbit (PTB) horseradish peroxidase conjugated secondary antibodies, diluted 1:2000 in 5 % milk TBST, were applied to the membranes for a period of 30-45 mins at room temperature, again with constant agitation. Membranes were then thoroughly rinsed for at least three 15-20 min periods in TBST solution. Protein-antibody complexes were detected using an enhanced chemiluminescence (ECL) technique according to the manufacturer’s instructions (Amersham) and visualised after exposure to Fuji RX X-ray film for periods of between 10 s and 30 mins.

2.8.11 Stripping and re-probing of western blots
Nitrocellulose membranes were stripped of existing protein-antibody interactions by incubation in a solution of 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 for 10 mins at 50°C. Membranes were then washed in TBST and re-probed with a different antibody as described above (Section 2.8.10).

2.8.12 Electrophoretic mobility shift assays (EMSAs)
Approximately 8.6x10^-8 nmoles labelled transcript (23,000cpm) (Section 2.7.4) were incubated in a 10 µl buffer mix containing 5 µl 5x transcription buffer (200 mM Tris-HCl pH 8.0, 40 mM MgCl2, 10 mM spermidine, 250 mM NaCl), 0.75 µl DTT (1M), 1.5 µl tRNA (10 mg/ml), 1 µl rATP (10 mM), 1 µl glycerol and 0.17 µl vanadyl ribonucleoside complexes (10 mM). Nuclear free cell extracts were diluted in 0.1% DEPC treated sterile,
distilled water to a final volume of 20 µl and then incubated with the transcript mix at room temperature for 10 mins. 3 µl 10×TBE loading buffer was added and samples were loaded directly onto 0.7% agarose gels made with 1×TBE filter sterilised buffer. Samples were then electrophoresed at 90 V for 2 hours in 1×TBE filter sterilised buffer. Gels were dried under vacuum at 60°C for 2 hours and exposed to Fuji-RX X-ray film at -70°C for 4-20 hours. Competition assays were performed as described above except that unlabelled RNA transcripts were added to nuclear free cell extract mixes prior to addition of the radiolabelled transcript mix.

2.8.13 Protein isolation from electrophoretic mobility shift assays (EMSAs)

An electrophoretic mobility shift assay was performed as described above but scaled up by a factor of 40. The whole sample was then loaded onto a preparative 0.7% agarose gel (1×TBE filter sterilised buffer) and electrophoresed at 90 V for 2 hours in 1×TBE filter sterilised buffer. Gels were then cut into 6 horizontal slices which were individually placed in dialysis tubing containing a minimal volume of 1×TBE. Proteins were electroeluted from the gel slices for 1 hour at 150 V. After reversing the current for 1 min proteins were recovered by precipitation with 10% TCA and centrifugation at 13,000 rpm for 10 mins. Pellets were washed 3 times in acetone and finally resuspended in 200 µl 1×SDS sample buffer prior to being boiled and loaded on to either 7.5% or 10% SDS-polyacrylamide gels.

2.8.14 UV cross-linking

5-10×10⁴ cpm of radiolabelled RNA transcript (Section 2.7.4) was incubated with 70 µg of CE in a 30 µl buffer mix containing 5 µl 5× transcription buffer (200 mM Tris-HCl pH 8.0, 40 mM MgCl₂, 10 mM spermidine, 250 mM NaCl), 0.75 µl DTT (1M), 1.5 µl tRNA (10 mg/ml), 1 µl rATP (10 mM), 1 µl glycerol and 0.1% DEPC treated sterile, distilled water for 10-15 mins at room temperature in a 96 well V-bottomed plate. Samples were then irradiated on ice for a period of 60 s using a short wave (254nm) UV-P Spot-Cure high intensity UV light source (800W/cm²) (UV Products Ltd, Cambridge, UK), at a distance of 8mm. 50 units of RNase T1 or 0.5 µg of RNase A was added to each of the samples which were then incubated for 20 mins at room temperature to allow degradation of any unprotected RNA species. An equal volume of 2× SDS sample buffer was added to the samples which were then heated for 5 mins at 90°C prior to separation by SDS-PAGE (10% or 7.5% gels). Gels were then stained using Coomassie, dried and the results visualised after exposure to Fuji RX X-ray film at -70°C for 40-160 hours.

2.8.15 In vitro translation

In vitro translation reactions were performed using either a standard rabbit reticulocyte lysate system (Promega) or a Flexi rabbit reticulocyte lysate system (Promega) according
to the manufacturer's recommendations. Briefly, transcripts were synthesised from
linearised vectors as described above (Section 2.7.4), passed through a G-50 Sephadex
column, extracted with phenol and chloroform, ethanol precipitated and then resuspended
in 15 µl of 0.1% DEPC treated sterile, distilled water. Capped transcripts were synthesised
in the same manner but in vitro transcription reactions were carried out in the presence of
7-methylguanosine triphosphoguanosine (mGpppG) (1 mM). RNA concentrations were
determined from A260nm values. A standard in vitro translation reaction was incubated at
30°C for 60-90 mins and contained 8.25 µl rabbit reticulocyte lysate, 0.25 µl amino acid
mixture lacking methionine (1 mM), 0.5 µl of 35S-methionine (1,200 Ci/mmol), 0.25 µl
RNasin ribonuclease inhibitor, RNA to a final concentration of 50 µg/ml made up to a total
volume of 12.5 µl with 0.1% DEPC treated water. Additionally, reactions carried out in
the Flexi rabbit reticulocyte lysate system were supplemented with 0.25 µl DTT (1 M) and
variable amounts of 2.5 M KCl to give final concentrations of between 0-150 mM KCl.
An equal volume of 2xSDS sample buffer was then added to the samples prior to
separation by SDS-PAGE (10% or 20% gels). Gels were then stained using Coomassie
and incubated in Amplify™ solution (Amersham) for 30 mins to increase the sensitivity
of detection. Gels were dried for 2 hours at 80°C and the results visualised after
fluorography, at -70°C, after exposure to Kodak XAR-5 film for periods of 16-40 hours.
CHAPTER 3

Characterisation and isolation of multiple myeloma cell lines
Characterisation and isolation of multiple myeloma cell lines

3.1 Introduction

The virtual confinement of the neoplastic clone in multiple myeloma to the bone marrow environment, often making the disease elusive to detection, has also precluded significant in vivo study of this malignancy. Therefore, to address this problem and permit further study in vitro, much effort has been employed in establishing human multiple myeloma cell lines (HMCLs). However, to date, although a wide variety of techniques have been utilised, no consistent highly reproducible clonogenic assay for multiple myeloma exists (reviewed in Barker et al., 1993). Indeed, many HMCLs are in fact lymphoblastoid cell lines (LCL) derived from MM patients as a result of Epstein-Barr virus (EBV) immortalisation of non-malignant B-cells and thus do not represent true malignant clones (Pellat-Deceunynk et al., 1995).

Several of the existing HMCLs have been in culture for between 5-30 years and accumulating numbers of phenotypic and genetic alterations will undoubtedly have been sustained within such a period. Characteristics observed in vitro may therefore not be legitimate representations of the in vivo situation. This is highlighted by studies examining oncogenic mutation frequencies which were found to vary considerably between freshly isolated patient material and established HMCL samples (Section 1.2.9) (Corradini et al., 1993; Portier et al., 1992; Neri et al., 1989). Furthermore, the results obtained from patient samples were also influenced by the disease stage emphasising the multifactorial nature of malignant transformation and the continual evolution of the neoplastic clone (Neri et al., 1993; Portier et al., 1992). Consequently, as the majority of HMCLs have been established from extra-medullary plasma cell proliferations, which are exclusively associated with terminal stages of the disease, oncogenic alterations observed from in vitro studies will encompass not only initiating events in myelomagenesis but also mutations associated with progression of the disease.

To eliminate, or at least substantially reduce all of the aforementioned variables, it was therefore decided to isolate and grow in vitro myeloma cells freshly explanted from MM patients. Accordingly, both peripheral blood and bone marrow aspirates obtained from MM patients were cultured in vitro in attempts to isolate new MM cell lines more typical of the in vivo state. In addition, existing MM cell lines were characterised for two cell surface antigens, CD38 and CD45 (Section 1.2.6). These antigens have previously been used to identify plasma cells (Billadeau et al., 1996; Witzig et al., 1996) and although not specific for myeloma cells, in combination, would be expected to confirm the myelomatous origin of the cell lines.
3.2 Results

3.2.1 Characterisation of human multiple myeloma cell lines

Expression of the cell surface antigens CD38 and CD45 in the multiple myeloma cell lines (GM2132, GM1311, GM1500, GM6923, U266) and the control cell lines (GM0892A, GM1953, GM03201, HL60, Colo320) was examined using directly conjugated fluorescently labelled antibodies raised against these two antigens. Green fluorescence was monitored for the CD38 antibody, which was conjugated to fluorescein isothiocyanate (FITC), whereas red fluorescence was monitored for the CD45 antibody due to the presence of a phycoerythrin (PE) fluorophore. CD38 and CD45 expression were visually analysed by immunofluorescence microscopy and quantitatively determined using the flow cytometric technique of fluorescent activated cell sorting (FACS). This latter method distinguishes cells on the basis of their light scattering and fluorescent properties thus allowing an accurate determination of both the number of cells expressing an antigen (percentage of positive cells) and the level of expression of that antigen (mean fluorescence intensity {mfi}) to be monitored.

Typical FACS profiles of CD45 expression and CD38 expression are shown in Figures 3.1 and 3.2. Such profiles were then subjected to statistical analysis using Lysis II software (Becton Dickinson) to calculate mfi values and the percentage of cells positive for a particular antigen. At least three independent experiments were performed and the average values obtained for each of the cell lines were calculated and are summarised in Table 3.1.

CD45 expression was found to vary considerably in both the control and MM cell lines but, as predicted, expression was confined to cells of haematopoietic origin with negligible CD45 expression levels being observed in the colon adenocarcinoma cell line Colo320. In the remaining four control cell lines 95-100% of cells displayed CD45 but expression levels varied from very low in the HL60 leukaemic cell line (mfi = 10.4) to very high in the GM03201 lymphoblastoid cell line (mfi = 61.1). As CD45 expression levels alter on differentiation this may reflect the different maturation states of these cell types. The same was found to be true for the MM cell lines in which CD45 expression was seen to range from negligible in the GM2132 cell line (mfi = 1.3), indicative of a fully differentiated myeloma plasma cell type to intermediate in the GM1311, GM1500, GM6923 and U266 cell lines indicative of more immature myelomatous cells.

The majority of cells from the MM cell lines GM2132, GM1311 and GM6923 were found to express CD38 (approximately 99%) although the levels differed from very strong
Figure 3.1: FACS profiles of CD45 expression. (x-axis: log fluorescence intensity, y-axis: number of events). Red peaks indicate background fluorescence obtained using a non-specific antibody conjugated with PE. Open peaks indicate the fluorescence obtained using a CD45 antibody conjugated with PE. GM2132, GM1311, GM1500, GM6923 and U266 are MM cell lines. GM1953, GM03201, GM0892A, HL60 and Colo320 are control cell lines.
Figure 3.2: FACS profiles of CD38 expression. (x-axis: log fluorescence intensity, y-axis: number of events). Red peaks indicate background fluorescence obtained using a non-specific antibody conjugated with FITC. Open peaks indicate the fluorescence obtained using a CD38 antibody conjugated with FITC. GM2132, GM1311, GM1500, GM6923 and U266 are MM cell lines. GM1953, GM03201, GM0892A, HL60 and Colo320 are control cell lines.
<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>CD45</th>
<th>CD38</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% +ve</td>
<td>mfi</td>
</tr>
<tr>
<td><strong>Multiple Myeloma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM2132</td>
<td>10.4</td>
<td>1.3</td>
</tr>
<tr>
<td>GM1311</td>
<td>99.7</td>
<td>42.1</td>
</tr>
<tr>
<td>GM1500</td>
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<tr>
<td>GM6923</td>
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</tr>
<tr>
<td>U266</td>
<td>66.9</td>
<td>21.0</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM1953</td>
<td>96.4</td>
<td>32.0</td>
</tr>
<tr>
<td>GM03201</td>
<td>100.0</td>
<td>61.1</td>
</tr>
<tr>
<td>GM0892A</td>
<td>95.6</td>
<td>38.2</td>
</tr>
<tr>
<td>HL60</td>
<td>94.7</td>
<td>10.4</td>
</tr>
<tr>
<td>Colo320</td>
<td>4.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Table 3.1**: Summary of FACS data obtained for the MM and control cell lines. Average values calculated from at least three independent experiments are shown. The percentage of the total number of cells positive for a particular antigen is indicated (% +ve). The level of expression is given by the mean fluorescence intensity value (mfi).
expression in the GM2132 cell line (mfi = 101.1) to weaker expression, comparable to that observed in two of the lymphoblastoid control cell lines, for the GM1311 cell line (mfi = 38.5). Only around 60% of the GM1500 cells expressed CD38 and the degree of expression of this antigen was also significantly reduced (mfi = 15.1). However, the most marked anomaly was observed in the U266 MM cell line in which less than 4% of cells expressed the CD38 antigen and expression levels were minimal (mfi = 0.4). Indeed, these values were comparable to the control adenocarcinoma cell line Colo320 which, due to its non B-cell origin displayed negligible CD38 expression. Conversely, the other control cell lines, all of lymphoid origin, expressed CD38, although the number of cells and their degree of CD38 expression again varied considerably, possibly reflecting the different maturation states of these cells. However, the different CD38 levels did not parallel the maturation state indicated from CD45 expression implying that the phenotypic expression of either one or both of these cell surface antigens may be affected by the immortalised nature of these cells.

The lack of CD38 expression in the U266 MM cell line was also clearly evident upon microscopical analysis by immunofluorescence (Figure 3.3). This would tend to imply that this cell line, one of the first HMCLs to be established in vitro (Nilsson et al., 1970), may have altered significantly during growth in vitro. Indeed, two forms of this cell line, pre-1984 and post 1984 with different growth characteristics have been reported (Hellman et al., 1988). Other studies have also noted disparities in the U266 cell line when compared to other HMCLs (Jernberg-Wiklund et al., 1992) which, taken together with the data presented above, would suggest that the U266 is an atypical MM cell line.

Another feature observed from immunofluorescent studies which was not discernible from FACS analysis was that in some of the MM cell lines, particularly evident in the GM2132 and GM1311 cell lines, CD38 staining of cells was not uniform, with large cells expressing high levels of CD38 and smaller cells displaying no CD38 expression. These smaller cells could represent an immature stage of myeloma cell development but as high levels of CD38 expression are normally also associated with early B-cell states and CD38 is widely expressed on B-lymphocytes this is unlikely. An alternative explanation is that these MM cell lines are non-clonal consisting of a mixed population of myeloma cells and accessory cells which are a prerequisite in propagation of the myeloma clone. These non-adherent cells could potentially expedite myeloma cell growth in vitro replacing the functions normally fulfilled by bone-marrow stromal cells.
Figure 3.3: Immunofluorescence analysis of CD38 expression. Cells were incubated with FITC conjugated CD38 antibody and then examined by fluorescence microscopy. (A) GM2132 MM cell line (x400). (B) GM1311 MM cell line (x200). (C) U266 MM cell line (x400). (D) GM6923 MM cell line (x200). (E) GM1953 control lymphoblastoid cell line (x200). (F) GM0892A control lymphoblastoid cell line (x200).
3.2.2 Isolation and characterisation of myeloma cells from MM patients

A total of 14 peripheral blood (PB) samples and 4 bone marrow (BM) aspirates were obtained from a total of 15 multiple myeloma patients from Leicester Royal Infirmary (Table 3.2). All presented different stages of the disease according to Salmon Durie classification (Durie & Salmon, 1975) and a number had been subject to a variety of diverse therapies (data restricted). A small specimen was taken from each patient sample at this stage and microscopically examined after Giemsa staining (Figure 3.4). The sample was then layered on to a Ficoll paque gradient, permitting separation of mononuclear cells, which after a further purification step on a similar gradient, were subsequently cultured in vitro. Morphological examination of a small proportion of this preparation was also carried out (Figure 3.4).

Prior to separation myeloma plasma cell types constituted between 0.4-1.0 % of cells in BM aspirates and were generally not observed in PB samples. These figures however increased to between 10-25 % in BM samples and 1-6 % in PB samples after mononuclear cell preparation.

3.2.3 In vitro growth of myeloma cells

Mononuclear cells isolated from MM patients were plated at different dilutions (1.7x10^5 cells/ml - 0.5x10^1 cells/ml) into semi-solid methylcellulose based medium. This provided a viscous environment more akin to the natural bone marrow milieu but also maintained the separation of clonal populations facilitating their subculture. The culture conditions utilised were modified from a method previously used for pluripotent haematopoietic cell growth (Takahashi et al., 1985). Addition of human plasma in these experiments however was found to specifically promote myeloma cell growth presumably by providing human haematopoietic growth factors absent in foetal calf serum (Takahashi et al., 1985) and accordingly the media was supplemented with human plasma. β-mercaptoethanol was also added as this has been shown to promote the development of B-cell colonies in vitro (Whitlock & Witte, 1982).

Both peripheral blood and bone marrow samples, in every case, gave rise to a spontaneous cell proliferation within a period of 6-14 days. However, the predominant cell types observed differed between PB and BM samples at high cell concentrations (>2x10^4 cells/ml). At lower concentrations (< 2x10^4 cells/ml) very small cell aggregates were observed and appeared identical from both sources (Figure 3.5). These aggregates were visible from around day 10 and persisted propagating in culture until around day 45 when their numbers began to decrease. Their widespread distribution throughout the
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Source of myeloma cells</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>Peripheral blood</td>
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<td>5</td>
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<td>6</td>
<td>M</td>
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<tr>
<td>7</td>
<td>F</td>
<td>Peripheral blood</td>
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<tr>
<td>8</td>
<td>M</td>
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<td>M</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>Bone marrow</td>
</tr>
</tbody>
</table>

**Table 3.2**: Summary of MM patient samples obtained from Leicester Royal Infirmary (courtesy of Dr Chapman).
Figure 3.4: Giemsa stains of MM patient samples. (A) Peripheral blood sample prior to mononuclear cell isolation. (B) Bone marrow aspirate sample prior to mononuclear cell isolation. (C) Peripheral blood sample after mononuclear cell isolation. (D) Bone marrow aspirate sample after mononuclear cell isolation. (x200 magnification).
Figure 3.5: *In vitro* growth of bone marrow and peripheral blood mononuclear cell preparations seeded at concentrations of $< 2 	imes 10^4$ cells/ml (x 100 magnification).
haematopoietic system, combined with their small size and high proliferative capacity would strongly imply that these cells therefore represent lymphocytes. Correspondingly, these cells were unresponsive to exogenous interleukin-6 (IL-6), a critical cytokine for myeloma plasma cell growth and hence subculturing of these cell types was not pursued.

Peripheral blood samples seeded at higher concentrations (>2×10⁴ cells/ml) gave rise to a mixed cell population within 2 weeks (Figure 3.6). Many cell types including macrophage, myeloid cells, lymphocytes and myeloma plasma cells were discernible, however the prevalence of mixed colonies arising from pluripotent precursor cells prevented the unequivocal identification of many cell types (Figure 3.6; Panel A). Confirmation of cell identity would require the removal of these cell types from the culture environment followed by microscopic examination after appropriate cytochemical staining.

Individual myeloma colonies from the peripheral blood cultures were subcultured into liquid RPMI-1640 media supplemented with IL-6 at approximately 20 day intervals. This resulted in some growth stimulation, allowing propagation of malignant plasma cell types in vitro up to 80 days (Figure 3.6; Panel B). However, throughout this period the population remained mixed. Elevated levels of generalised cell death were observed but with successive subdivisions the proportion of myeloma plasma cells (verified by immunofluorescence of λ/k light chains) increased. There are several possible reasons for this. Firstly, the malignant plasma cell types were longer lived than myeloid haematopoietic cells which began to assume a granular appearance and started dying after about 35 days in culture (Figure 3.6; Panel C). This could indicate that either these myeloid cells were unresponsive to IL-6 or because of their terminally differentiated nature possess a finite lifespan. Accordingly, IL-6 could therefore be functioning to stimulate the proliferation of plasma cell types or prevent cell death induced by apoptosis. However, the failure of these malignant plasma cells to persist in vitro for longer than 80 days, combined with the fact that the population remained mixed on subdivisions, would disfavour the aforementioned roles of IL-6 in myeloma cell growth and instead support the hypothesis that IL-6 promotes the differentiation of precursor cell types. This is also consistent with the fact that only small numbers of malignant plasma cell types existed in the original mononuclear cell preparations.

Growth in bone marrow cultures elicited a very different morphology consisting of a stromal cell layer on which large macrophage and smaller myeloma plasma cell types, distinguishable by their eccentric nuclei, were clustered (Figure 3.6; Panel D). This adherent cell network remained visible for approximately 40-50 days and within this period was endowed with a high regenerative capacity upon disturbance or division. Myeloma plasma cells tightly adhered to this network impeding subculturing of individual
Figure 3.6: *In vitro* growth of mononuclear cell preparations seeded at concentrations of $>2 \times 10^4$ cells/ml. Panels A - C are peripheral blood samples. Panels D - F are bone marrow samples.

(A) 14 - 20 day culture; mixed colonies prevailed some of which contained myeloma plasma cell types (x 400).

(B) 25 - 30 day subculture supplemented with IL-6; many cell types were observed but increased numbers of small round myeloma plasma cells were visible (x 100).

(C) 35 - 80 day subculture; larger myeloid cells became granular in appearance and died leaving small clusters of myeloma plasma cell types (x 100).

(D) 14 - 20 day culture; stromal cells on which myeloma plasma cells and larger macrophage were clustered were visible (x 400).

(E) 25 - 30 day subculture supplemented with IL-6; stromal cells rapidly regenerated and a massive proliferation of myeloma plasma cells was observed (x 100).

(F) 70 - 140 day subculture; stromal cells were no longer visible but a 3-D network consisting of malignant plasma cell types and extracellular matrix components remained slowly growing in culture (x 100).
plasma cells. Indeed, plasma cells that became detached from the stromal cell network displayed no proliferative activity and died.

Subculturing into liquid RPMI-1640 media was again performed at 20 day intervals, although cultures were only supplemented with IL-6 upon the first division. This however resulted in a massive proliferation of plasma cell types upon the stromal cell layer (Figure 3.6; Panel E). Small areas of red colouration within the media, arising from haemoglobin breakdown, also indicated the presence of haemocytes in these cultures. Upon subsequent divisions however these cells were lost with myeloma plasma cell types predominating. The division potential of these malignant cells was reduced with successive subdivisions but small aggregates containing 10-20 myeloma plasma cells were observed between 40-70 days. These small myeloma colonies appeared more widely dispersed due to the absence of the stromal cells, but remained attached to an invisible support probably constructed from extracellular matrix components. Larger cell types independent of the myeloma plasma cell clumps were also seen. Further attempts to isolate malignant plasma cell types from within these structures proved unfruitful, succeeding only in detaching the extracellular matrix component from the culture dish (Figure 3.6; Panel F). Malignant plasma cell types within this three dimensional matrix of cells and extracellular matrix components continued to proliferate, albeit slowly, and were maintained in vitro for a period of 5 months.

3.2.4 CD38 enrichment of peripheral blood samples

Although the above results clearly demonstrated the superior nature of bone marrow aspirates in the in vitro growth of malignant plasma cell types, primarily due to the elevated numbers of myeloma plasma cells from the original preparation and the presence of the bone marrow stromal cells, peripheral blood samples were more attainable. It was therefore decided to adapt the method used for growth of PB samples by specifically selecting mononuclear cells expressing CD38 in an attempt to reduce the heterogeneity of the cell types observed. Accordingly, using a flow cytometric technique and antibodies directed against CD38 a PB mononuclear cell preparation enriched in CD38 expressing cells was isolated and cultured in vitro at concentrations of 3x10^4 cells/ml. The results are shown in Figure 3.7.

CD38 enrichment of PB samples visibly reduced the heterogeneity of the cell types present and proliferation of malignant plasma cell types was observed within 4-10 days of in vitro culture. This was a considerably shorter period than previously seen but with successive divisions this augmented proliferative potential diminished suggesting that the observed stimulatory effect was possibly incurred as a direct result of antibody
Figure 3.7: *In vitro* growth of peripheral blood mononuclear cell preparations enriched for CD38 expressing cells. (A) 4 - 10 day culture; the population was fairly heterogeneous with myeloma plasma cell types predominating (x 100). (B) 14 - 28 day culture; small clusters of 6 - 14 malignant plasma cells were seen (x 100). (C) Myeloma plasma cell cluster magnified (x 400).
conjugation. After approximately 14 days small aggregates and linear arrays of myeloma plasma cell types were observed (Figure 3.7; Panels B & C). These persisted in culture for around 28 days but no further growth stimulation was observed implying that other factors are necessary for malignant plasma cell growth in vitro.

3.2.5 Growth of peripheral blood samples on bone marrow stromal cell lines

One possible requirement absent from peripheral blood samples which was demonstrated to be important in malignant plasma cell growth from BM samples was the stromal cell network. These cells essentially provide two functions which stimulate the proliferation of myeloma plasma cells. Firstly they produce cytokines, such as IL-6 and secondly they permit intercellular adhesion interactions. Indeed, the significance of the latter, resulting in the abrogation of myeloma plasma cell growth was observed when malignant plasma cell types in BM cultures were isolated from the stromal cell layer. Similar phenomenon have been reported previously whereby separation of haematopoietic progenitor cells from bone marrow stromal cells (BMSCs) by thin agar resulted in a decline in the number of primitive cells present in the culture (Kierney & Dorshkind, 1987). Furthermore, treatment of progenitor cells with conditioned media harvested from long term bone marrow cultures was found to be insufficient to stimulate cell growth implying that cell to cell contact is required for in vitro proliferation (Shadduck et al., 1983). Adherence between BMSCs and plasma cells has also been shown to be an important factor in immunoglobulin secretion (Roldan & Brieva, 1991). PB mononuclear cells seeded at concentrations of approximately 3x10^4 cells/ml were therefore co-cultured with immortalised bone marrow stromal cell lines CRL7541 and CRL7544 established from MM patients (Figure 3.8).

Within 3 days of co-culture small clusters of cells were observed to associate with the BMSCs. However it was not clear if myeloma plasma cell proliferation occurred, as after 4-6 days a general cell necrosis was observed. The reasons for this are not clear but may have been due to the vastly different growth rates or immunological incompatibilities of the cell types involved.
**Figure 3.8**: *In vitro* growth of mononuclear cell preparations from peripheral blood samples cultured on the immortalised MM bone marrow stromal cell line CRL7541. (A) Bone marrow stromal cell line CRL7541 (x100). (B) 3 day co-culture of CRL7541 stromal cell line with a mononuclear cell preparation from a peripheral blood sample (x100).
3.3 Summary

FACS analysis examining CD38 and CD45 cell surface antigen expression confirmed the nature of all the multiple myeloma cell lines, with the exception of U266. This cell line has altered significantly upon *in vitro* growth and disparities in this cell line when compared to other HMCLs have previously been noted (Jernberg-Wiklund *et al.*, 1992). Thus, the U266 cell line appears to be an atypical MM cell line. FACS data was also utilised to select GM2132 and GM03201 as the most representative multiple myeloma and control lymphoblastoid cell lines respectively, on which subsequent work was focused (Chapters 5 & 6).

Immunofluorescence studies examining CD38 expression revealed that the MM cell lines, particularly GM2132 and GM1311, consisted of a mixed population of large CD38 positive cells and smaller CD38 negative cells. This would tend to imply that these cell lines are non-clonal containing myeloma cells and accessory cells. It could therefore be postulated that these non-myeloma cells may play a substitute role for the bone marrow stromal cells and expedite myeloma cell growth *in vitro*. This is also consistent with the fact that the majority of HMCLs have been established from extra-medullary plasmacytomas eg. pleural fluid or ascites fluid. Extravasation of the myeloma cells from the bone marrow would thus require evasion of the mandatory stromal/myeloma cell to cell contacts, necessary for their early development, and then re-localisation of myeloma cells to other remote sites. Additional cell types may therefore cooperate in either or both of these processes and provide conditions conducive to myeloma cell growth.

*In vitro* cell growth of myeloma cells was achieved for a period of 80 days in samples obtained from peripheral blood. However the population remained mixed even after subculturing of "myeloma" colonies in the presence of IL-6 and further growth stimulation by the addition of exogenous IL-6 was not achieved. This was probably because the initial mononuclear cell preparation contained only a small proportion of malignant plasma cell types (< 6%) and IL-6 was not primarily functioning to induce their proliferation but rather to promote B-cell differentiation and prevent apoptosis of these myeloma plasma cell types. This is also consistent with the fact that with increased time in culture cell numbers would decline due to the finite lifespan and terminally differentiated nature of the majority of peripheral blood cell types. This would be offset by increasing numbers of other cell types due to the IL-6 induced maturation of precursor cells however, as only a limited number of such progenitors would be present the effects would not be sustained indefinitely as observed.
To reduce the heterogeneity of the cell types observed in the PB samples mononuclear cell preparations were sorted into CD38 positive and CD38 negative populations using a flow cytometric technique. PB cultures enriched for CD38 cells displayed a marked reduction in cell heterogeneity and myeloma plasma cell growth was greatly favoured. High division rates of the malignant plasma cell types initially observed were not sustained suggesting that this arose as a direct result of antibody ligation. Furthermore, the proliferative capacity of these myeloma plasma cells ceased after approximately 30 days in culture implying that additional factors were required for growth. One possible factor which was absent was stromal cell contacts, however, attempts to grow myeloma cells from PB samples on immortalised BMSCs proved unsuccessful probably due to incompatibilities of the cell types involved.

Growth of malignant plasma cell types in culture from BM samples was more productive with cells being maintained in vitro for up to 5 months, without the requirements of exogenous cytokines or conditioned media. This is considerably longer than has previously been achieved using similar methods and sources of material possibly by the inclusion of human plasma and β-mercaptoethanol which both appear to promote B-cell growth. Moreover, prolific proliferation of myeloma plasma cell types was obtained after treatment with exogenous IL-6, confirming the importance of this cytokine in the furtherance of the malignant clone. Bone marrow stromal cells in these cultures were also found to play a critical role in myelomagenesis as abrogation of plasma cell proliferation was observed if these cells were detached from the stromal cell layer. This is consistent with the findings of previous studies and reinforces the vital nature of stromal / myeloma cell to cell contacts in myeloma cell growth.

In conclusion, although no new MM cell lines were obtained, in vitro culture was achieved and allowed sufficient expansion of the malignant plasma cell clone which would greatly facilitate further study. Previously this would not have been possible due to limited cell numbers. By further refinement of the growth conditions eg. CD38 enrichment combined with IL-6 treatment it should be possible to produce MM cell lines from BM aspirates from MM patients, independent of the disease state and therefore more representative of the in vivo situation.
CHAPTER 4

Sequence analysis of c-myc exon 1 from control and multiple myeloma cell lines
Sequence analysis of \textit{c-myc} exon 1 from control and multiple myeloma cell lines

4.1 Introduction

From an early stage \textit{c-myc} exon 1 was postulated to control the translational efficiency of its cognate mRNA (Saito \textit{et al.}, 1983). Several lines of evidence support this hypothesis. Firstly, the extensive size of the untranslated exon 1 region of \textit{c-myc} coupled to the high degree of nucleotide sequence conservation observed between species implied that it had an important function. Moreover, breakpoints within exon 1, detected in 70\% of murine plasmacytomas and 50\% of human lymphomas, are also associated with deregulated \textit{c-myc} expression. Its involvement in regulating translation was confirmed when the 5'UTR fused to reporter constructs was found to repress translation (Darveau \textit{et al.}, 1985). This inhibitory effect was mapped to within a 240 nucleotide sequence capable of preventing translation of heterologous genes in certain \textit{in vivo} and \textit{in vitro} systems (Parkin \textit{et al.}, 1988).

This correlation of exon 1 involvement in regulating \textit{c-myc} translational efficiency prompted the examination of this region in the multiple myeloma cell lines, to determine if an altered nucleotide sequence could be responsible for the observed increase in \textit{c-myc} translation. \textit{c-myc} exon 1 was therefore cloned and sequenced from five multiple myeloma cell lines and four control cell lines.

4.2 Results

4.2.1 Cloning \textit{c-myc} exon 1

Chromosomal DNA was prepared from each of the five MM cell lines (GM2132, GM1311, GM1500, U266, GM6923) and four control cell lines (GM03201, GM0892A, GM1953, HL60) and then amplified by the polymerase chain reaction (PCR) using primers specific for human \textit{c-myc} exon 1 (FP1/FP2). Both these primers were specifically designed with restriction sites in their 5' ends to facilitate further manipulation (\textit{BamHI} and \textit{EcoRI}). The plasmid pMC41, which contains a full length genomic clone of \textit{c-myc}, was also included as a positive control. In all cases a single 605 bp fragment, as predicted from the nucleotide sequence, was generated after PCR amplification (Figure 4.1). This indicated that none of the cell lines examined contained any gross structural abnormalities of the \textit{c-myc} exon 1 region.
Figure 4.1: PCR amplification of genomic cell line DNA with *c-myc* exon 1 specific primers (FP1 & FP2) produced a 605 bp product. M; 100 bp markers. Lanes 1-4; multiple myeloma cell lines; GM2132, GM1311, U266, GM6923. Lanes 5-8; control cell lines; GM1953, GM03201, GM0892A and HL60. Lanes 9 & 10; negative controls without DNA or oligonucleotide primers. Lane 11; pMC41 plasmid DNA was used as a positive control.
The PCR product from each reaction was purified using a glassmilk and NaI procedure (GeneClean II) and digested with the restriction enzymes BamHI and EcoRI. After a further purification the products were then ligated into M13mp18 or M13mp19, similarly digested with BamHI and EcoRI and purified, and used to transfect E.coli JM109. Transfectants were selected by disruption to the lac Z gene resulting in white plaques and verified for the presence of an insert by BamHI/EcoRI restriction digestion of "miniprep" DNA (Figure 4.2).

4.2.2 Sequencing c-myc exon 1

It was deemed necessary to sequence at least 4 clones from each cell line to eliminate sequence disparities arising simply due to inaccuracies of Taq DNA polymerase. Single-strand DNA was therefore prepared from positive clones containing a 597 bp insert and sequenced using a series of primers and T7 DNA polymerase (Figure 4.3). Utilisation of both M13mp18 and M13mp19 vectors enabled nucleotide data to be generated from both the coding and non-coding strands. This method thereby alleviated reading errors introduced by compressions as a consequence of the high GC content of the region.

A total of 46 clones derived from a number of separate PCR reactions were sequenced and compared to the published sequence (Entrez : HUMMYC). The deviations observed in the control cell lines and the MM cell lines are summarised in Tables 4.1 and 4.2 respectively. Their distribution within exon 1 is shown in Figure 4.4.

Sequence variations were observed in both the control and multiple myeloma cell lines. However, out of a total of 25.1 kb sequenced, only 12 nucleotide positions were different, giving a mutation frequency of 0.05%. This corroborates that the 5'UTR is subject to a series of restraints, implying it has an important underlying function. Most of the sequence differences found were randomly distributed throughout the exon apart from a clustering of base substitutions around positions 2675 and 2750 in the control cell lines and the MM cell lines respectively. These sequence alterations may therefore represent either random errors introduced by the PCR amplification process or reflect genuine polymorphisms within this region.

The most salient feature detected by sequencing was the prevalence of a C→T transition observed in all but one of the multiple myeloma cell lines (Figure 4.5). The absence of this sequence alteration in the U266 MM cell line however may be more than coincidental as this cell line has previously been shown not to express high c-myc levels (Jernberg-Wiklund et al., 1992). Combined with the FACS data obtained in Chapter 3 this
Figure 4.2: (A) Strategy used to clone *c-myc* exon 1. Briefly, the 605 bp *c-myc* exon 1 fragment generated by PCR amplification was digested with *BamHI* and *EcoRI* and cloned into either M13mp18 or M13mp19 similarly digested with *BamHI* and *EcoRI*. (B) Plasmid DNA was prepared from transfectants and digested with *BamHI* and *EcoRI* (Lanes 3, 5 & 7). Lanes 2, 4 & 6 contain undigested plasmid. Lanes 1 & 8 contain λ *HindIII* and 100 bp markers respectively. Any positive clones identified to contain a 597 bp fragment were subsequently sequenced.
Figure 4.3: Schematic diagram showing the distribution of primers used to sequence \emph{c-myc} exon 1 (not to scale).
<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>VECTOR</th>
<th>CLONE</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
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<td>GM1953</td>
<td>M13mp18</td>
<td>5-1</td>
<td>C → A 2673</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
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<td></td>
<td>M13mp19</td>
<td>5C</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>5D</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>5F</td>
<td>Wt</td>
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<td></td>
<td>M13mp19</td>
<td>6F</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>6G</td>
<td>Wt</td>
</tr>
<tr>
<td>GM0892A</td>
<td>M13mp18</td>
<td>7-1</td>
<td>C → T 2679</td>
</tr>
<tr>
<td></td>
<td>M13mp18</td>
<td>7-2</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>7A</td>
<td>C → T 2556</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>7B</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>7C</td>
<td>C → T 2796</td>
</tr>
<tr>
<td>HL60</td>
<td>M13mp18</td>
<td>8-1</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp18</td>
<td>8-2</td>
<td>A → G 2771</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>8F</td>
<td>Wt</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
<td>8G</td>
<td>Wt</td>
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<tr>
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<td>M13mp19</td>
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<td>pMC41</td>
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<td>C → T 2756</td>
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<tr>
<td></td>
<td>M13mp19</td>
<td>11A</td>
<td>C → T 2756 A → G 2677</td>
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<tr>
<td></td>
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<td>11B</td>
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<td></td>
<td>M13mp19</td>
<td>11C</td>
<td>C → T 2756</td>
</tr>
</tbody>
</table>

**Table 4.1**: Table summarising the positive clones isolated and sequenced from the four control cell lines; GM1953, GM03201, GM0892A, HL60 and the plasmid pMC41 which carries a genomic clone of c-myc.
<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>VECTOR</th>
<th>CLONE</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
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<td>1-1</td>
<td>C → T 2756</td>
</tr>
<tr>
<td></td>
<td>M13mp19</td>
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<td>T → C 2752</td>
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<tr>
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<td>M13mp19</td>
<td>1B</td>
<td>Wt</td>
</tr>
<tr>
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<td>M13mp19</td>
<td>1D</td>
<td>Wt</td>
</tr>
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<td>2-2</td>
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</tr>
<tr>
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<td>C → T 2756</td>
</tr>
<tr>
<td></td>
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<tr>
<td>U266</td>
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<td>M13mp19</td>
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</tr>
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<td></td>
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<td></td>
<td>M13mp19</td>
<td>12F</td>
<td>Wt</td>
</tr>
</tbody>
</table>

Table 4.2: Table summarising the positive clones isolated and sequenced from the five multiple myeloma cell lines; GM2132, GM1311, U266, GM6923 and GM1500.
| 1. all mts |  |  |  |  |  |  |  |  |  |  |  |
|------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| 20         | 80               | 100              | 120              | 140              | 160              | 180              | 200              | 220              | 240              | 260              |
| 2294       |                  |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| 2420       | 2500             | 2520             | 2540             | 2560             | 2600             | 2620             | 2640             | 2660             | 2680             | 2700             |
| 2720       | 2740             | 2760             | 2780             | 2800             | 2820             | 2840             | 2860             | 2880             | 2900             | 2920             |
| 2940       | 2960             | 2980             | 300              | 320              | 340              | 360              |                  |                  |                  |                  |
| 380        | 400              | 420              | 440              | 460              | 480              |                  |                  |                  |                  |                  |
| 2794       |                  |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| 2780       |                  |                  |                  |                  |                  |                  |                  |                  |                  |                  |

**Figure 4.4**: Diagram showing the distribution of the sequence alterations observed from sequencing *c-myc* exon 1. The upper sequence is that of human *c-myc* exon 1 obtained from Entrez (HUMMYC). The lower sequence shows the positions of all the deviations (multiple myeloma and control cell lines) which were obtained from sequencing analysis. (See also Tables 4.1 & 4.2).
Figure 4.5: Nucleotide sequence of four representative clones around position 2756 of c-myc exon 1. (A) M13mpl8 clones 2-2 (MM) and 7-2 (control) show the coding strand sequence. (B) M13mpl9 clones 2D (MM) and 7A (control) show the non-coding strand sequence. The conserved nucleotide substitution (C-T) observed at position 2756 in some multiple myeloma clones is evident.
information would tend to suggest that U266 is an atypical MM cell line. Hence use of this cell line was discontinued.

The mutation was also observed in control pMC41 plasmid, which was derived from a human foetal liver library (Watt et al., 1983b). The sequence from this genomic clone has previously been compared to a cDNA clone derived from a leukaemic cell line (K562) and the same sequence disparity was noted (Watt et al., 1983b). However, the significance in this case is not clear.

4.2.3 Expression of the C→T mutation

9 out of 18 myeloma clones displayed the C→T transition at position 2756 indicating that all of the cell lines were heterozygous for this mutation. Therefore, to determine if both alleles were expressed, using a reverse transcription PCR (RT-PCR) approach cDNA was prepared and cloned into M13mp19 BamHI/EcoRI by the same method as outlined above. Five clones from each cell line, obtained from a number of different PCR reactions, were examined to determine if they expressed a wild type (Wt) (2756:C) or mutant (Mt) (2756:T) sequence. The results are summarised in Table 4.3.

Consistent with the results obtained from genomic sequencing none of the control cell lines expressed the mutation. In both the genomic and cDNA sequencing as the clones were derived from a number of independent PCR reactions this would tend to confirm that the mutation observed in the MM cell lines is genuine and did not arise from cross-contamination from the positive control.

The mutant allele was detected only in two of the multiple myeloma cell lines, GM6923 and GM1500 with frequencies of 40% and 80% respectively. In these cases the detection of both alleles however indicated that allelic exclusion whereby the wild type allele is silenced, as often observed in Burkitt's lymphoma (Tachibana et al., 1993), did not occur. The lack of preferential expression of the mutant allele would also strongly support the view that the mutation does not directly affect transcriptional processes.

The absence of the mutation in the GM2132 and GM1311 MM cell lines would tend to imply that the mutant allele is not expressed. However, as only four clones from each cell line were examined it is possible that the mutation is expressed only at a very low frequency. Alternatively, as the FP1/FP2 primer pair used for RT-PCR amplification would detect transcripts originating only from the P0 promoter, differential promoter usage, particularly from either of the major promoters P1 or P2, could influence transcript
### Table 4.3: Summary detailing the number of clones isolated and sequenced from both genomic and cDNA samples. The number of wild type (Wt) or mutant (Mt) clones with regard to the sequence at position 2756 is also indicated. The proportion of clones that contain the mutant sequence is expressed as a percentage of the total number of clones isolated for a given cell line.

<table>
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<th>cDNA</th>
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<tr>
<td></td>
<td>Wt</td>
<td>Mt</td>
<td>%Mt</td>
<td>Wt</td>
<td>Mt</td>
<td>%Mt</td>
</tr>
<tr>
<td>Multiple Myeloma</td>
<td></td>
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<td></td>
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<td></td>
</tr>
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</tr>
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<td></td>
</tr>
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<td>3</td>
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<td>40</td>
</tr>
<tr>
<td>Control</td>
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</tr>
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<td>GM1953</td>
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<tr>
<td>pMC41</td>
<td>0</td>
<td>4</td>
<td>100</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>46</td>
<td></td>
<td></td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The proportion of clones that contain the mutant sequence is calculated as follows: 

\[
\text{Proportion of Mt} = \left( \frac{\text{Mt clones}}{\text{Total clones}} \right) \times 100\%
\]

For example, in the Multiple Myeloma GM2132, the proportion of mutant clones is calculated as: 

\[
\text{Proportion of Mt} = \left( \frac{1}{4+1} \right) \times 100\% = 20\%
\]
production and hence alter the apparent expression of the mutant allele in these two cell lines, although previous reports have observed enhanced transcription from the PO promoter in MM cells (Travis et al., 1994; Hoover et al., 1994). Indeed, this was one reason for using the FP1/FP2 primer pair. Another main reason for choosing the aforementioned primer pair was to eliminate possible transcriptional affects of the mutation. The rationale underlying this was the fact that as the PO promoter lies 982 bp upstream from the mutation effects on transcription were expected to be minimal, when compared to the P1 and P2 promoters which lie 266 and 428 bp upstream of the mutation respectively. Also as the P0 promoter accounts only for a small fraction of c-myc transcripts it would be possible to distinguish between allelic exclusion effects of the mutation as opposed to altered promoter usage specifically associated with production of the mutant allele. Clearly, as the mutation does not prevent transcription from the wild type allele the former of these is inapplicable. However, it does not rule out the possibility that the mutation could alter transcription from the two major promoters.

4.2.4 Screening for the mutant allele in MM cell lines

To further assess the expression of the mutant allele a screening procedure was devised by virtue of the fact that the mutation generates an additional Taq I restriction site (Figure 4.6). Genomic and cDNA was therefore amplified by PCR, using exon 1 specific primers (FP1/FP2) and subsequently digested with the restriction enzyme Taq I, following a glassmilk/Nal purification step. cDNA was also amplified using a FP2/FP2501 primer pair capable of yielding a 388 bp product detecting transcripts originating from all three promoters; PO, P1 and P2. Digest products were separated by agarose gel electrophoresis, blotted on to nitrocellulose membranes and probed using the radiolabelled oligonucleotide FP2610 to increase the sensitivity of detection. Three representative digests are shown in Figure 4.7.

Comparing the results of the genomic digest with those of the cDNA digest, amplified with the FP2/FP2501 primer pair, both the genomic and cDNA hybridisation patterns obtained were identical. This strongly supports that the mutation is non-effective at a transcriptional level although an alteration in promoter utilisation between P2 and P1, without a concomitant change in transcriptional activity, cannot be discounted.

The smaller fragment, characteristic of the mutation, was not detected in any of the four control cell lines confirming the data obtained from sequencing. However, this fragment was clearly detectable only in the GM6923 MM cell line and was not present in the other three MM lines; GM2132, GM1311 and GM1500. This result was surprising as although it parallels the lack of expression of the mutation obtained from cDNA sequencing in the
Figure 4.6: Taq I restriction digest pattern of c-myc exon 1 predicted from PCR amplification with either the FP1/FP2 or the FP2/FP2501 primer pairs. The C-T point mutation observed in the multiple myeloma cell lines served to create an additional Taq I restriction site and hence alter the observed restriction digest pattern.
Figure 4.7: Taq I restriction digestion of PCR amplified cell line genomic and cDNA samples blotted onto nitrocellulose and probed using the radiolabelled primer FP2610. (A) Genomic DNA amplified using the FP1/FP2 primer pair. (B) cDNA amplified using the FP1/FP2 primer pair. (C) cDNA amplified using the FP2/FP2501 primer pair. All lanes, except the first containing undigested control pMC41 plasmid, were treated with Taq I.
GM2132 and GM1311 cell lines in every other case the mutant allele was detected by sequencing. One possible explanation is that these MM cell lines are non-clonal, as discussed in Chapter 3.

PCR amplification of such a mixed cell population would give rise to c-myc sequences from both myeloma and non-myeloma cells. Thus, assuming the non-myeloma cells do not contain the mutation, coupled with the heterozygous nature of the myeloma cells, a disproportionate number of cells would appear wild type. Mutant sequences could still be detected by sequencing, albeit at lower frequency, however, restriction analysis with Taq I, giving rise to the characteristic smaller fragment, can occur only if both DNA strands annealed after the PCR reaction are the mutant sequence. For a heterozygous population this would contribute a maximal 25% of the total amount of DNA. In a non-clonal population therefore this frequency would be significantly reduced thus preventing detection of the mutation. Consistent with this, very faint mutant fragments were observed in all three of these MM cell lines when lanes were grossly overloaded (data not shown). Differences between individual PCR reactions are unlikely to account for this discrepancy as identical restriction patterns were obtained on several occasions.

Accordingly, this also implies that the GM6923 MM cell line is clonal and expresses the mutation. An alternative hypothesis is that any accessory cells could additionally carry the same point mutation. Morphological examination combined with the uniform immunofluorescent staining of cells with CD38 and CD45 antibodies (Section 3.2.1) however support that all the cells within this population are clonal and disfavour the possibility that the mutation originates from a non-myeloma accessory cell.

In the GM1500 cell line the mutation was detected in both genomic and cDNA clones from sequencing analysis. However the mutant fragment was not visible by restriction digest in the genomic samples or cDNA samples amplified by the FP2/FP2501 primer pair, but the 361 bp indicative of the mutation was observed in the cDNA digest amplified by the FP1/FP2 primer pair. This explains the apparent discrepancy in mutant expression observed between the cDNA sequencing and cDNA restriction digest (FP2/FP2501) and suggests that in this cell line there is enhanced utilisation of the PO promoter which is specifically associated with expression of the mutant allele. The mutation itself is unlikely to have caused this effect as this was not observed in any of the other MM cell lines.

It thus appears that in the GM1500 MM cell line an additional mutation, in the mutant allele, lying outwith the region sequenced, is specifically activating PO transcription. As transcription from PO is minimal this effect is masked when examining cDNA using primers which detect transcripts from all promoters. It follows therefore that although no expression of the mutant sequence was detected in the GM2132 and GM1311 MM cell
lines, if transcription from P1, which normally accounts for approximately 10-20% of transcripts, is enhanced, then again this may not be detected. However, as the mutation was found in all of these MM cell lines (GM2132, GM1311, GM1500, GM6923) from genomic sequencing but not detected in them all by the aforementioned screening method the sensitivity of this latter method for detecting expression of the mutant allele is questionable.

4.2.5 Screening for the mutant allele in patient material

Although the screening method outlined above did not detect the mutant allele in some of the MM cell lines it was decided to amplify genomic DNA (FP1/FP2 primer pair) isolated from either bone marrow from MM patients or peripheral blood from control patients and carry out a Taq I restriction digest. The results are shown in Figure 4.8.

The 361 bp fragment indicative of the mutation was not observed in any of the peripheral blood samples isolated from control patients (Figure 4.8; Panel A) but was detected in the GM6923 and pMC41 positive controls, indicating that the screening method can be employed to detect the mutation in some cases. This is further supported by the fact that the mutant fragment was observed in all three bone marrow samples isolated from MM patients (Figure 4.8; Panel B). However, although this data would strongly imply that the mutation may be widespread in MM, one negative control sample, PCR amplified in the same set of reactions, also contained the 361 bp fragment (data not shown). It therefore cannot be concluded with certainty that the mutation is associated with multiple myeloma.

The absence of the mutant fragment in the four patient control samples tentatively suggests that this C→T sequence alteration is not prevalent within the general population, although the insensitivity of the screening method used, as outlined above, could account for this result. However, if the mutation merely represented a polymorphism at this locus then individuals either heterozygous or homozygous for this particular marker would be anticipated amongst the general population. Accordingly, even though the control sample size is very small the probability of not detecting either genotype is remote. The C→T base substitution is therefore very unlikely to correspond to a polymorphism of the c-myc locus but instead reflect a bone fide mutation which may be associated with multiple myeloma.

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Figure 4.8: Taq I restriction digests of DNA samples PCR amplified using the FP1/FP2 primer pair, separated by agarose gel electrophoresis (2.0% agarose) and stained with ethidium bromide. (A) Peripheral blood samples isolated from control patients. (B) Bone marrow samples isolated from multiple myeloma patients. GM6923 DNA and pMC41 DNA were used as positive controls for the presence of the C-T point mutation. GM1953 DNA was used as a negative control. The position of the 361 bp fragment created by the C-T point mutation is indicated.
4.2.6 Effects of point mutations on MYCHEXI coding potential

c-myc exon 1 has the coding potential for a 188 amino acid polypeptide, MYCHEXI, of unknown function (Gazin et al., 1984). However, due to its upstream locality to the main open reading frame it was postulated that it could play a role in governing c-myc expression (Gazin et al., 1984). The effect on MYCHEXI coding potential of the mutations observed by genomic sequencing was therefore examined. The results are summarised in Table 4.4 and shown diagramatically in Figure 4.9.

In the control cell lines all five point mutations observed would change the coding potential of MYCHEXI. In three cases, which are all clustered together in a very proline rich segment, a proline residue is substituted by either a serine or threonine residue. Another case involves a change from an isoleucine residue to a valine and the final alteration would substitute a tryptophan aromatic amino acid in the normal place of a basic arginine residue.

Six of the seven point mutations detected in the MM cell lines would also serve to alter the MYCHEXI amino acid sequence. Two of these involve substitution of glycine residues by either serine or arginine and two involve substitution by proline of a leucine and a serine codon. The additional mutations which were observed in a single clone are an asparagine for a glycine change and replacement of threonine by alanine.

The effects that any of the aforementioned substitutions would have on MYCHEXI are not clear as the function of polypeptide is unknown. In most cases however the substitutions would be predicted to distort the polypeptide chain. The clustering of sequence alterations in the control cell lines around position 2675 were associated with replacement of cyclic amino acids with non-cyclic amino acids. The clustering of base substitutions in the MM cell lines around position 2750 were in contrast not associated with a particular distinguishing alteration in amino acid composition. Taken together this could imply that the region around 2675 reflects a polymorphic site whereas the site in the vicinity of position 2750 constitutes a mutational hotspot. It is equally plausible that the sequence alterations observed are errors introduced by PCR amplification and the effects on MYCHEXI coding potential are inconsequential. Evidence to support this comes from the fact that an additional mutation observed in the control pMC41 plasmid occurs at position 2677.

The consistent 2756 C→T point mutation observed in the MM cell lines was found to occur in the third base of a codon altering a TCC triplet to a TCT codon. Thus, the coding potential for a serine residue was retained by this conservative substitution. Accordingly,
<table>
<thead>
<tr>
<th>CELL LINE</th>
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<th>SEQUENCE</th>
<th>Amino Acid coding potential</th>
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<td></td>
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<td>2556 CGG→ TGG</td>
<td>Arg→ Trp</td>
</tr>
<tr>
<td></td>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
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</tr>
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<td>2449 GAT→ GGT</td>
<td>Asp→ Gly</td>
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<tr>
<td></td>
<td>4B</td>
<td>2745 ACG→ GCG</td>
<td>Thr→ Ala</td>
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<tr>
<td></td>
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</tr>
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<td>12B</td>
<td>2821 TCT→ CCT</td>
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<td>Ser→ Ser</td>
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<td>Ser→ Ser</td>
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<tr>
<td>GM1500</td>
<td>12E</td>
<td>2756 TCC→ TCT</td>
<td>Ser→ Ser</td>
</tr>
</tbody>
</table>

*Table 4.4*: Summary of sequence alterations observed in the control and multiple myeloma cell lines and the effects on the amino acid coding potential of MYCHEXI.
**Figure 4.9**: c-myc exon 1 sequence showing the amino acid coding potential of MYCHEXI. The upper amino acid sequence is the wild type protein sequence. The lower amino acid sequence shows the effects of the point mutations, observed from sequencing, on MYCHEXI coding potential. (See also Table 4.4).
it is highly improbable that MYCHEXI polypeptide is directly involved in deregulating c-myc translation.

4.2.7 Effect of the C→T mutation on secondary structure predictions

c-myc exon 1 is very GC rich (64%) and consequently predicted to be highly structured. No secondary structure data has been published however. It was therefore of interest not only to predict secondary structures of the c-myc 5'UTR but also to determine if the C→T point mutation was likely to affect the folding patterns. The MFold program (Zucker, 1989) was therefore applied to the c-myc wild type and mutant sequences from position 2289 - 2882 (1 - 593) (Mutation at position 467). This predicts the optimal and suboptimal secondary structures, within a specified energy increment, for an RNA molecule. The results obtained were analysed in the form of p-num plots and predicted secondary structure representations.

P-num plots

The p-num plot graphically presents the number of possible pairing partners (y-axis) for each nucleotide in a sequence (x-axis). Regions with no pairing partners (y=0) are therefore predicted to be single-stranded regions of RNA. Similarly, the secondary structure of regions with only one possible pairing partner can be reliably predicted but those with multiple potential base pairings are more problematic with many confirmations possible. P-num plots for both the wild type and mutant sequences are shown in Figure 4.10.

Both the mutant and wild type sequences have large numbers of potential base pairings for each nucleotide implying that the whole 5'UTR is likely to be found not as a linear RNA molecule but in higher order structures that are possibly dynamic in nature. The wild type sequence was predicted to have only one single stranded (ss) region whereas the mutant sequence was predicted to contain four ss regions. Examining the mutation site (467) in more detail it can be seen that the Mt-T base is capable of forming 15 potential pairings compared to 22 possible interactions predicted for the Wt-C nucleotide. From these results it therefore appears that this single nucleotide substitution would reduce the number of possible pairings and hence restrict the number of permitted RNA conformations by imposing a more defined structure on the mutant sequence.
Figure 4.10: P-num plot graphs obtained using Zucker's MFold program. The horizontal axis is the nucleotide sequence and the height on the vertical axis indicates the number of possible different pairing partners predicted by the program. (A) P-num plot of the Wt \(c\)-myc exon 1 sequence from nucleotides 2289-2882 (1-593). (B) As for A but the Mt sequence. (C) Magnification of the 467 region of the Wt sequence (467=C). (D) magnification of the 467 region of the Mt sequence (467=T). [Energy increment = 5.7 kcal/mol].
Secondary structure predictions

From the p-num plot, due to all the potential pairing partners for each nucleotide thousands of possible RNA structures could be generated. However, using the RNA fold program of Zucker it is possible to determine the optimal (lowest free energy) and suboptimal energies (lower free energies within a few percent of the minimum energy). For the wild type sequence the optimal folding energy was calculated to be -194 kcal/mol and that of the mutant was -194.4 kcal/mol. The default energy increment is 10.2 kcal/mol. The program therefore predicts any secondary structure within this range ie. for the Wt sequence between -204.2 and -194 kcal/mol and for the Mt sequence between -204.6 and -194.2 kcal/mol. This still generates many possible structures but only the most stable were examined. The results are shown in Figure 4.11.

In general if all the structures are compared it is possible to bisect the 5'UTR into two domains. A 5' domain from approximately nucleotides 1-365 and a 3' domain from nucleotides 366-593. In structures C, D and E this 3' domain is identical consisting of a basic "flower shaped" 7 stem loop structure. Similar stem loops of this region are also observed in structures A and B although the overall arrangement is different. The 5' domain of all of the structures is very similar consisting of a very long base paired stem which branches out at the top and is capable of forming a number of different conformations. This is calculated to have a free energy of approximately -130 kcal/mol. The high degree of conservation observed in this 5' region would imply that a limited number of folds are favoured.

For every pair of secondary structures examined the Mt sequence has a slightly lower free energy indicating that it forms a more stable structure. This is clearly seen in A2, C2, D2 and E2 where in the Wt the 467 nucleotide is found in an unpaired region but in the Mt an additional short stem loop is formed encompassing this site (-0.4 kcal/mol) (Figure 4.12). A similar increase in base pairing is seen if structures B1 and B2 are compared but in this case the mutation also serves to reduce the size of one stem loop and to increase the length of another. The mutation is therefore predicted to distort the local secondary structure but to have no repercussions on the overall RNA conformation.

4.3 Summary

c-myc exon 1 was sequenced from a total of 42 genomic and 33 cDNA clones derived from five MM cell lines and four control cell lines. Four of these MM cell lines were found to contain a conserved C→T point mutation at position 2756, however the
Figure 4.11: Secondary structure predictions of c-myc exon 1 obtained from the MFold program of Zucker (Zucker, 1989). A1-E1 represent predictions from the Wt sequence (467=C). A2-E2 represent predictions for the Mt sequence (467=T).
Figure 4.12: Detailed predicted secondary structure around position 2756 of c-myc exon 1. (A) Wild type sequence. (B) Mutant sequence.
frequency with which this was expressed varied considerably. The absence of this mutation in the U266 MM cell line may correlate with the fact that this cell line does not have high \textit{c-myc} levels (Jernberg-Wiklund \textit{et al.}, 1992). In addition the FACS data obtained in Chapter 3 would tend to support that this is an atypical MM cell line.

Preliminary examination using a relatively insensitive \textit{Taq I} restriction digest screening method demonstrated the presence of this mutation in the 3 MM patient samples examined. However, from the small numbers analysed and a false positive result it is not possible to conclude with any certainty that the C$\rightarrow$T mutation is associated with multiple myeloma. Notwithstanding, as this mutation was not observed in any of the control cell lines or four control patient samples this strongly suggests that the base alteration is not widespread amongst the general population and argues against this sequence deviation occurring as a result of \textit{in vitro} culture or merely reflecting a polymorphism of the \textit{c-myc} locus at this site. Moreover, all MM samples examined thus far have been heterozygous for the mutation which could imply that a homozygous mutation may be deleterious. However, this could be substantiated only by extensive examination of larger patient samples and verified by \textit{in vitro} studies.

The mutation was not found to alter the coding potential of MYCHEXI. Hence, the MYCHEXI polypeptide is very unlikely to be responsible for the increased translation observed in the MM cell lines. Correspondingly, this also discounts a direct involvement of MYCHEXI in regulating \textit{c-myc} translation.

c-\textit{myc} exon 1 is predicted to be highly structured with a free energy of approximately $-200$ kcal/mol. It thus would confer severe restrictions on the scanning mechanism of translation. However, it should be noted that the 5'UTR would not normally be found in isolation and could assume very different conformations \textit{in vivo} with other parts of the mRNA molecule. It would therefore be necessary to confirm the predicted secondary structures by nuclease mapping before any definitive conclusions could be drawn. In general, the 5'UTR can be bisected into a 5' and 3' domain, with the 5' end favouring a very long stable stem loop structure ($-129.8$ kcal/mol) and the 3' end forming a multiple stem loop structure. The C$\rightarrow$T point mutation is predicted to introduce an additional short stem loop structure into this 3' domain. However as this has an energy of $-0.4$ kcal/mol the effects on a scanning ribosome presumably would be negligible. Similarly, although the mutation affects the local secondary structure the overall conformation is minimally altered and the mutation \textit{per se} is therefore unlikely to be responsible for the observed increase in translation. Thus, in all probability, if the mutation does influence translation additional factors must be involved.
CHAPTER 5

Investigation of proteins which bind the 5' untranslated region of c-myc
Investigation of proteins which bind the 5' untranslated
region of c-myc

5.1 Introduction

The 5' untranslated regions (UTRs) of a number of proto-oncogenes have been shown to inhibit the translation of heterologous reporter genes in some in vitro systems. This is in accordance with Kozak's rules which would predict that their extreme length, high degree of predicted secondary structure and the prevalence of upstream initiation codons would reduce their translational efficiencies. However, in other in vitro systems and certain in vivo systems no repressive effect on translation was observed suggesting that, in addition to the standard translational machinery, other cell specific trans-activating factors could also be involved. c-myc exon 1 is no exception to this displaying little effect and severe inhibition on reporter translation depending on the particular in vitro or in vivo system examined (Lazarus et al., 1988; Parkin et al., 1988; Lazarus, 1992; Fraser et al., 1996). Accordingly, it would not be implausible to speculate that the translational efficiency of c-myc could be modulated by the binding of factors, protein or RNA, to its 5' untranslated region (exon 1). Such factors could either act to repress or activate translation by altering secondary structures or by modification of the translational apparatus.

The paradigm of translational regulation by motifs found within the 5'UTR is undoubtedly ferritin mRNA. In this case a 28 nucleotide stem loop structure, termed the iron responsive element (IRE), specifically binds the iron regulatory protein (IRP) to repress translation. Under iron depleted conditions, however, the binding affinity of IRP is reduced, thereby alleviating the translational repression and allowing production of ferritin. In addition to ferritin mRNA the 5'UTRs of other mRNAs including poly(A) binding protein and thymidylate synthase have been shown to repress translation but the mechanisms remain ill-defined.

Translational activation by specific protein factors has, so far, been shown to occur only in picornaviral transcripts by virtue of the internal mechanism of ribosome entry they utilise. However, with the discovery of cellular mRNAs containing IRESes, particularly the oncogene FGF-2, this would tend to indicate that translational activator proteins are likely to exist.

It was therefore decided to determine whether protein factors were capable of binding the 5'UTR of c-myc and to investigate if the 2756 T point mutation observed in the MM cell lines displayed altered binding characteristics. Three independent methods, all using the whole 600 nucleotide 5' UTR, were used to examine this parameter. The rationale for
using such a large RNA was that any secondary or tertiary structure interactions required for protein factor binding would be maintained and thus be more representative of the in vivo state.

5.2 Results

5.2.1 Generation of c-myc 5'UTR and control transcripts

In order to examine proteins capable of binding to the c-myc 5'UTR mRNA it was first necessary to clone the region into a vector from which "run off" transcripts could be made by in vitro transcription. pBluescript SK was chosen as a suitable vector as the multiple cloning site is flanked by both the T3 and T7 bacteriophage promoters. The c-myc 5'UTRs from both a wild type clone (6F) and a mutant clone (1-1) (Table 4.1 & 4.2) were isolated from the appropriate M13mp18/19 sequencing vectors by restriction digest with BamHI and EcoRI. The resulting 605 bp fragment was gel isolated and purified using NaI and glassmilk and subsequently ligated into pBluescript SK similarly digested with BamHI and EcoRI. E. coli JM109 ampicillin resistant transformants containing a disrupted lac Z gene were screened for the presence of the insert using a BamHI/EcoRI restriction digest. Positive plasmids termed pSK-Wt and pSK-Mt were isolated (Figure 5.1). These plasmids were additionally digested with TaqI to verify the presence or absence of the mutation (Figure 5.2).

Both plasmids were linearised downstream of the inserts by HindIII restriction digestion which, after a phenol/chloroform purification step, enabled Wt and Mt transcripts from the coding strand to be generated by in vitro transcription using T3 RNA polymerase. Transcripts were radiolabelled at this stage by the substitution of either 32P-labelled UTP or GTP instead of the unlabelled nucleotide in the in vitro transcription reaction. The size and integrity of the transcripts were verified by visual examination after separation by agarose gel electrophoresis and ethidium bromide staining. Both gave rise to transcripts of 654 nucleotides comprising of 605 nucleotides derived from the c-myc 5'UTR and 49 nucleotides of additional pBluescript SK vector sequence.

In addition to the Mt and Wt transcripts two similarly sized transcripts were used as control transcripts in protein factor binding. The first, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was formed by restriction digest of the plasmid pSKGAPDH with EcoRI and HindIII. This generated a 702 bp fragment which was gel purified and then cloned into pBluescript SK similarly digested with EcoRI and HindIII. Linearisation of this construct, pSKGAP:E/H, with HindIII and in vitro transcription using T3 RNA polymerase produced a 770 nucleotide transcript (Figure 5.3). This RNA, as it contained
Figure 5.1: (A) Construction of pSK-Wt and pSK-Mt vectors. c-myc exon 1 was removed from the M13mp18/19 sequencing vectors (Wt; clone 6F) (Mt; clone 1-1) by restriction digestion with BamHI and EcoRI. The resulting 605 bp fragment was gel purified and then ligated into the plasmid pBluescript SK similarly digested with BamHI and EcoRI. (B) Restriction digests of pBluescript SK transcription vectors. Lanes 2-5; pBluescript SK. Lanes 6-9; pSK-Wt. Lanes 10-13; pSK-Mt. In each case samples are loaded in the order; undigested plasmid, HindIII digest, BamHI/EcoRI digest, XhoI digest. Lanes 1 & 14 contain λ HindIII and 100 bp markers respectively.
Figure 5.2: *Taq I* restriction digests of pBluescript SK vectors. Samples are loaded in the order of undigested plasmid (U) and *Taq I* digested samples (T). The pSK-Wt and pSK-Mt plasmids gave rise to different restriction patterns confirming the presence of the C-T point mutation in the pSK-Mt vector.
Figure 5.3: (A) Construction of pSKGAP:E/H transcription vector. The plasmid pSKGAPDH was digested with the restriction enzymes EcoRI and HindIII releasing a 702 bp fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This fragment was gel purified and then ligated into the vector pBluescript SK digested with EcoRI and HindIII to create pSKGAP:E/H containing a shortened version of GAPDH. (B) Restriction digests of pSKGAP:E/H. Lane 1: λ HindIII. Lane 2: undigested pSKGAP:E/H. Lane 3: HindIII digest. Lane 4: EcoRI/HindIII digest.
part of the GAPDH coding sequence and was predicted to be relatively unstructured, served as a non-specific unstructured transcript of a similar size.

The other control RNA was generated from the plasmid pJ0HRV10-605. This plasmid contains the human rhinovirus (HRV) IRES and was used as a control for a highly structured RNA which is known to bind a number of proteins. In this case the plasmid was linearised with BamHI and in vitro transcribed using T7 RNA polymerase. The resulting product was approximately 650 nucleotides long. Both control RNAs were radiolabelled as described above.

5.2.2 Electrophoretic mobility shift assays : Cell extract titrations

To analyse whether proteins were capable of binding to the c-myc 5'UTR electrophoretic mobility shift assays (EMSAs) were performed whereby radiolabelled transcripts were incubated in the presence of cellular proteins. The transcripts and complexes that they formed with the cellular proteins were then resolved by electrophoresis on 0.7% agarose gels and the results visualised by autoradiography after the gels were dried. Any proteins binding to the transcripts would cause an increase in molecular weight correspondingly manifested as a decrease in the electrophoretic mobility of the radiolabelled RNA.

Accordingly, nuclear free cell extracts (CEs) were prepared from both a MM cell line, GM2132, and a control lymphoblastoid cell line, GM03201. These two cell lines were chosen as the most representative from each class as determined from FACS analysis (Section 3.2.1). The GM2132 cell line additionally had been shown to contain the highest level c-myc protein of all the myeloma cell lines and hence could potentially contain enhanced levels of any putative trans-regulatory factors (Figure 1.13). Radiolabelled transcripts for the Mt, Wt, GAPDH or IRES sequences were incubated with increasing concentrations of GM2132 CE or GM03201 CE in standard buffer conditions for 10 minutes. Samples were then separated by agarose gel electrophoresis, the gels dried and autoradiographed. The results of these electrophoretic mobility shift assays are shown in Figure 5.4.

Different electrophoretic mobility shift patterns were obtained for the c-myc 5'UTR transcripts and both the control RNAs implying that each had a characteristic pattern determined by the binding of different proteins. Proteins were responsible for this shift as no change in electrophoretic mobility was observed if cell extracts were first treated with proteinase K or boiled for 10 minutes prior to incubation (Figure 5.4). In each case addition of increasing amounts of cell extract resulted in the presence of less unbound transcript which was associated with an elevated signal in much higher molecular weight
Figure 5.4: Electrophoretic mobility assays showing cell extract titrations using either myeloma (GM2132) or control (GM03201) cell extracts. Equivalent amounts of Wt, Mt, GAPDH and IRES transcripts were incubated with CE as indicated. Samples were then separated by electrophoresis on 0.7% agarose gels, dried and the results visualised by autoradiography.
complexes. With the Wt, Mt and IRES transcripts lower molecular weight complexes however were also enhanced, suggesting that these may represent binding of specific protein factors whereas the higher molecular weight complexes may be associated with non-specific general RNA binding components.

This statement is further endorsed by comparing the electrophoretic mobility shift patterns obtained with the GM2132 CEs with those from the GM03201 CEs. For all four transcripts the second shift, as labelled for the Wt and Mt RNAs, was higher with the GM2132 CE than the GM03201 CE. This would tend to suggest that in this MM cell line there is an altered repertoire of RNA binding proteins. Alternatively, it is possible that there are different ratios or levels of general RNA binding proteins within these cells, perhaps reflecting either the different differentiation states or the tumourogenic nature of these B-cells.

The electrophoretic mobility shift patterns obtained with the Wt and Mt transcripts were very similar indicating that both RNA species probably bind the same proteins. However, for any given CE concentration, incubated with the same concentration of radiolabelled RNA, a higher proportion of the Mt transcript was present in a bound state compared to the equivalent Wt transcript. Thus, it appears that there is enhanced binding of proteins to the mutant transcript. Protein binding assays for the Wt and Mt transcripts were also performed under different salt conditions; 0-200 mM NaCl and 0-20 mM MgCl₂ concentrations (data not shown). However, no differences in the characteristic EMSAs were observed indicating that protein binding to c-myc 5'UTR is not fundamentally sensitive to Na⁺ or Mg²⁺ concentrations.

5.2.3 Electrophoretic mobility shift assays: Competition experiments

To further assess if the mutant sequence displayed an altered binding affinity for proteins a series of competition experiments were carried out. Unlabelled Mt and Wt transcripts were used as specific competitors and unlabelled GAPDH was used as a non-specific competitor. The results obtained using GM2132 CE are shown in Figure 5.5.

The mutant transcript was found to bind proteins with approximately a ten fold increased affinity (Figure 5.5; Panel A). This is clearly illustrated using radiolabelled Wt transcripts and comparing the effects of addition of unlabelled Wt and Mt transcripts. A 30 fold molar excess of unlabelled Wt transcript was required to abolish the electrophoretic mobility shift pattern, whereas only a 3 fold molar excess of unlabelled Mt transcript was required for the same competitive interaction. The reciprocal case is true for the radiolabelled Mt transcript whereby a significant increase in unbound RNA was observed
Figure 5.5: Competitive EMSAs. (A) 75 μg GM2132 cell extract was incubated with equivalent amounts of radiolabelled transcript and increasing concentrations of unlabelled transcript as indicated. Samples were then separated by agarose gel electrophoresis, dried and exposed to X-ray film. (B) The above results were densometrically scanned and then plotted graphically as the percentage of bound radiolabelled transcript of the total amount of radiolabelled transcript present. In each case the first character (*) denotes the radiolabelled transcript used.
only at a 300 fold molar excess with Wt unlabelled competitor and at a 30 fold molar excess of Mt unlabelled competitor. Again a ten fold difference in binding affinity was seen but labelled Mt RNA still persisted in higher molecular weight complexes, even at higher concentrations, suggesting that the mutation, in addition to exhibiting greatly enhanced protein binding characteristics, may display altered kinetics of interaction. The differences in binding affinities between the mutant and wild type transcripts is also clearly evident when the results are plotted graphically and the fraction of bound transcript is expressed as a percentage of the total amount of radiolabelled transcript present (Figure 5.5; Panel B).

Although not shown, similar results were obtained using GM03201 CEs suggesting that the difference observed in the binding characteristics is primarily a function of the transcript sequence rather than the proteins binding to it. The addition of non-specific unlabelled GAPDH transcripts did not affect binding except at very high concentrations implying that specific protein factors bind the c-myc 5'UTR.

Competition experiments were also performed in the presence of two non-specific RNA competitors; yeast tRNA and heparin (Figure 5.6). Addition of the latter at concentrations of greater than 1 μg significantly reduced all binding and was therefore omitted from binding assays. Addition of yeast tRNA, however, had little affect on binding, although at higher concentrations a slight enhancement in higher molecular weight complexes was observed. No differences in the effects of either of these two competitors were seen between the Wt and Mt transcripts.

5.2.4 Examination of proteins involved in EMSAs

Electrophoretic mobility shift assays were scaled up by a factor of 40 for both Wt and Mt transcripts prior to separation on preparative 0.7% agarose gels. Gels were then horizontally cut into six 1 cm gel slices, starting immediately below the first dye front, as only unbound RNA runs below this region. The proteins contained in each gel slice were then electroeluted, TCA precipitated, separated by SDS-PAGE and visualised by silver staining. The results of both Wt and Mt transcripts individually incubated with either GM2132 CE or GM03201 CE are shown in Figure 5.7. Similar results were obtained reproducibly although slight variations in protein composition were observed between gel slices, for a given CE and transcript, due to experimental variation. Direct qualitative comparisons between individual gel slices therefore cannot be regarded as absolute and similarly quantitative comparisons can only be made relative to other proteins in a particular gel slice. The technique however is specific for individual transcripts as the protein banding patterns obtained were different if GAPDH or IRES transcripts were used
Figure 5.6: Competitive EMSAs using non-specific RNA competitors yeast tRNA and heparin. 45 µg GM03201 CE was incubated with equivalent amounts of radiolabelled transcript (Wt or Mt) in the presence of differing amounts of yeast tRNA or heparin as indicated. Samples were then resolved by agarose gel electrophoresis and the results visualised by autoradiography. Identical results were obtained using GM2132 CEs.
Figure 5.7 : Isolation of proteins from EMSAs. (A) Schematic diagram outlining the method used for isolating proteins from EMSAs. Transcripts were incubated with CEs for 10 mins and then separated on 0.7% agarose gels. Gels were then cut horizontally into six slices between the two dye fronts as indicated. Proteins in each fraction were recovered by electroelution and TCA precipitation, prior to separation by SDS-PAGE. (B) - (E) The results of SDS-PAGE of proteins, visualised by silver staining, using different RNA transcripts (Wt & Mt) and CEs (GM2132 & GM03201). (B) GM2132 CE and Wt transcript. (C) GM03201 CE and Wt transcript. (D) GM2132 CE and Mt transcript. (E) GM03201 CE and Mt transcript. Fractions 1-6 as shown. M; molecular weight markers as indicated in kD.
Figure 5.8: Isolation of proteins from EMSAs. (A) GM2132 CE was incubated with buffer in the absence of any RNA transcripts and then resolved by agarose gel electrophoresis (0.7% agarose gel). Gels were then cut horizontally into six slices between the two dye fronts as shown in Figure 5.7 and the proteins in each fraction were recovered by electroelution and TCA precipitation. Samples were separated by SDS-PAGE and the proteins visualised by silver staining. (B) As for A but IRES transcripts were incubated with GM2132 CE for 10 mins prior to agarose gel electrophoresis. Fractions 1-6 as detailed in Figure 5.7. M: molecular weight markers as indicated in kD.
(Figure 5.8 & data not shown). Similarly, a control experiment conducted in the absence of any RNA transcript also produced a different protein banding pattern (Figure 5.8). These proteins which represent non-specific protein factors which arise simply as a result of their inherent electrophoretic mobilities can therefore be eliminated as RNA binding factors.

Comparing all of the panels of Figure 5.7 several points can be noted. Firstly, in general the Wt and Mt transcripts appear to bind the same proteins. Secondly, two prominent high molecular weight proteins differ between the GM2132 CEs (98 kD, 86 kD) and the GM03201 CEs (104 kD, 91 kD). Thirdly, many more proteins were observed in fractions 4-6. However, as similar results were obtained using the control transcripts and this region corresponds to higher molecular weight complexes this suggests that these proteins represent non-specific binding factors. This is also substantiated by the fact that a number of these proteins were also observed in the no RNA control (Figure 5.8). Accordingly, attention focused on gel fractions 1-3 which were therefore more likely to contain factors specifically associated with the first and second shifts.

Fraction 1 predominantly contained five proteins of 61, 58, 45, 43 and 33 kD. Additionally the Mt transcript was associated with a 38 kD protein and the Wt transcript displayed an extra band of around 66 kD. The mutant transcript also displayed enhanced binding to the 58/61 kD doublet relative to the wild type transcript (Figure 5.7; Panel D). This doublet was also visible in fraction 2. However, the lower 58 kD form was stronger with the Mt transcript whereas Wt transcripts exhibited elevated levels of the 61 kD protein. Similarly, a lower proportion of the 45 and 43 kD proteins is observed in fraction 2 compared to fraction 1 for the GM2132 CEs but the opposite is seen in the GM03201 CEs. However, the significance of these two observations is unclear.

In addition to the proteins observed in fraction 1, fraction 2 also contained two other proteins of 134 and 78 kD. Three higher molecular weight proteins were also observed; a 98 kD protein, a 106 kD protein specific to the GM2132 CE and a 109 kD protein specific to the GM03201 CE. The first of these, the 98 kD, was present at elevated levels compared to the higher molecular weight protein (106/109 kD), in the GM2132 CEs. Furthermore if the GM2132 CEs are compared (Figure 5.7: Panels B & D) then the mutant transcript shows greatly enhanced binding to this 98 kD protein.

A larger number of proteins were observed in fraction 3, the majority of which were ubiquitously present and were also detected in fractions 1 and 2. Proteins of 160, 150, 56, 49, and 40 kD were additionally present. No striking differences were observed between the mutant and wild type transcripts in this fraction, however, a large number of differences were seen between the two cell extracts. As mentioned above, the two
prominent bands in the GM2132 CE were 98 and 86 kD whereas those in the GM03201 CE were 104 and 91 kD. Specific GM2132 associated proteins of 85, 82, 73 and 68 kD were also observed compared to proteins of 84, 76 and 66 kD which were GM03201 CE specific. These differences between the cell extracts may account for the apparent disparity observed in the second shift.

In conclusion both the Wt and Mt transcripts appear to bind the same proteins although consistently higher levels of three species, with molecular weights of 98, 58 and 38 kD, were observed with the Mt transcript. This would tend to suggest that the mutation may be altering the binding affinity of these proteins. Furthermore, the GM2132 myeloma cell extract was found to contain much higher levels of the 98 kD protein compared to the control GM03201 cell extract. Other differences were also observed between the GM2132 CEs and GM03201 CEs implying that these different stages of B-cell differentiation express an altered array of polypeptides. Alternatively, the malignant nature of the myeloma clone could be responsible for the differences observed. The majority of proteins found using this modified EMSA technique however appear to be conserved c-myc 5'UTR binding factors. Some of these proteins probably therefore represent specific RNA binding proteins to this region but a large number of them may be general RNA binding proteins.

5.2.5 Immunological investigation of proteins with the potential to bind the c-myc 5'UTR

Although the above EMSA method identified a large number of candidate c-myc 5'UTR binding proteins their identities were uncertain. It was therefore decided to examine nuclear free cell extracts for proteins which could potentially be involved in c-myc 5'UTR binding. Accordingly, both GM2132 CEs and GM03201 CEs were separated by SDS-PAGE, electroblotted on to nitrocellulose and probed with a panel of different antibodies. Four protein species were investigated; c-myc, actin, La and polypyrimidine tract binding protein (PTB). The first two had molecular weights similar to proteins identified using the EMSA protein isolation technique and the latter two were proteins known to bind the highly structured IRES RNA structures of picornaviruses. The results of immunodetection of all of these proteins are shown in Figure 5.9.

As predicted, due to its nuclear localisation, no c-myc was detected in either cell extract. Although very unlikely it was postulated that the c-myc protein, with a molecular weight of approximately 62 kD, could have been binding to its 5'UTR and autoregulating its own translation. However, the lack of c-myc protein in the cell extracts strongly argues that this is not the case. Actin, in contrast, was very strongly expressed in both cell extracts.
Figure 5.9: Western blot analysis of control (GM03201) and MM (GM2132) nuclear free cell extracts. (A) Blot of 10% SDS-PAGE gel probed with c-myc antibody (9E10). HL60 whole cell preparation was loaded as a positive control. (B) Blot of 7.5% SDS-PAGE gel probed with actin antibody. (C) Blot of 7.5% SDS-PAGE gel probed with La antibody. (D) Blot of 7.5% SDS-PAGE gel probed with PTB antibody.
This protein has a molecular weight of 45 kD which, combined with its reported weak RNA binding activity, could account for the 45 kD protein observed in the EMSA protein isolation experiments.

La and PTB were expressed both in the myeloma GM2132 CE and the control GM03201 CE, although expression of the 47 kD La protein was greater than PTB expression. A small variation in the PTB doublet (p56/57) distribution was also observed, with the myeloma cell extracts displaying predominately the upper isoform whereas the control cell lines had larger quantities of the lower isoform. The significance of this however is unclear as the function of these two isoforms is unknown. As both these proteins were expressed in the cell extracts and are known to have an RNA binding capacity it was decided to also determine if they were present in specific fractions obtained after gel isolation using the modified EMSA technique. Experiments were carried out as described above (Section 5.2.4) but after separation by SDS-PAGE gels were electroblotted on to nitrocellulose and probed with antibodies specific either for La or PTB. The results are shown in Figure 5.10.

La and PTB were detected in both the GM2132 and the GM03201 cell extracts and were found in similar fractions irrespective of the CE used or whether the EMSA protein isolation was carried out using a Wt or Mt transcript. La was distributed in fractions 2, 3 and 4 but was most prominent in fraction 3. PTB expression was detected in fractions 3, 4, 5 and 6, with highest level expression in fractions 4 or 5. It thus appears that the c-myc 5'UTR can bind both the La and PTB proteins, but the latter of the two is most likely to be a non-specific RNA binding factor. This may also be the case for the La protein, being detected in earlier fractions associated with more specific binding simply as a result of its high binding affinity for RNA. Non specific RNA binding, or at least binding at a site remote from position 2756, is also implicated by the fact that the distribution of expression of both these factors is paralleled with the Wt and Mt transcripts.

5.2.6 North western analysis

To further assess the relationship between specific and non-specific RNA binding factors a series of north western blots were carried out. Different concentrations of nuclear free cell extracts from both the control, GM03201 and myeloma GM2132 cell lines were therefore separated by SDS-PAGE and then electroblotted on to nitrocellulose membranes. Proteins were then allowed to partially renature on the membranes under the appropriate buffer conditions and then probed using radiolabelled transcripts. The results obtained using the Wt and Mt transcripts and the two control transcripts are shown in Figure 5.11 and summarised in Table 5.1. In addition north western analysis using the Wt and Mt
Figure 5.10: Western blot analysis of proteins isolated from EMSAs. (A) Proteins were separated by SDS-PAGE (7.5% gels) and then silver stained. (B) Duplicate gels were blotted on to nitrocellulose and then probed with antibodies for La and PTB. Lanes 1-6 correspond to gel fractions as detailed in Figure 5.7. Results shown are for GM2132 CE with Mt c-myc exon 1 transcript and GM03201 CE with c-myc exon 1 Wt transcript.
Figure 5.11: North western analysis of multiple myeloma (GM2132) and control (GM03201) cell extracts. Different concentrations of CEs, as indicated, were separated by SDS-PAGE, blotted on to nitrocellulose and then probed using equivalent amounts of radiolabelled RNA transcripts. (A) Wt transcript. (B) Mt transcript. (C) GAPDH transcript. (D) Wt transcript with 2µg of unlabelled GAPDH transcript as non-specific competitor RNA. (E) Mt transcript with 2µg of unlabelled GAPDH transcript as non-specific competitor RNA. (F) IRES transcript.
<table>
<thead>
<tr>
<th>RNA transcript (*;labelled)</th>
<th>Polypeptides (kD)</th>
<th>GM2132 CE specific (kD)</th>
<th>GM03201 CE specific (kD)</th>
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</thead>
<tbody>
<tr>
<td>Wt*</td>
<td>180, 160, 138, 125, 120, 115, 98, 82, 79, 68, 61, 58, 46, 42, 38</td>
<td>86</td>
<td>90, 76, 70</td>
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<tr>
<td>Wt* &amp; GAPDH</td>
<td>138, 82, 61, (79), (68), 46</td>
<td>86</td>
<td>90, (76), (70)</td>
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<tr>
<td>Mt*</td>
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<td>86</td>
<td>90, (76), (70)</td>
</tr>
<tr>
<td>Mt* &amp; GAPDH</td>
<td>160, 138, 98, 82, (79), (68), 61, 57, 46, 38</td>
<td>86</td>
<td>90, 76, 70</td>
</tr>
<tr>
<td>GAPDH*</td>
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<td>116, 103, 86, 76, 68, 36</td>
<td>104, 90, 66</td>
</tr>
<tr>
<td>IRES*</td>
<td>160, 140, 125, 98, 79, 72, 61, 54, 46, 33, 30</td>
<td>86, 76, 68, 38</td>
<td>90, 66, 49</td>
</tr>
</tbody>
</table>

**Table 5.1**: Table summarising the proteins observed to bind RNA transcripts from the north western data shown in Figure 5.11. Numbers in brackets indicate species to which only minimal binding was observed.
transcripts was performed in the presence of an excess of unlabelled GAPDH transcript as a non-specific competitor.

Using each of the RNA transcripts singly a large number of RNA binding proteins were observed and although each one gave rise to a characteristic pattern of proteins many were common to all of the transcripts. This suggests that they either represent non-specific RNA-binding proteins or bind RNA simply as a result of their high levels of expression. The latter of these is particularly evident at low protein concentrations. Similar to the situation observed using the modified EMSA technique a number of differences were seen between the two cell extracts. However, in general, the majority of these were not transcript specific implying that the myeloma cell lines have an altered spectrum of general RNA protein binding factors.

The most informative data for protein factor binding to the Wt and Mt transcripts was obtained using unlabelled GAPDH competitor (Figure 5.11; Panels D & E). This significantly reduced the number of proteins binding to both the c-myc 5'UTR transcripts although the effect was most marked for the Wt transcripts. Again this implies that the Mt transcript has a greater affinity for protein binding which is consistent with the original EMSA data.

Predominantly, six proteins were found to bind the Wt transcript. 138, 82, 61 and 46 kD proteins were found in both GM2132 and GM03201 CEs whereas proteins of 90 and 86 kD appeared to be GM03201 CE and GM2132 CE specific respectively. Minimal binding to four other species of 79, 68, 76 and 70 kD, was also observed, the latter two of which were GM03201 CE specific.

All of the aforementioned proteins were also found to bind the Mt transcript albeit with higher affinity. This non-specific elevation in binding capacity of the Mt transcript was also accompanied by the binding of four additional proteins of 160, 98, 57 and 38 kD which were not detected with the Wt transcript. However, longer exposures of the northern western probed with the Wt transcript, in conjunction with the single transcript northern western data, would tend to indicate that the Wt transcript is also capable of binding these factors although with significantly lower affinity. The proteins identified using this technique strongly support the data obtained from the EMSA protein isolation experiments.
5.2.7 UV cross-linking analysis

In addition to the EMSA protein isolation experiments and north western analysis a third complimentary approach using UV cross-linking was performed to verify that the proteins identified by the previous two methods were indeed capable of directly interacting with the c-myc 5'UTR RNA. Accordingly, $^{32}$P-GTP radiolabelled Wt and Mt transcripts were incubated with GM2132 myeloma and GM03201 control cell extracts, the proteins cross-linked to the RNA using short wave UV light and the unprotected RNA digested with either RNase A or RNase T1. The proteins were then separated by SDS-PAGE and proteins binding RNA fragments were visualised by autoradiography after gels were dried. In addition UV cross-linking assays were performed using transcripts radiolabelled with $^{32}$P-CTP. The main rationale for this was the fact that as differences observed in proteins binding to the Mt transcript were likely to reside around the C rich position 2756, radiolabelling of C nucleotides would facilitate detection of such RNA fragments. The results of UV cross-linking studies using both GTP and CTP labelled transcripts are shown in Figure 5.12.

From the UV cross-linking experiments two distinguishing features were observed. Firstly, the pattern of bands obtained with each of the radiolabelled transcripts was distinct indicating that particular proteins displayed preferential binding to either G or C rich sequences. The second discernible feature was that the Wt and Mt transcripts gave rise to different bands implying that they do in fact bind different proteins \textit{in vitro}. This would also tend to suggest that the altered binding of proteins to the mutant transcript is not only restricted to the C-rich region around the point mutation but also has the potential to alter the binding characteristics at more distal sites, possibly via modifications in secondary structure interactions. No clear differences were observed between the two cell extracts substantiating that the mutation is sufficient to alter the binding of components found in both the multiple myeloma and control cell extracts. Results differed depending on the RNase treatment used due to the selective cleavage specificities of the two enzymes involved.

Wt transcripts radiolabelled with GTP were primarily associated with three proteins of around 78, 58 and 43 kD. Two of these, the 78 and 43 kD species, were also detected using CTP radiolabelled Wt transcripts. Mt transcripts radiolabelled with GTP cross-linked to three proteins of 43, 40 and 38 kD. This 38 kD protein was also observed using CTP radiolabelled Mt transcripts in addition to two less intense bands of around 98 and 43 kD. It would thus appear that the Wt transcript principally binds proteins of 78 and 58 kD whereas the Mt transcript is associated with proteins of 98, 40 and 38 kD. Both transcripts appear to bind a 43 kD protein.
Figure 5.12: UV-cross linking analysis. Briefly, RNAs were incubated with 70 µg CE, UV irradiated on ice and treated with RNase T1 or RNase A as indicated. Samples were then separated by SDS-PAGE, gels dried and exposed to X-ray film. (A) Wt GTP radiolabelled RNA transcript. (B) Mt GTP labelled RNA transcript. (C) Wt CTP radiolabelled RNA transcript. (D) Mt CTP radiolabelled RNA transcript. Lanes 1 & 3 GM2132 CE. Lanes 2 & 4 GM03201 CE. Molecular weights are shown in kD.
Taken together these results would tend to imply that only a few proteins are directly associated with the RNA from the \textit{c-myc} 5'UTR. Correspondingly, RNA-binding proteins detected using the other two methods may interact with the RNA in a more non-specific or indirect manner. Alternatively, due to the nature of the UV cross-linking method utilised, a number of proteins may not have been identified because they either cannot be cross-linked or the distribution of radiolabelled nucleotides is insufficient for their detection. This method did however confirm the previous findings that the Mt transcript can bind different proteins, specifically identified as 38 and 98 kD proteins, when compared to the Wt sequence.

5.3 Summary

To investigate proteins capable of binding to the \textit{c-myc} 5'UTR three complementary approaches were employed; EMSAs, north western analysis and UV cross-linking. All methods utilised 600 nucleotide radiolabelled transcripts derived from both the wild type and mutant sequences and generated a number of possible proteins with RNA binding potential. These are summarised in Table 5.2.

The majority of proteins identified using the EMSA protein isolation and north western techniques were also found to be able to bind the control IRES and GAPDH RNAs. These proteins are therefore likely to represent either general RNA binding factors or alternatively bind RNA non-specifically as a result of high expression levels. As the \textit{c-myc} 5'UTR transcripts were approximately 600 nucleotides in length it was not unexpected that such a large number of RNA binding proteins were obtained.

Some differences in these general RNA binding factors were also detected between the myeloma GM2132 and control GM03201 cell extracts. This probably accounts for the slight discrepancies in the electrophoretic mobility shift patterns obtained and may reflect the different differentiated states of these B-cell clones. Alternatively, the dissimilar proteins observed in the myeloma cell extracts may be attributable to the malignant phenotype of these cells.

The Wt and Mt transcripts displayed similar protein binding potential as predicted by virtue of the fact that only one nucleotide out of 600 was altered. However, the data obtained from the EMSAs implied that the Mt sequence conferred approximately a ten fold enhancement in RNA binding affinity to the \textit{c-myc} 5'UTR transcript compared to the Wt sequence. This was confirmed using north western techniques in the presence of non-specific competitor RNA. This technique also identified six proteins displaying an augmented affinity for both \textit{c-myc} 5'UTR transcripts; namely proteins of 138, 90, 86, 82,
<table>
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<th>Method</th>
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<th>Enhanced binding to Wt transcript (kD)</th>
<th>Enhanced binding to Mt transcript (kD)</th>
<th>GM2132 CE specific (kD)</th>
<th>GM03201 CE specific (kD)</th>
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<td>EMSA protein isolation</td>
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<td>98, 58, 38</td>
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<td>North western analysis</td>
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<td>160, 98, 57, 38</td>
<td>86</td>
<td></td>
<td>90, (76), (70)</td>
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<td>UV cross-linking; GTP</td>
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<td>78, 58</td>
<td>40, 38</td>
<td></td>
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<tr>
<td>UV cross-linking; CTP</td>
<td>43</td>
<td>78</td>
<td>98, 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUMMARY</td>
<td>78, 61, 43</td>
<td>98, 38, 86</td>
<td>90 (76)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.2**: Summary of the proteins identified to bind the c-myc 5' UTR using the three different methods employed. Proteins that showed preferential binding to either the Wt or Mt sequences are indicated. Proteins that appeared to be restricted to a particular CE are also shown. Numbers in brackets represent proteins that displayed only minimal binding.
61 and 46 kD. The 90 kD protein being GM03201 CE specific and the 86 kD GM2132 specific.

Additionally, the Mt transcript bound four other proteins of 160, 98, 57 and 38 kD. The three lower molecular weight polypeptides were also identified in EMSA protein isolation experiments by virtue of their increased levels. Similarly, proteins of 98 and 38 kD were found in UV cross-linking experiments using the Mt transcript. It therefore appears that the C→T point mutation is sufficient to enhance the binding of principally two proteins of 98 and 38 kD to the c-myc 5'UTR. Consequently one would predict that these proteins bind around this 2756 site possibly due to a modification in the secondary structure, which subsequently could be transmitted to more distal sites, or simply as a result of the altered nucleotide sequence.

Two proteins, La and PTB, were immunologically detected as proteins capable of binding to the c-myc 5'UTR. These proteins are more likely to represent general RNA binding factors as opposed to proteins specifically associated with c-myc exon. However, this would require further verification using purified recombinant proteins and smaller segments of the 5'UTR in binding assays.

The identities of the other proteins recognised as candidate c-myc 5'UTR binding proteins remains undetermined. Protein isolation EMSAs were scaled up and protein microsequencing of a number of proteins (98, 62, 45 and 38 kD) was carried out to further characterise these species. However, only the 45 kD protein was positively identified as actin, confirming the western blot data. No protein sequence data was obtained for the other polypeptides as they were present in insufficient quantities and were all N-terminally acetylated.

In conclusion the c-myc 5'UTR appears capable of binding a number of proteins some of which may be specifically associated with c-myc transcripts and some of which are ubiquitous RNA binding factors. Moreover, the single nucleotide substitution observed in the multiple myeloma cell lines substantially alters the protein binding characteristics to this region specifically enhancing the recruitment of two protein factors of 38 and 98 kD. The functional significance of this modification in trans-acting factors however is uncertain, although one could speculate that it could affect the initiation of translation. It may also be of interest to note that both the 38 and 98 kD proteins did interact with the HRV control IRES RNA and two proteins of similar sizes have been implicated in IRES function (Borman et al., 1993).
CHAPTER 6

Effects of c-myc exon 1 on the in vitro translation of heterologous genes
Effects of c-myc exon 1 on the in vitro translation of heterologous genes

6.1 Introduction

A number of previous studies have examined the effects of c-myc exon 1 sequences on translation of heterologous genes. However, results have often been contradictory, being dependent on the species examined and the particular translation system utilised. Murine c-myc exon 1 has been shown to inhibit the translation of reporter genes both in several in vitro systems (wheat germ lysates and rabbit reticulocyte lysates) and after microinjection into Xenopus laevis oocytes (Darveau et al., 1985; Parkin et al., 1988). However, it was not found to affect the translation of heterologous genes in HeLa cell extracts or after transfection into established cell lines (Darveau et al., 1985; Parkin et al., 1988; Lazarus et al., 1988). Similarly, Xenopus laevis c-myc I first exon sequences and human c-myc exon 1 exhibited inhibitory effects on translation after microinjection into Xenopus oocytes but, at least in the case of Xenopus exon 1 containing reporters, this block to translation was removed after fertilisation (Lazarus, 1992; Fraser et al., 1996). The inhibitory effect of murine c-myc exon 1 on reporter translation has also been reported to be lost upon fertilisation of Xenopus oocytes (Lazarus et al., 1988). In vitro translation studies using human c-myc exon 1 sequences have not been reported but transfection of reporter constructs bearing this region into both COS and NIH3T3 cells have been found to repress reporter translation (Ray et al., 1989; Butnick et al., 1985).

It was therefore decided to examine in vitro the translation of reporter constructs containing the human c-myc exon 1 region and determine if the mutation, detected in the multiple myeloma cell lines, could be functionally responsible for the observed alteration in c-myc translation. Accordingly, in vitro translation of both monocistronic and dicistronic reporter constructs were performed in rabbit reticulocyte lysates.

6.2 Results

6.2.1 Construction of monocistronic reporter constructs

To examine c-myc exon 1 effects on in vitro translation the luciferase reporter gene was cloned downstream of the c-myc exon 1 sequences (Wt and Mt) previously cloned into the pBluescript vector pSK-Wt and pSK-Mt (Section 5.2.1). Thus, the 1.6 kb luciferase gene fragment was removed from the vector pGL3 by restriction digestion with NcoI and XbaI and gel purified using a glassmilk and NaI procedure. The ends of the fragment were then made flush by treatment with the Klenow fragment of DNA polymerase I which was
subsequently heat inactivated. Both the pSK-Wt and pSK-Mt vectors were digested with
HincII and then treated with alkaline phosphatase to prevent religation of the vector. After
phenol and chloroform extractions, followed by ethanol precipitation, the luciferase gene
fragment was ligated into these vectors. *E. coli* JM109 transformants were selected by
ampicillin resistance and checked for luciferase gene insertion by restriction digestion
with *SacI* and *XhoI*. Correct orientation of the luciferase gene was confirmed by *HincII*
digestion (Figure 6.1). The two resultant plasmids were termed pWt-luc and pMt-luc. In
addition the plasmid pGEM-luc, which contains no extra sequence upstream of the
luciferase gene, was used as a positive control.

6.2.2 *In vitro* translation of monocistronic reporter constructs

The pWt-luc and pMt-luc plasmids, containing the luciferase reporter gene, were
linearised using *Acc65I* and transcribed *in vitro* using T3 RNA polymerase. The control
plasmid pGEM-luc was linearised with *XhoI* and then *in vitro* transcribed using S6 RNA
polymerase. Reactions were performed either in the presence or absence of 7-
methylguanosine triphosphoguanosine (mGpppG). Unincorporated nucleotides were then
removed by passing the samples through a G-50 spun column and extracted with phenol
and chloroform prior to ethanol precipitation. *In vitro* translation reactions in rabbit
reticulocyte lysates were carried out using RNAs at a concentration of 50 μg/ml in the
presence of 35S-methionine under the standard conditions described (Section 2.8.15).
Samples were then separated by SDS-PAGE, and after coomasie staining and destaining
gels, were treated with fluorographic enhancer. Gels were dried, exposed to X-ray film
and the results visualised after fluorography. The results obtained using the three
constructs are shown in Figure 6.2.

Using the control construct a 62 kD protein corresponding to luciferase was observed with
both the uncapped and capped transcripts. However, the amount of product was
significantly enhanced using capped transcripts consistent with Kozak's rules (Section
1.4.6). This was in direct contrast to both the Wt and Mt reporter constructs in which only
minimal luciferase production was observed and no magnification in the luciferase signal
was apparent upon capping of these transcripts. mGpppG was therefore omitted from
subsequent transcription reactions. From these results it would also appear that the human
c-myc exon 1 region is inhibitory to translation, at least in this system.

Although no differences in luciferase expression were discernible between the Wt and Mt
transcripts, because of the extreme inhibitory effect and hence exceptionally low levels of
luciferase product, subtle alterations in the translational efficiencies between the two
cannot be discounted. It is clear that neither produce a 32 kD protein representative of
Figure 6.1: (A) Construction of monocistronic luciferase reporter constructs. The 1.6 kb luciferase gene was removed from the vector pGL3 by restriction digestion with Nco I / Xba I and the ends filled in using the Klenow fragment of DNA polymerase I. The luciferase gene was then cloned into the Hinc II site of pSK-Wt and pSK-Mt c-myc exon 1 containing vectors to form the two plasmids pWt-luc and pMt-luc. (B) Restriction digests of monocistronic luciferase reporter constructs. Lanes 2-4; pSK-Wt. Lanes 5-7; pWt-luc. Lanes 8-10; pMt-luc. In each case samples were loaded in the order; undigested plasmid, SacI/XhoI digest, SacI/HincII digest. Lanes 1 & 11 contain λ HindIII and 100 bp markers respectively.
Figure 6.2: \textit{In vitro} translation of monocistronic luciferase reporter constructs. Vectors pWt/Mt-luc and pGEM-luc were linearised, transcribed \textit{in vitro} using either T3 or S6 RNA polymerase and then \textit{in vitro} translated in a rabbit reticulocyte lysate system incorporating radiolabelled methionine. The products were separated by SDS-PAGE (10\% polyacrylamide gel) and the results visualised by autoradiography. U; uncapped transcripts. C; capped transcripts.
MYCHEXI, indicating that this protein is not translated *in vitro* using these constructs in this *in vitro* translation system.

### 6.2.3 *In vitro* translation of monocistronic reporter constructs supplemented with HeLa cell extracts

To further investigate the translational inhibition observed with *c-myc* exon 1 constructs, *in vitro* translation assays were performed in rabbit reticulocyte lysates supplemented with different concentrations of HeLa cell extracts. The results are shown in Figure 6.3.

Addition of HeLa cell extracts did not alter the production of luciferase from the control transcripts, although at higher HeLa cell extract concentrations the number of spurious background products increased due to increased frequencies of random initiation events. This was also observed at high concentrations with the Wt and Mt transcripts including an intense band of approximately 40 kD. As a similar sized protein was observed in the control construct it is unlikely to represent MYCHEXI.

Again, no differences were observed between the Wt and Mt sequences with both inhibiting luciferase translation, although increased expression of luciferase was observed with higher concentrations of HeLa cell extracts. This would tend to imply that HeLa cell extracts supply trans-acting factors that are required to derepress the translational inhibition imposed by the *c-myc* 5'UTR. These factors are presumed to be either absent or present in limiting quantities in the rabbit reticulocyte lysate and hence normally, translational repression is observed. However, as luciferase levels were not specifically augmented relative to other spurious translation products it would appear that none of these putative trans-acting factors are specific for relieving the repressive effects of *c-myc* exon 1 sequences on translation. Hence, *c-myc* exon 1, either Wt or Mt sequence, severely inhibits translation of heterologous genes *in vitro*.

### 6.2.4 Secondary structure predictions and translation

Using the MFold program secondary structures of *c-myc* exon 1 were calculated to have energies of approximately -200 kcal/mol (Section 4.2.7). Consequently, it would be predicted that this region would impose severe restrictions on the scanning mechanism of translation, which is consistent with the data obtained. Furthermore, although the mutation was predicted to introduce an additional short stem loop region into the *c-myc* 5'UTR, as the energy of this structure is only -0.4 kcal/mol, it would be unlikely to alter the translational efficiency of the scanning ribosome complex, which is again consistent
Figure 6.3: *In vitro* translation of monocistronic luciferase reporter constructs supplemented with different concentrations of HeLa cell extracts as indicated. Reactions were carried out in rabbit reticulocyte lysates supplemented with radiolabelled methionine under equivalent ionic concentrations. Products were separated by SDS-PAGE (10 % polyacrylamide gels) and dried gels subsequently exposed to X-ray film.
with the data obtained. However, this raises two questions. Firstly, as the c-myc exon 1 region is predicted to be highly stable, how is c-myc translation normally achieved in vivo? Secondly, as the mutation per se is unlikely to affect ribosome scanning, but may bind different protein factors, how could this be involved in the augmented translation of c-myc?

The first of these, in part, could be alleviated by the use of different promoters (Figure 6.4) which give rise to different energy structures. However, the lower energies of the P1 and P2 transcripts compared to those from P0 may be insurmountable by the presence of stem loops of -103.7 kcal/mol (P1) and -26.1 kcal/mol (P2) within 12 nucleotides of the cap structure. It would be predicted that both these structures would prevent the association of the 43S pre-initiation complex, or at least in the case of P2 transcripts, severely restrict this interaction. Moreover, the inclusion of this large stem loop of approximately 100 kcal/mol in P0 transcripts irrespective of the other upstream structures (not shown) would in itself be sufficient to inhibit ribosome progression.

It would thus appear, both from secondary structure predictions and in vitro experiments, that translation of c-myc transcripts by the conventional scanning mechanism is highly inefficient. These results taken together with the facts that trans-acting factors only partially eliminated the inhibition and no enhanced translation was observed using capped mRNAs suggested that a cap-independent mechanism of internal initiation could be functioning for c-myc translation. Therefore to examine if c-myc exon 1 was capable of directing the internal entry of ribosomes the region was cloned into a dicistronic construct.

### 6.2.5 Construction of dicistronic reporter constructs

pSK-Wt and pSK-Mt vectors (Section 5.2.1) were digested with the restriction enzymes BamHI and EcoRI, the 605 bp c-myc exon 1 fragment gel purified and treated with the Klenow fragment of DNA polymerase I to make the ends flush. The enzyme was then heat inactivated. The dicistronic vector pXLJ'0 was digested with SnaBI, treated with alkaline phosphatase and then extracted with phenol and chloroform prior to ethanol precipitation. The c-myc fragment was then ligated into the linearised pXLJ'0 vector and transformed into E. coli JM109. Ampicillin resistant colonies were examined for the presence of an insert of approximately 700 bp by restriction digestion with SalI and BamHI. The orientation of c-myc exon 1 positive colonies was then checked by PvuII restriction digestion. Correspondingly, two Wt dicistronic constructs were made pXL-Wt(+) in which the c-myc exon 1 fragment was in the correct 5'-3' orientation and pXL-Wt(-) in which the exon 1 region was in the reverse 3'-5' orientation. Similarly two Mt constructs were made; pXL-Mt(+) and pXL-Mt(-) (Figure 6.5).
Figure 6.4: Detailed secondary structure of the 5' region of the c-myc 5'UTR as predicted from the MFold program of Zucker, 1989. The positions of the P0, P1 and P2 promoters are indicated.
Figure 6.5: (A) Construction of dicistronic reporter constructs. *c-myc* exon 1 was removed from the pSK-Wt and pSK-Mt constructs by restriction digestion with *BamHl* and *EcoRI*. The resulting 605 bp fragments were gel purified and then treated with the Klenow fragment of DNA polymerase I prior to ligation into the *SnaBI* site of the dicistronic vector pXLJ'0. Two constructs for each *c-myc* exon 1 sequence were formed; (+) constructs in which *c-myc* exon 1 was in the correct 5'-3' orientation and (-) constructs in which *c-myc* exon 1 was in the reverse 3'-5' orientation. (B) Restriction digests of plasmid constructs as indicated. For each set of plasmids samples are loaded in the order: undigested plasmid, *SalI/BamHI* digest, *PvuII* digest. Lanes 1 & 17 contain λ *HindIII* and 100 bp markers respectively.
Additionally, the vector pXLJ'0 with no insert was used as a negative control and the plasmid pXLJ0DA1099 containing the Theiler's murine encephalomyocarditis virus (TMEV) IRES was used as a positive control of internal initiation. In all cases the upstream cistron encodes the cyclin B2 protein from *Xenopus laevis* and the downstream cistron encodes a modified version of the non-structural protein (NS') from influenza virus. However, in the positive TMEV IRES control construct the NS' product is extended by 11 amino acids due to the presence of the authentic TMEV initiation codon in frame with that of the NS' protein.

### 6.2.6 *In vitro* translation of dicistronic reporter constructs

The pXLJ'0 series of constructs were linearised downstream of both cistrons by restriction digestion using *EcoRI* and transcribed *in vitro* using T7 RNA polymerase. Unincorporated nucleotides were removed by centrifugation through G-50 columns. Samples were then extracted with phenol and chloroform and ethanol precipitated prior to translation in rabbit reticulocyte lysates. Lysates were programmed with equimolar quantities of uncapped RNA transcripts (50 μg/ml) and *in vitro* assays were performed under standard conditions. The results of *in vitro* translation of all the constructs is shown in Figure 6.6. In each case it is necessary to consider relative amounts of translation from each cistron rather than absolute quantities observed.

As predicted the pXLJ'0 negative control construct produced predominantly cyclin B2 product whereas the positive TMEV IRES control construct primarily produced the downstream NS' product. All of the dicistronic vectors containing *c-myc* exon 1 sequences produced much larger quantities of the upstream cyclin B2 product compared to the NS' cistron. However, the amount of the downstream cistron varied depending on whether sequences were in the correct (+) or reverse (-) orientations, suggesting the existence of a functional difference between the two. Sequences in the reverse orientation were found to decrease levels of the NS' product, compared to those of the negative control. This is most likely to be explained by the fact that addition of 605 nucleotides of structured intercistronic sequence was an impediment to ribosome progression and hence a reduced frequency of readthrough was observed. It would therefore follow that the same 605 nucleotide fragment in the correct orientation would also be expected to reduce readthrough from the NS' cistron. However, both the Wt and Mt sequences in the correct orientation increased the level of the NS' cistron relative to the cyclin B2 product when compared with the negative control. This would tend to suggest that the *c-myc* exon 1 sequence in the correct orientation is capable of promoting a low level of internal ribosome entry.
Figure 6.6: *In vitro* translation of dicistronic pXLJ’0 reporter constructs. Vectors pXL-Wt(+), pXL-Wt(-), pXL-Mt(+), pXL-Mt(-), pXLJ’0 (negative control) and pXLJ0DA1099 (positive IRES control) were linearised with Eco RI, transcribed *in vitro* using T7 RNA polymerase and then *in vitro* translated in a rabbit reticulocyte lysate system incorporating radiolabelled methionine. The products were resolved by SDS-PAGE (20% polyacrylamide gel) and dried gels were exposed to X-ray film.
Other possibilities to explain this discrepancy between c-myc exon 1 orientations eg. that it may be arising from the different arrangements of sequential nucleotides, cannot be discounted. However, as neither orientation contains short uORFs, which could potentially alter downstream translation, and a stem loop of -100 kcal/mol is predicted to be inserted immediately downstream of the cyclin B2 cistron in (+) c-myc constructs, internal initiation is the most plausible explanation for the observed data.

In vitro translation experiments were repeated several times and consistently gave similar results for a given batch of rabbit reticulocyte lysate. Accordingly the percentages of the total amount of protein for the cyclin B2 and NS' products were quantified after phosphorimager analysis (Molecular Dynamics; ImageQuant) and plotted graphically for each construct. The average values for one set of experiments are shown in Figure 6.7.

In the negative control approximately 85 % of translation occurred from the cyclin B2 cistron with readthrough of the NS' cistron being responsible for only 15 % of the total protein production. In both c-myc exon 1 reverse orientation constructs this frequency of NS' production dropped to around 10 % but increased in the correct orientation constructs to levels of 30 % and 55 % for the Wt and Mt constructs respectively. These values were very much lower than the TMEV IRES positive control construct (95 % NS' production) but demonstrates that c-myc exon 1 contains an IRES element and furthermore, that the Mt sequence is approximately 1.8 fold stronger in promoting the internal entry of ribosomes.

6.2.7 In vitro translation of dicistronic constructs supplemented with HeLa cell extracts

As mentioned above, although elevated, the levels of the downstream NS' cistron observed with c-myc exon 1 sequences in the correct orientation were minimal compared to those seen using the positive control TMEV IRES. Therefore to examine if rabbit reticulocyte lysates were deficient in trans-acting factors required for efficient c-myc internal ribosome entry, in vitro translation assays were supplemented with HeLa cell extracts. The results using various dicistronic constructs with different HeLa cell extract concentrations are shown in Figure 6.8.

Addition of increasing amounts of HeLa cell extracts in all cases was found to inhibit the productivity of both the cyclin B2 and NS' cistrons equally and to generally increase the number of background products as a result of non-specific initiation events. This suggests that the HeLa cell extracts contain either proteases or RNases which degrade protein or RNA factors and hence alter the specific protein synthesis capacity of the rabbit reticulocyte lysates. Alternatively, factors present in the HeLa cell extracts actively
Figure 6.7: Graph showing the relative translational efficiencies from the upstream cyclin B2 cistron and the downstream NS' cistron for each of the pXLJ'0 dicistronic reporter constructs. Translation products were analysed using a Molecular Dynamics Phosphorimager; the relative amounts of the cyclin B2 and NS' proteins were calculated and expressed as a percentage of the total amount of translation observed for each construct. Average values obtained from a series of experiments using the same batch of rabbit reticulocyte lysate are plotted.
Figure 6.8: *In vitro* translation of dicistronic pXLJ'0 reporter constructs supplemented with different concentrations of HeLa cell extracts as indicated. *In vitro* translation reactions were carried out in rabbit reticulocyte lysates in the presence of radiolabelled methionine under equivalent ionic conditions. Products were resolved by SDS-PAGE (20% polyacrylamide gels) and dried gels subsequently exposed to X-ray film. Constructs as detailed in Figure 6.6.
repress translation from standard initiation sites and enhance translation from non-canonical sites. Factors specifically required for promoting the internal entry of ribosomes to c-myc exon 1 sequences however appeared to be lacking.

6.2.8 *In vitro* translation of dicistronic constructs supplemented with MM cell extracts

To further examine if other specific trans-acting factors could be required to promote c-myc internal ribosome entry *in vitro* translation reactions, carried out in rabbit reticulocyte lysates, were supplemented with increasing concentrations of GM2132 myeloma cell extract. The results obtained for the Mt c-myc exon 1 dicistronic construct pXL-Mt(+) and the positive control TMEV IRES construct pXLJ0DA1099 are shown in Figure 6.9.

Addition of increasing amounts of GM2132 myeloma CE was found to slightly decrease NS' production in the positive TMEV IRES control although the ratio of the NS':cyclin B2 proteins did not significantly alter. In the c-myc exon 1 Mt construct, however, NS' levels increased, relative to cyclin B2 production, as progressively more myeloma CE was added. This was not accompanied by elevated levels of background translation products as observed with HeLa cell extracts. Taken together, this would tend to suggest that specific factors found within the myeloma cell extract can augment the IRES function of the c-myc 5'UTR. It remains uncertain if this phenomenon is restricted to only the Mt sequence and whether the same affect would be achieved using GM03201 control cell extracts, as these variables were not tested.

6.2.9 *In vitro* translation of dicistronic constructs using different KCl concentrations

The translational fidelity of both uncapped mRNAs and IRES mediated transcripts has been reported to be highly dependent on the concentration of KCl within the rabbit reticulocyte lysate system, with every transcript displaying a different optimal KCl concentration for translation (Jackson, 1991). Indeed, high K+ ion concentrations, which serve to non-specifically increase mRNA secondary structures have been shown to inhibit the translation of most mRNAs except heat shock proteins (Zapata *et al.*, 1991). It was therefore decided to examine the effects of increasing KCl concentrations on the *in vitro* translation of the various dicistronic constructs. Accordingly, rabbit reticulocyte lysates ([KCl] of approx. 80 mM) were supplemented with KCl to final concentrations of around 100 and 120 mM. The results of these experiments are shown in Figure 6.10.
Figure 6.9: *In vitro* translation of dicistronic pXLJ'0 reporter constructs supplemented with different concentrations of GM2132 MM cell extracts as indicated. *In vitro* translation reactions were carried out in rabbit reticulocyte lysates in the presence of radiolabelled methionine. Products were resolved by SDS-PAGE (20 % polyacrylamide gels) and dried gels subsequently exposed to X-ray film.
Figure 6.10: In vitro translation of dicistronic pXLJ’0 reporter constructs with varying KCl concentrations. A standard rabbit reticulocyte lysate system was supplemented with KCl to give final concentrations of approximately 80 - 120 mM. Products were then resolved by SDS-PAGE (20% polyacrylamide) and the results visualised by autoradiography. The results shown were obtained using a single batch of rabbit reticulocyte lysate.
In the negative control construct higher concentrations of KCl were found to inhibit the translation of both cistrons with only residual levels of protein synthesis at 120 mM KCl. This was in direct contrast to the positive TMEV IRES control construct in which 120 mM KCl concentrations were found to stimulate internal initiation, greatly enhancing translation from the downstream cistron and correspondingly reducing the translation from the upstream cyclin B2 cistron. No similar switch in initiation was observed with either the Wt (+) or Mt (+) c-myc exon 1 sequences, with increased KCl concentrations causing a marked inhibition in translation of both cistrons in the Mt construct but having negligible effect with the Wt sequence. The reason for this dissimilarity is uncertain. Both reverse orientation c-myc constructs, Wt (-) and Mt (-), also displayed an inhibition in translation at higher KCl concentrations but again no change in the cyclin B2: NS' ratio was observed.

It would thus appear that the KCl concentration markedly affects translational efficiencies being generally repressive at high concentrations possibly by stabilisation of inappropriate secondary structures or perturbing interactions with factors involved in the initiation of translation. Variations in KCl concentrations can also explain the small inconsistencies in the translational efficiencies observed between different batches of rabbit reticulocyte lysates. These findings also prompted examination of the translational efficiencies at lower KCl concentrations. For this a different reticulocyte lysate system containing much lower KCl concentrations was utilised (Flexi RRLS; Promega). The results are shown in Figure 6.11.

Similar to the previous findings, KCl concentrations of between 40 and 80 mM had little effect on the translational efficiency of either cistron in the negative control construct and in the positive TMEV IRES control construct increasing KCl concentrations were accompanied by a shift in production of the downstream NS' product. More interestingly, the Wt and Mt dicistronic constructs containing c-myc exon 1 in the correct orientation also displayed a switch of preferential cistron translation which was dependent on KCl concentration. Thus, for the Mt construct at concentrations of <40 mM KCl translation occurred primarily from the downstream cistron whereas at concentrations of 60 mM KCl, approximately equal quantities of both cistrons were observed. Further increases in KCl concentrations resulted in the preferential translation of the upstream cyclin B2 cistron. This pattern was similarly observed with the Wt construct except that the shift from the NS' cistron to the cyclin B2 cistron occurred at a lower KCl concentration. No corresponding switch in cistron production was observed in the reverse orientation c-myc exon 1 constructs, Wt (-) and Mt (-), implying that production of the downstream NS' cistron at low KCl concentrations is not simply an artefact of the presence of an additional 605 nucleotide sequence.
**Figure 6.11**: *In vitro* translation of dicistronic pXLJ'0 reporter constructs with varying KCl concentrations. A Flexi rabbit reticulocyte lysate system (Promega) was supplemented with KCl to give final concentrations of approximately 0 - 120 mM. Products were then resolved by SDS-PAGE (20% polyacrylamide) and the results visualised by autoradiography. The results shown were obtained using a single batch of rabbit reticulocyte lysate.
For the *c-myc* exon 1 Wt and Mt dicistronic constructs and the positive TMEV IRES control the percentage of translation occurring from the downstream NS' cistron was calculated by laser densitometry (Molecular Dynamics; ImageQuant). These values were then plotted against the KCl concentration of the rabbit reticulocyte lysate mixture as shown in Figure 6.12. From this it can clearly be seen that internal initiation is favoured at high KCl concentrations by the TMEV IRES but low KCl concentrations enhance the IRES capability of *c-myc* exon 1. Furthermore, the Mt sequence exhibits an augmented capacity to promote the internal entry of ribosomes, compared to that of the Wt sequence, and this is particularly evident at lower KCl concentrations. However, the percentages of translation values obtained are not absolute and vary from those obtained in Figure 6.7 principally for two reasons. Firstly, the methods used to quantify the images were different; phosphorimager analysis versus laser densitometry. Secondly, starting KCl concentrations were not defined and substantial variations between different batches of rabbit reticulocyte lysates were seen. Therefore, although translation reactions were supplemented with equivalent amounts of KCl, considerable deviations in the final KCl concentration would be attained.

In conclusion *c-myc* exon 1 is capable of directing the internal initiation of translation and this function is more pronounced at lower KCl concentrations (<approx. 80 mM). Moreover, for all KCl concentrations tested the Mt sequence has an augmented capacity to direct the internal initiation of translation. This would tend to imply that there is a functional difference between the translational efficiencies of the Wt and Mt *c-myc* exon 1 sequences.

### 6.3 Summary

The effects of human *c-myc* exon 1 on translation of heterologous reporter genes was examined *in vitro* using a rabbit reticulocyte lysate system. Using monocistronic constructs it was found that *c-myc* exon 1 sequences, cloned upstream of a luciferase reporter gene, were severely inhibitory to translation, consistent with previous data obtained using murine *c-myc* exon 1 (Darveau *et al*., 1985; Parkin *et al*., 1988). This inhibition was partially relieved by addition of HeLa cell extracts, however, the effects were non-specific as the increase in luciferase production was accompanied by elevated levels of background translation. No apparent differences were observed between the Wt and Mt sequences but, due to the exceptionally low levels of reporter gene translation, small disparities in translational efficiencies between the two constructs cannot be dismissed. Capping of these *c-myc* exon 1 transcripts did not appreciably increase luciferase production.
Figure 6.12: Graph showing the effects of KCl concentrations on the production of the downstream NS' protein for the dicistronic reporter constructs pXL-Wt(+), pXL-Mt(+) and pXLJ0DA1099. Translation products were quantified by laser densitometry (Molecular Dynamics; ImageQuant), the relative amounts of the NS' protein calculated and expressed as a percentage of the total amount of translation observed for each construct. The data plotted were obtained from a series of experiments using the same batch of rabbit reticulocyte lysate.
Consideration of hypothetical secondary structures of \textit{c-myc} exon 1 with regard to translational efficiencies suggested a repressive role in translation and provided a theoretical explanation for the experimental data obtained \textit{in vitro}. These results taken together with the lack of enhanced translation observed using either capped transcripts, or by the addition of putative trans-acting factors, suggested that a cap-independent mechanism of internal initiation could be speculated to function for \textit{c-myc} translation.

Accordingly, \textit{c-myc} exon 1 sequences were cloned between two cistrons in a dicistronic reporter construct and the translational efficiencies from each examined under different conditions \textit{in vitro}, again using a rabbit reticulocyte lysate system. Insertion of the 605 nucleotide \textit{c-myc} exon 1 sequence in the opposite orientation was found to decrease the amount of readthrough from the downstream cistron. However, under standard conditions, the percentage of product from the downstream cistron relative to the total amount of translation increased if the \textit{c-myc} exon 1 sequence was in the correct orientation. Furthermore, the mutant sequence consistently produced a higher proportion of the downstream product relative to that of the Wt sequence. This would tend to imply that \textit{c-myc} exon 1 is capable of directing a low level of internal ribosome entry and the Mt sequence displays an approximately 1.8 fold enhanced capacity for this function, under the conditions examined.

Addition of postulated trans-acting factors from HeLa cell extracts were not found to alter the translation from either cistron in the \textit{c-myc} containing constructs. However, enhanced translation of the downstream cistron was observed upon \textit{in vitro} translation of Mt \textit{c-myc} exon 1 constructs supplemented with GM2132 myeloma cell extracts. This MM cell line may therefore contain specific factors necessary for efficient \textit{c-myc} internal ribosome entry. It remains to be determined as to whether the effect is restricted to only the Mt \textit{c-myc} exon 1 sequence and is strictly dependent on MM plasma cell proteins.

The equilibrium of cistron production from the \textit{c-myc} dicistronic constructs was found to be highly dependent on KCl concentrations within the rabbit reticulocyte lysate system. Thus, at low KCl concentrations the downstream product predominated whereas at higher KCl concentrations production of the upstream cistron was greater. Furthermore, for every concentration of KCl examined, the Mt sequence displayed approximately a 1-4 fold elevation in production of the downstream cistron when compared to the Wt sequence. Thus, it would appear that at low KCl concentrations \textit{c-myc} exon 1 is capable of directing the internal entry of ribosomes but this function becomes diminished at higher KCl concentrations. However, the Mt sequence, possibly via small alterations in secondary structure, exhibits a stronger capacity to promote the internal entry of ribosomes and consequently is capable of sustaining augmented levels of internal initiation of translation at higher KCl concentrations relative to the Wt sequence. It is therefore possible to
speculate that within a cellular environment KCl concentrations may be such as to permit a higher proportion of internal initiation from c-myc mRNAs containing the Mt sequence resulting in increased translation and augmented c-myc protein levels.

In conclusion it thus appears that the C→T mutation, observed in the multiple myeloma cell lines, is functionally capable of altering the translational efficiency of c-myc transcripts and could therefore, in part, be responsible for the elevated levels of c-myc protein observed.
CHAPTER 7

Discussion
Aberrant translational control of the c-myc oncogene has been postulated to explain the disparate levels of c-myc mRNA and protein observed in cell lines derived from patients with multiple myeloma. To date, no other genetic marker has been consistently found associated with the malignant phenotype and thus further investigation into this putative control mechanism of c-myc expression was warranted.

Cell lines were first examined for the expression of two cell surface antigens, CD38 and CD45, which have previously been used to identify myeloma plasma cells (Billadeau et al., 1996; Witzig et al., 1996). This attested to the myelomatous origin of all the MM cell lines, with the exception of U266, but could not rule out the possibility that other genetic alterations may have accumulated with prolonged growth in vitro. Attempts to isolate new MM cell lines were unsuccessful. However, malignant plasma cell types derived from bone marrow samples were expanded and maintained in culture for up to 5 months. This would greatly facilitate further study, which previously would have been precluded due to insufficient cell numbers, and would significantly reduce the frequency of mutations arising as a result of in vitro adaptation. In addition, by refining the growth conditions further, perhaps using a combination of CD38 enrichment, IL-6 treatment and bone marrow stromal cell feeder layers, it may become possible to routinely establish multiple myeloma cell lines from MM patient material rather than from extra-medullary sources.

To determine if an altered nucleotide sequence could be responsible for the increase in c-myc translation, c-myc exon 1, a region previously implicated in translational control, was sequenced from five MM cell lines and four control cell lines. This revealed a consistent C→T transition at position 2756 in four of the MM cell lines, the disparity again being found in the U266 cell line. Such anomalous behaviour of this cell line has been reported in previous studies examining c-myc protein levels (Jernberg-Wiklund et al., 1992). In this case, unlike the other MM cell lines examined, U266 did not contain high levels of the c-myc protein but instead had elevated levels of the L-myc protein (Jernberg-Wiklund et al., 1992). The absence of the C→T nucleotide substitution in this cell line may therefore not be coincidental and tentatively suggests that there is a correlation between the C→T sequence alteration and enhanced translation of the c-myc message.

It is possible that the C→T transition could represent a polymorphism of the c-myc locus at this site. However, as the mutation was not observed in any of the control cell lines examined, nor was it seen in control patient samples, it does not appear to be prevalent amongst the general population. Moreover, the lack of MM cell lines homozygous for the mutation could itself imply that the mutation is deleterious.
Preliminary data obtained using an insensitive PCR restriction digestion technique also detected this mutation in MM patient samples. However, because the sample size was very small and a false positive result was obtained, it remains uncertain as to whether the mutation is associated with the malignant phenotype. To prove conclusively that the C→T mutation is linked with MM much larger numbers of patients would require to be screened using more sensitive techniques eg. single strand conformational polymorphism (SSCP) or single nucleotide primer extension (Snupe). These PCR based methods are capable of detecting low level sequence variations, even against wild type backgrounds, and hence would eliminate problems encountered with non-clonal populations. It may also be of interest to use such techniques to examine familial cases of multiple myeloma to determine if the C→T mutation could be a predisposing factor in development of the disease.

As initiation of protein synthesis is the rate limiting step of translation, factors binding to the c-myc 5'UTR could potentially regulate translation. This was therefore investigated using a variety of techniques which all revealed similar findings. Firstly, the Mt sequence displays an approximately 10 fold greater binding affinity for proteins when compared to the Wt 5'UTR sequence. Secondly, both the Wt and Mt c-myc 5'UTRs bind a number of proteins (78, 61, 43 kD) (also see Table 5.2), but the Mt sequence shows greatly enhanced binding to two proteins of 98 and 38 kD. Thirdly, the control lymphoblastoid cell line (GM03201) and the MM cell line (GM2132) show some variation in protein binding factors. This may either reflect the different differentiation states of these B-cell clones or be a consequence of the tumourogenic nature of the myeloma cells. It would thus appear that the c-myc 5'UTR is capable of binding a number of protein factors and these differ slightly depending on the cell type involved. Moreover, the Mt sequence displays enhanced binding in particular to two proteins of 38 and 98 kD.

The identities of the majority of the protein factors found to bind the c-myc 5'UTR remain undetermined as they were not present in sufficient quantities to permit protein sequencing. By using large scale protein preparations, in combination with RNA affinity columns, though, it should be possible to isolate large enough quantities to obtain protein sequence data. This information can then be used to search sequence databases to determine if the proteins correspond to known genes or whether they are encoded by novel cDNAs, which would require further investigation. It also remains uncertain as to whether any of the binding proteins identified are purely c-myc transcript specific or represent more generalised RNA binding factors, although the latter of these two scenarios is more likely and consistent with the data obtained.

It would be of interest to map the sites to which these proteins bind within the 5'UTR, although one would predict that the 38 and 98 kD species would bind around position
2756. By using smaller regions of the 5'UTR and nuclease mapping studies it may be possible to define these boundaries more precisely, although it may also be necessary to consider that long range interactions may influence binding.

From computer modelling studies the secondary conformation of *c-myc* exon 1 is predicted to be highly structured with an energy of approximately -200 kcal/mol. This arrangement, due to the presence of many large stem loops, would impose severe restrictions on the scanning ribosome complex, which is consistent with the extreme inhibitory effects of *c-myc* exon 1 on reporter gene translation observed *in vitro*. Consequently, it was not possible to determine if there were discernible differences in translational efficiencies between the Wt and Mt sequences. However, although the mutant is predicted to increase the secondary structure by forming a short stem loop (-0.4 kcal/mol) the effects on the global secondary structure would be minimal and hence effects on a scanning ribosome complex would also be expected to be negligible. Thus, from this model, the mutation *per se* would be unlikely to alter the translational efficiency of *c-myc*. Nevertheless, it should also be noted at this point that the actual secondary structure of the *c-myc* 5'UTR may be very different from those predicted. Therefore confirmation of this would require secondary structure mapping *in vitro* using a variety of methylation/nuclease techniques. This itself however may pose problems particularly if the secondary structure interactions are dynamic in nature. In addition, as the *c-myc* 5'UTR *in vivo* is not normally found in isolation, secondary structure interactions with other regions of the *c-myc* mRNA could be important in determining the overall structure and hence "translatability" of the message.

The *in vitro* translation system used to examine protein synthesis rates creates an artificial environment for translation where there is no competition between messages and thus is dissimilar to that found within a natural cellular context. mRNAs that would normally compete poorly for translation initiation factors would therefore be expected to be translated. However, as the *c-myc* 5'UTR inhibited the translation of heterologous reporter genes, this would tend to imply that other factors are necessary for efficient *c-myc* translation. Accordingly, rabbit reticulocyte lysates were supplemented with HeLa cell extracts or myeloma cell extracts, but only the latter of these were able to partially remove the suppressive effects on translation. Thus it is apparent that myeloma cell extracts contain factors necessary to promote *c-myc* translation *in vitro*.

Effects of the *c-myc* 5'UTR on translation of heterologous reporter genes were not examined *in vivo*. It would therefore be of interest to transiently transfect various cell types with wild type and mutant constructs to determine whether: 1) The cellular background influences *c-myc* reporter gene translation. 2) If the Wt and Mt sequence
differentially affect translation. 3) Whether the human c-myc 5'UTR is also inhibitory to translation in vivo.

The extensive size of the c-myc 5'UTR, coupled with its inhibitory effects on translation and predicted secondary structure, prompted the examination of this region in dicistronic reporter constructs. From this it was found that the c-myc 5'UTR is capable of directing a low level of translation mediated by internal initiation which was favoured at low KCl concentrations. As KCl stabilises secondary structure this would tend to suggest that a dynamic or flexible structure is required for this function. Moreover, the mutant sequence displays a 1-4 fold enhancement in internally initiated translation when compared to the Wt sequence presumably by adopting a conformation more conducive to internal initiation. Thus it appears that the Wt and Mt sequences are functionally distinct and, furthermore, that the C→T mutation is sufficient to cause an increase in translation. This augmented translation is not adequate to account for the 10-25 fold elevation in c-myc protein levels observed in the MM cell lines, however, in combination with the effects of other protein factors in these cell lines, enhanced translation of c-myc mRNA could occur. It is not clear if the effects on translation of the mutation are limited only to myeloma cells or whether greatly augmented c-myc protein levels could similarly be achieved in control lymphoblastoid cell lines transfected with Mt c-myc constructs.

Several lines of evidence corroborate that the 5'UTR of c-myc could contain an internal ribosome entry segment (IRES). Firstly, the extensive size of exon 1, its high degree of sequence conservation and large amounts of predicted secondary structure make it comparable to picornaviruses. Secondly, it displays regions of complementarity to the 18S ribosomal RNA (Matveeva & Shabalina, 1993). Thirdly, c-myc transcripts have been reported to localise to nucleoli, the sites of ribosome assembly (Bond & Wold, 1993). Fourthly, 17 upstream CUG codons are found within the c-myc 5'UTR which may be akin to the upstream AUG codons found within picornaviral transcripts. Finally, the discovery of an IRES element in a mammalian cellular transcript is not unprecedented. Indeed, IRESes have been identified in immunoglobulin heavy chain binding protein (BiP), fibroblast growth factor 2 (FGF-2) and eIF-4G (Macejak & Sarnow, 1991; Vagner et al., 1995; Gan & Rhoads, 1996).

To verify that the c-myc 5'UTR contains an IRES, or IRES like element, it would be necessary to transiently transfect c-myc dicistronic reporter constructs into cell lines. Again both the Wt and Mt sequences could be compared and the affects of different cell types investigated. In addition, as there are two possible translation start sites it would be of interest to determine if both Myc isoforms utilise the same methods of translational initiation. Internal initiation could possibly favour the production of a particular isoform, for example under certain conditions, due to the production of specific protein factors and
therefore activate/repress a different subset of genes. Indeed, production of MYCHEXI necessitates the requirement for the scanning mechanism of initiation to also function. Alternatively, promoter usage which would dictate the 5' leader sequence could also influence the mode of initiation of translation. Consequently, examination of c-myc promoter usage and the ratio of Myc 1:Myc 2 in the MM cell lines could provide further clues to the mechanism of the aberrant control of the c-myc gene observed. It would also be necessary to define the minimal internal ribosome entry segment and determine the factors required for full IRES activity by reconstituting the system in vitro and adding back purified factors eg. PTB and La.

Further studies on the MM cell lines have shown that c-myc mRNA is 3.4 times more associated with polysomes in these cell lines than control cell lines (Paulin et al., 1996). As this is not accompanied by an increase in polyosme size or an altered translational elongation rate there appears to be increased mobilisation of the c-myc message in these cell lines. It is possible therefore to envisage a situation as depicted in Figure 7.1. In the control lymphoblastoid cell lines c-myc protein levels are low as the c-myc mRNA is inefficiently translated by: 1) The scanning mechanism of translation due to the length and secondary structure of the mRNA. 2) A mechanism of internal initiation as the appropriate protein binding factors are either absent, present in insufficient quantities or IRES activity is suppressed by other binding factors which presumably normally bind the mRNA in mRNP particles. However in the MM cell lines c-myc protein levels are elevated because: 1) A C—>T point mutation in the c-myc 5'UTR causes adoption of a secondary structure more conducive to protein factor binding, specifically enhancing the binding of a 38 kD and a 98 kD species. It may be of significance that two proteins of similar molecular weights have been implicated in HRV IRES functioning (Borman et al., 1993). 2) There are increased levels of some protein binding factors eg. 98 kD protein, which promote internal initiation and there are decreased levels of other protein factors which could repress IRES function. Enhanced binding of more IRES activating proteins would thus serve to recruit more c-myc mRNA to ribosomes and hence result in increased translation of the c-myc mRNA.

If this hypothesis is then extrapolated further and related to the malignancy it is possible that the C—>T mutation in c-myc could be an initiating event in multiple myeloma. Normally as plasma cells are terminally differentiated cells Myc levels would presumably be very low. However acquisition of the C—>T point mutation could enhance the IRES capacity of c-myc resulting in a small increase in Myc protein levels either in the presence or absence of additional protein factors. This would impart some growth advantage to the cell by either increasing its proliferative capacity or, more likely, preventing apoptotic cell death. In addition cells may also become associated with the bone marrow stromal cells. In this connection, EBV immortalised B-cell lines transformed with c-myc have been
Figure 7.1: Model to explain the increased translation of c-myc observed in multiple myeloma cell lines. (A) In the control cell lines there is inefficient translation of c-myc mRNA due to: 1) its secondary structure, 2) there are proteins present which sequester the message in mRNP particles suppressing translation, 3) mobilising proteins are present but not in sufficient quantities to stimulate translation. (B) In the MM cell lines a C-T point mutation in the 5'UTR permits the mRNA to adopt a different secondary structure which enhances the binding of protein factors. In addition, there may be increased levels of mobilising factors (e.g., 98kD) and decreased levels of repressive factors in these cell lines. Thus, taken together, all these elements serve to increase the recruitment of c-myc mRNA to ribosomes resulting in elevated c-Myc levels. Furthermore, preliminary evidence indicates that this translational control mechanism may function using a method of internal initiation.
shown to possess up-regulated levels of CD38 and a number of other adhesion molecules (Cutrona et al., 1995). Within this environment the clone could gradually develop and acquire further mutations allowing propagation and expansion of the neoplastic clone.

In conclusion aberrant translational control of the c-myc gene in multiple myeloma provides a novel and alternative mechanism for deregulating c-myc expression as distinct from the currently recognised methods of gene amplification and chromosomal translocation. Primarily, increased translation of the c-myc gene occurs through a C→T point mutation at position 2756 lying within the exon 1 region. This enhances and/or alters protein factors binding to the 5'UTR and serves to increase the apparent ability of c-myc mRNA to promote the internal initiation of translation. In addition, in the MM cell lines this capacity to direct internal ribosome entry may be magnified further by modifications in the protein factors present aggrandizing the levels of c-myc protein. It would therefore be of interest to determine if other cell lines, which also contain high levels of c-myc protein, similarly show a translational deregulation of the c-myc gene and furthermore, to examine if the above mechanism is a common phenomenon or unique and restricted only to multiple myeloma.
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PUBLICATIONS
arising from this work


Aberrant translational control of the c-myc gene in multiple myeloma

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We demonstrate a 10- to 25-fold increase in the amount of c-myc protein in several independent cell lines derived from patients with multiple myeloma (MM). This is not accompanied by a corresponding increase in the overall level of the c-myc mRNA. There is, however, a 3.4-fold increase in the amount of c-myc mRNA associated with the polysomes in these cell lines without any detectable change in either the polysome size or the rate of translation elongation, thus suggesting that there is an increase in the extent of mobilisation of c-myc mRNA to the polysomes in MM. Analysis of the 5' untranslated region of c-myc has revealed the presence of a mutation, in all of the MM cell lines examined, in a region which has been implicated previously in the translational control of this mRNA species. These data suggest aberrant translational control of the c-myc gene in cell lines derived from patients with MM, which may contribute towards pathogenesis of the disease.

Keywords: c-myc; translational control; multiple myeloma

Introduction

Multiple myeloma (MM) is a disorder which is characterised by both the expansion of a plasma cell type in the bone marrow and local osteolysis. The disease is normally fatal with death occurring from infection and/or haemorrhage between 3 and 5 years after initial disease presentation (Niesvizky et al., 1993).

Interactions between a number of cell types are important in the development of MM. During the initial stages of the disease, the survival, proliferation and attachment of plasma cell types is supported by the bone marrow stromal cells. However, despite extensive studies of the plasma cell types which are thought to be involved in the pathogenesis of MM, the origin of the malignant cell type in this disease is still unclear. Theoretically, these cells could arise either from de-regulation of fully differentiated plasma cells or from pre-plasma cells that retain their proliferation potential and have escaped the differentiation pathways. Both MM plasma cells and normal plasma cells express the cell surface antigen CD38 with MM plasma cells expressing CD38 to a greater degree (Hata et al., 1993). In addition, some MM plasma cell lines have higher than normal levels of CD56 and CD45 (Van Camp et al., 1990; Barker et al., 1992). Heterogeneity within the population of normal plasma cells has limited the accurate definition of MM plasma cells, and a cellular differentiation antigen which is specific to all plasma cell types and all cases of MM has yet to be identified (Barker et al., 1992).

Consistent changes in either the activation of oncogenes or inactivation of tumour suppressor genes have also not been found in MM plasma cells. This is hampered by the fact that there are differences in oncogene expression between MM cell lines and freshly isolated patient material. Mutations in both the Ki- or N-ras oncogenes are associated with the later stages of MM and occur in in vitro cultured cell lines with a variable frequency. In one study N- and Ki-ras mutations were detected in 47% of patient samples (Portier et al., 1992) whilst in a second study 9% of patient samples showed N- and Ki-ras mutations (Corradini et al., 1993). In a third study 27% patients showed N- and Ki-ras mutations at diagnosis (Neri et al., 1989) with the number of mutations increasing to 46% after treatment, suggesting that a degree of clonal selection had occurred as a result of the chemotherapeutic regimens used (Neri et al., 1989). In addition, mutations have been detected in the p53 tumour suppressor gene in freshly derived plasma cells, which differ to those that have been subject to in vitro culture. For example, GC to AT transition mutations within exons 5 and 7 of the p53 gene occur at a frequency of 20% in freshly isolated patient material. This increases to 80% in studies of MM cell lines, which again probably reflects clonal selection in vitro (Portier et al., 1992; Mazars et al., 1992). In patient samples, p53 mutations are found primarily in the terminal stages of the disease and are unlikely to be relevant to the initiation of MM (Neri et al., 1993).

There is an increase in the level of the bcl-2 protein and its cognate mRNA in both MM cell lines and freshly obtained material from MM patients such that the amount of bcl-2 mRNA and protein in MM is of an equivalent level to that found in follicular lymphoma cell lines, which generally possess a 14;18 chromosomal translocation at the bcl-2 locus (Petterson et al., 1992). However, it is unclear whether this high expression is either associated with the malignant phenotype or is a reflection of the level of bcl-2 expression which is typical of normal plasma cells.

The apparent absence of c-myc de-regulation in MM has always been enigmatic particularly in comparison with other human B-cell neoplasias which are often associated with high levels of c-myc expression (Croce et al., 1992).
and Nowell, 1985). In studies of a total of 58 MM patients only two examples of c-myc associated chromosomal translocations and seven of increased c-myc mRNA stability were observed (Selvanyagam et al., 1988; Lewis and McKenzie, 1984). A complex translocation involving the third exon of the c-myc gene resulting in stabilisation of the c-myc mRNA has also been reported in one case of MM (Hollis et al., 1988).

In addition to these mechanisms mentioned above the expression of the c-myc protein can also be modulated by translational control. There are several lines of evidence which suggest that this occurs; (i) c-myc exon 1, which is predominantly non-coding, is well conserved amongst species suggesting that it may have a functional role (Saito et al., 1983; Watt et al., 1983); (ii) Exon 1 contains cis acting nucleotide sequences that can inhibit translation of heterologous genes, both in vitro and in certain in vivo systems (Parkin et al., 1988); (iii) Xenopus c-myc-1 mRNA possesses a region in its 5'UTR between the c-myc promoters P0 and P1 which is important in regulating translation (Lazarus, 1992); (iv) a protein has been isolated which binds mRNA derived from the region between P1 and P2 (Parkin and Sonenberg, 1989); (v) In both Xenopus oogenesis (Godeau et al., 1986; Taylor et al., 1986) and mouse erythroleukaemia cells induced to differentiate with DMSO (Spotts and Hann, 1990), the rate of synthesis of the c-myc protein is not coupled to that of the level of c-myc mRNA and finally (vi) we have shown recently that there is a translational upregulation of the c-myc oncogene in cell lines derived from patients with Bloom's syndrome (West et al., 1995). These data lead us to investigate the possibility that there may be aberrant regulation of c-myc in MM occurring at the level of translation of the message.

We demonstrate here that in MM cell lines the c-myc protein levels are elevated 10–25-fold, without either a concomitant increase in the half life of the protein or the level of the c-myc mRNA. We have shown, using sucrose density analysis, that there is an increase in the polysomal association of the c-myc mRNA in the MM cell lines. This increase does not appear to be due to an elevation in the rate of initiation or elongation of translation, which suggests that instead there is an increase in the mobilisation rate of the c-myc message from inactive mRNA to the polysomes. Examination of the 5' untranslated region of c-myc has shown that in the MM cell lines analysed there is a specific mutation in a region which has been implicated previously in translational control (Parkin et al., 1988).

Results

High levels of the c-myc protein in cell lines derived from patients with MM

The amount of c-myc protein was found to be elevated in 4 MM derived cell lines (GM2132, GM1311, GM1500, GM06923) as determined by ELISA (Figure 1A). This concentration corresponds to between approximately 100 x 10^3 and 250 x 10^3 molecules of c-myc protein/cell in MM and 5 x 10^3 to 10 x 10^3 molecules/cell in the B-cell lymphoblastoid cell lines. In two cases (GM2132 and GM06923) the c-myc protein levels were comparable to those observed in COLO320DM, a cell line derived from a colon adenocarcinoma which has a translocation and an approximately 20-fold amplification of the c-myc gene (Schwaab et al., 1986) and correspondingly high levels of c-myc mRNA and protein. The results obtained by ELISA were supported by quantitative immunoprecipitation (Figure 1B).

This increase in the c-myc protein in MM is in agreement with a study on freshly derived plasma cell types from patients with MM (Greil et al., 1991). The latter demonstrated a qualitative increase in the level of c-myc protein in 92% of cases examined, whereas our study shows a quantitative 10–25-fold increase in the level of this protein in all cell lines investigated when compared to that observed in lymphoblastoid cell lines.

One explanation for the increase in the levels of the c-myc protein could lie in stabilisation of the c-myc protein, for example by mutation, post translational modification or defects in the proteolytic systems that are involved in c-myc protein turnover.

![Figure 1](image-url)
required for its rapid turn-over. To test this hypothesis, the half life of the c-myc protein was determined. In common with other proteins which appear to have regulatory functions, the c-myc protein has a short half life corresponding to approximately 20–30 min (Hann and Eisenman, 1984). Thus a pulse chase analysis was performed on the cell lines derived from MM patients in order to analyse the half life of the c-myc protein in this system. No differences were observed in the half life of the c-myc protein in MM cell lines when compared to controls (Figure 2A–C) and the half life was found to be approximately 20 min which is consistent with values already obtained for other systems (Hann and Eisenman, 1984). For the c-myc protein to attain a level of 100 pg/mg of cell lysate (the lowest amount observed in the MM cell lines), purely on the bases of stability, the half life would be required to increase to approximately 160 min. It is therefore unlikely that such a mechanism accounts for the high level of expression of the c-myc protein in MM.

We are not aware of any reports which describe a consistently high level of c-myc mRNA in cell lines derived from patients with MM, however, to eliminate this possibility in the system used here Northern analysis was performed to determine the amount of c-myc mRNA in the MM cell lines.

**MM plasma cells do not express high levels of c-myc mRNA**

The absolute amounts of c-myc mRNA and GAPDH in both the MM and lymphoblastoid cell lines were determined by Northern RNA hybridisation analysis (Figure 3A). The relative amounts of c-myc and GAPDH mRNA were determined using a Molecular Dynamics phosphorimager and normalised to that found in the cell line GM1953 where the c-myc mRNA:GAPDH mRNA was considered to equal one (Figure 3B). The respective ratios for the remaining cell lines were: GM0892, 1.5; GM2132, 2.5; GM1500, 0.6; GM1311, 0.86; GM06923, 1.5. For COLO 320 DM the ratio was 36, indicating the gross overexpression of c-myc mRNA in this cell line. From these data we conclude that there is not a generalised excess of c-myc mRNA in these MM cell lines. In addition, there is not a detectable alteration in the size of the c-myc transcript between control and MM cell lines suggesting that there is no translocation of the c-myc gene 3' of the promoter.

One possible explanation for the dissociation of the amount of c-myc protein, produced by the MM cells, from the level of c-myc mRNA could be that in MM cells there is an increase in the rate of initiation of translation of this message. Therefore to investigate the...
this possibility, the degree of association of the \textit{c-myc} mRNA with the polysomes was determined in both the MM cell lines and the control cell lines.

\textit{The c-myc mRNA is associated with the polysomes to a greater extent in the cell lines derived from MM patients than in the control cell lines.} 

Cytoplasmic mRNAs are found in two distinct states; those which are actively being translated by ribosomes, in polysomes; and those which are not active, in mRNP particles. Regulation of the proportion of active to non-active mRNAs (or mobilisation), is one method by which the degree of translation of a particular message can be regulated. This is distinct from the level of translational control which results from changes in re-initiation rates on the polysomes (Hershey, 1991) since alterations in re-initiation events causes a change in the size of the polysomes. Any change in polysome size will be proportional to the initiation rate and inversely proportional to the elongation rate. Hence, at a constant elongation rate, an increase in the rate of initiation will result in larger polysomes (Hershey, 1991). An increase in the proportion of the \textit{c-myc} mRNA on the polysomes would indicate an increase in \textit{c-myc} mRNP mobilisation, whilst an increase in polysome size would suggest that there was a greater number of re-initiation events.

Polysomes and monosomes from MM and control cell lines were separated by sucrose density centrifugation and the amount of \textit{c-myc} mRNA associated with the polysomes was determined. The polysome peaks were assigned by treatment of cells with puromycin, a specific inhibitor of elongation of nascent polypeptide chains and is thus a compound which can be used to dissociate polysomes (Figure 4). The GAPDH polysomes (fractions 6 - 10 Figure 4A) and \textit{c-myc} polysomes (fractions 5 - 9 Figure 4C) are clearly lost upon puromycin treatment (Figure 4B and D respectively). Polysome profiles of \textit{c-myc} and GAPDH message that are representative of those obtained with both the control cell lines and the MM cell lines are shown using GM1953 and GM2132 as examples respectively (Figure 5). To analyse these data, the amount of either \textit{c-myc} or GAPDH mRNA associated with the polysomes and monosomes was determined and expressed as a ratio for both the \textit{c-myc} and GAPDH mRNAs (Table 1). These ratios were subsequently expressed relative to each other for each cell line i.e. \textit{c-myc} mRNA (polysomal)/\textit{c-myc} mRNA (monosomal) divided by GAPDH mRNA (polysomal)/GAPDH mRNA (monosomal) to give an

![Figure 4](image-url)
indication of the relative excess association of the translated c-myc mRNA to the translated GAPDH mRNA. These experiments were performed on three independent occasions and the mean determined in order to give the data indicated in Table 1. Overall, the MM cell lines have an average 3.4-fold increased ratio (2.77/0.82) of polysomal-associated c-myc, clearly indicating that there is aberrant translation of the c-myc mRNA in MM. This large increase in the degree of polysome associated c-myc mRNA could account for the 10–25 increase in the amount of the c-myc protein produced. There is no evidence for a precise linear correlation between these two determinants and even a small shift in the degree of polysomal association of a message can result in a very large increase in the synthesis of the corresponding protein. For example, increased translation of HSP70 mRNA following heat-shock showed that a 10-fold increase in HSP70 protein expression was only accompanied by a shift in the percentage of the mRNA found in a translationally repressed form on the polysomes from 77% to 68% (Theodorakis et al., 1988).

We do not observe a difference in polysome size between the control and MM cell lines examined. There is a slight variation in the position of the polysome peaks from one experiment to another, however, there is no gross alteration in polysome size between the MM and control cell lines. The polysome peaks associated with the c-myc message in all cases were found in fractions 6–8 (Figure 5 and our unpublished data) and this strongly suggests that the higher level of polysomal c-myc mRNA does not result from an increase in the re-initiation rate of translation of the c-myc mRNA. The aberrant translational control in MM is thus likely to reside either at the level of c-myc mRNA mobilisation or result from an increased c-myc mRNA elongation rate. Obviously the former will be difficult to measure until a factors involved in regulating c-myc mRNA mobilisation can be identified, but the latter can be measured by making use of the specific inhibitor of elongation aurintricarboxylic acid.

Elongation rates of the c-myc message in MM cell lines and control cell lines

To determine the elongation rates of translation of the c-myc message, the MM and control cells were incubated with the initiation inhibitor aurintricarboxylic acid, samples were withdrawn at predetermined times and as before, sucrose gradient density centrifugation was performed to analyse the ratio of the c-myc mRNA on the polysomes/monosomes. These data indicate that the rate of translational

![Figure 5](image-url)
A berrant translational control of c-myc

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Table 1  Summary of the results from polysome gradient analysis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>c-myc mRNA (polysomal)</th>
<th>GAPDH mRNA (polysomal)</th>
<th>c-myc/ GAPDH mRNA ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloma</td>
<td>0.90 ± 0.14</td>
<td>0.35 ± 0.08</td>
<td>2.57</td>
</tr>
<tr>
<td>GM06923</td>
<td>1.00 ± 0.17</td>
<td>0.36 ± 0.11</td>
<td>2.70</td>
</tr>
<tr>
<td>GM1500</td>
<td>1.12 ± 0.10</td>
<td>0.51 ± 0.04</td>
<td>2.20</td>
</tr>
<tr>
<td>GM2132</td>
<td>0.63 ± 0.07</td>
<td>0.20 ± 0.03</td>
<td>3.25</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>2.77</td>
</tr>
<tr>
<td>Control</td>
<td>0.91 ± 0.11</td>
<td>1.10 ± 0.09</td>
<td>0.82</td>
</tr>
<tr>
<td>GM0892</td>
<td>0.30 ± 0.05</td>
<td>0.30 ± 0.06</td>
<td>0.91</td>
</tr>
<tr>
<td>GM1953</td>
<td>0.20 ± 0.01</td>
<td>0.29 ± 0.06</td>
<td>0.69</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
</tbody>
</table>

To obtain the polysome:monosome mRNA ratio, the sum of fractions in each designated peak (as in figures 4 and 5) were calculated for both the c-myc and GAPDH mRNA species. The polysomes:monosome ratio for associated c-myc mRNA was then expressed relative to the polysome:monosome ratio for GAPDH (A/B). These data represent the mean of three independent experiments ± one standard deviation.

Table 2  Sequencing of c-myc 5' UTR from MM and control cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cell lines</td>
<td>2750, 2760, 2770</td>
</tr>
<tr>
<td>GM0892</td>
<td>ACT CTC CCG ACG CGC GGA GGC</td>
</tr>
<tr>
<td>MM cell lines</td>
<td>2750, 2760, 2770</td>
</tr>
<tr>
<td>GM1311, GM2132</td>
<td>ACT CTC TCG ACG CGC GGA GGC</td>
</tr>
<tr>
<td>GM06923, GM1500</td>
<td>ACT CTC GCG ACG CGC GGA GGC</td>
</tr>
</tbody>
</table>

In each case approximately 10 independent clones from PCR and RT – PCR were sequenced (numbering as in Gazin et al., 1984).

Exon 1 of the c-myc gene is predominantly non-coding (Watt et al., 1983) and has been identified as a possible modulator of the translational efficiency of the c-myc mRNA (Darveau et al., 1985; Parkin et al., 1988). Exon 1 contains within it cis acting nucleotide sequences that can inhibit translation of heterologous genes, both in vitro and in certain in vivo systems (Parkin et al., 1988). Therefore, to determine whether there was any alteration in this region of DNA in the MM cell lines c-myc exon 1 from MM and control cell lines was amplified from genomic DNA and mRNA by both PCR and RT-PCR. The fragments were subcloned into MP13MP19 and in each case approximately 10 independent clones derived from a number of separate PCR reactions were sequenced. In all four cell lines derived from patients with MM a specific mutation was located at position 2756 (Gazin et al., 1984); a C-T transition. No other consistent alteration was detected (Table 2). This mutation is not present in 100% of the DNA samples isolated thus implying that the MM cell lines are heterozygous for the mutation. Hence, in clones of c-myc exon 1 obtained from the MM derived cell lines the mutation was found to occur at a frequency of 60% in GM06923, 44% in GM1500, 20% in GM2132 and 55% in GM1311 whilst the mutation was never observed in any of the clones obtained from the control cell lines. This alteration is not present in the 5' end of a genomic c-myc clone isolated from the leukaemic cell line K562 (Watt et al., 1982) and we have not found this mutation in other cell types which have high c-myc mRNA and protein levels and which are of tumour origin e.g. HL60 (our unpublished data).
Discussion

These data provide evidence that c-myc expression in MM plasma cells is regulated in part by enhanced translation. Compared to control cells, the MM cell lines have a greatly increased amount of c-myc protein as determined by ELISA and immunoprecipitation, which cannot be accounted for by either an increase in protein stability or an elevated level of the c-myc mRNA. By analysis of the association of c-myc mRNA with polysomes we demonstrate that there is a greater amount of c-myc mRNA in a translatable form when compared to control cells, and this could be important in disease pathogenesis in MM.

Since there is no apparent increase in polysome size, we believe that an increase in the initiation rate of the c-myc mRNA is unlikely. Similarly the elongation rate of the c-myc message in the cell lines derived from patients with MM appears to be identical to that observed in the cell lines derived from healthy individuals, thus implying the aberrant regulation does not occur at the elongation stage of the translation process. Therefore this mechanism is clearly different to that described for the translational regulation of HSP70 where the message is regulated at the level of elongation. These data suggest that there could be an increase in the mobilisation of the c-myc message such that in the MM cell lines there is a shift in the proportion of the c-myc mRNA that is found in the active polysomes compared to inactive mRNPs. Translational control of this type has been described for several other systems. For example a general shift of mRNA from polysomes to monosomes without a corresponding increase in polysome size has been observed in the development of sea urchins and in the maturation of Xenopus oocytes (Davidson, 1986). A shift in the opposite direction i.e. of the mRNA from mRNPs to polysomes has been described in mammalian cells in several instances, e.g. insulin message is mobilised onto the polysomes when glucose starved islet cells are treated with glucose (Welsh et al., 1986). The co-ordinated regulation of the levels of ferritin protein with iron availability is perhaps one of the best understood translational control systems which is mediated by mRNA mobilisation. Here a repressor protein (the iron response binding protein) binds to the iron response element (a stem loop structure), which is present in the 5' untranslated region of the ferritin mRNA and blocks translation of this message (Klausner et al., 1993).

However, many mechanisms which regulate the translation of individual or specific groups of mRNAs are not well defined although the presence of secondary structure in untranslated regions (UTR) of mRNA appears to be important since many cellular mRNAs encoding proto-oncogenes, transcription factors, growth factors and growth factor receptors contain unusually long, highly structured 5'UTRs (Gray and Hentze, 1994). These regions of secondary structure in 5'UTRs are often associated with a negative control of translation. It has been shown that mRNAs with excessive secondary structure are discriminated against by the translational machinery and are generally only poorly translated (Kozak, 1986), yet the overexpression of the initiation factor eIF-4E (the cap binding protein) may relieve this inhibition (Koromilas et al., 1992). Examples of 3'UTRs which repress translation include basic fibroblast growth factor (FGF) where deletion of the 5'UTR results in overexpression of one of the isoforms (Prats et al., 1992); when the platelet-driven growth factor 5'UTR is placed into a CAT reporter vector, it confers a 30-40-fold reduction of chloramphenicol acetyl transferase (CAT) mRNA translation in transfected COS cells (Johannes and Berger, 1992); mutations in the ornithine decarboxylase (ODC) 5'UTR which reduce the stability of a predicted stem loop cause an increase in the translational efficiency of ODC mRNA (Rao et al., 1988). Finally for c-myc, the 5'UTR has been shown to affect the translation of the c-myc message since full length mRNAs have lower translation efficiencies than those which lack exon 1 (Darveau et al., 1985). This negative regulation has been mapped to a 240 nucleotide element which, when placed at the 5' end of heterologous genes, will inhibit the translation of the corresponding mRNAs in several in vitro systems (Parkin et al., 1988). The C-T mutation which we have observed in all of the MM cell lines could cause an alteration in the translational control of c-myc. For example this mutation may result in structural changes such that the mRNA becomes accessible to the translation machinery or affect affinity of binding of specific regulatory factors. However, the C-T mutation in position 2756 has also been detected in a genomic clone obtained from a foetal liver (Watt et al., 1983) and it is not therefore unique to MM. Experiments are underway to test whether this mutation in the c-myc 5'UTR effects translation in both in vivo and in vitro systems.

In addition the increase rate of translation of c-myc in the MM cell lines could also result from either the enhanced expression of a protein which causes the mobilisation of the c-myc message or the insufficient production of a c-myc repressor protein. One would predict that the latter situation is probably more likely, since positive regulation of translation by mRNA binding proteins is a rare phenomenon and to date there are no unambiguous examples of mRNA binding proteins that directly activate translation. Moreover, the scanning mechanism of initiation would probably impose severe constraints on such a system (Kozak, 1992).

Our data imply a direct link between a disease state and an altered control of translation. The plasma cell is primarily required for production of immunoglobulin and as such, its translation machinery is very well adapted for high volume protein production. Thus aberrations in this system, e.g. to more efficiently translate transformation-associated genes such as c-myc could conceivably contribute in the pathway to malignancy in MM. Thus we propose that c-myc is deregulated in MM, a change in common with many other B cell lineage tumours. However in contrast to many of the latter, which for example exhibit c-myc chromosomal translocations and changes in c-myc mRNA stability, in MM c-myc protein is overexpressed via a translational control mechanism.

Materials and methods

Cell culture

The cell lines GM2132, GM06923, GM1500, U266 (post 1984), GM1311 (all derived from MM patients), GM1953, GM0892A (lymphoblastoid cell lines derived from healthy...
N a ;H P O j pH 7.2 and 7% sodium dodecyl sulphate at
0, 5, 15 and 30 min, resuspended in polysome buffer and
applied to 15-45% sucrose gradients as described above.

b lo ts were hybridised in a buffer containing 250 o f growth media, treated w ith 0.1 m.vt aurintricarboxylic
messages, a total o f 4 x 10^6 cells were resuspended in 1 ml
was dried, resuspended in NaOH and applied to a Zeta
clearances. For each cell line, the resulting fragments from the PCR were digested with
and dN TPs) and 20µM MgCl2, 10 mM HEPES buffer at pH 7.4, 0.5% Nonidet P-40, 100 µg/ml cycloheximide and 10 mM vanadyl ribonu-
cleoside complexes. When necessary, the cell suspensions were treated with puromycin (2µg/ml, Sigma) for 10 min
at 37°C. Cell lysates were centrifuged for 5 min at 12 000
to pellet the nuclei, which were discarded and the
centrifuged at a 184 000
gradient prepared in the above buffer but excluding cyclohexim de and NP40. The gradients were then
continuously during fractionation. For each cell line,
the results were norm alised to 100% at r = 0 and
the half life determined.

Protein analysis
Enzyme linked immunosorbant assays (ELISAs) were
performed as previously described (Sullivan et al., 1989).
This detection method utilises two anti-peptide antibodies
which recognise different epitopes on the human c-myc
protein. Three ELISAs were performed on three indepen-
dent occasions. To confirm these results quantitative
immunoprecipitation was performed using a rabbit
polyconal antibody raised against the recombinant c-myc
protein. Exponentially growing cell cultures were preincu-
bated in methionine free media for 30 min, labelled with [35S]methionine (0.25–0.5 mCi) for 15 min and lysates
were prepared for immunoprecipitation. After electrophoresis,
gels were fluorographed in 16% sodium salicylate.
No immunoprecipitation of c-myc was observed if lysates were
treated with either preimmune rabbit serum or if the c-myc
antibody was preincubated with the immunogen (not
shown). The half life of the c-myc proteins in the various
cell lines was determined by labelling the cultures with
[35S]methionine as described above, harvesting the cells at
predetermined time intervals, followed by immunoprecip­i-
tation. The c-myc proteins were scanned with a densit-
ometer, the results were normalised to 100% at t = 0 and
the half life determined.

Polymerase chain reaction
Primers, 5'-GCGGGATCCCGCGGGCTTATATGC
-3' and 5'-GTTGAAATCTTCTGTTTTT
-3' were synthesised and used to amplify c-myc exon 1 (2289–2881). PCR
reactions contained 10 µl PCR buffer (Advanced Biosystems), 10 µl
MgCl2 (25 mM), 1 µl dNTPs (10 mM of each dATP, dCTP, dGTP, dTTP), 1 unit of Taq DNA polymerase (Advanced
biosystems), 0.7 µg DNA, (or 0.2 µg cDNA obtained by
incubating total cellular RNA with reverse transcriptase
and dNTPs) and 20 µM of each oligonucleotide. The PCRs
were carried out in a Perkin Elmer Cetus DNA thermal
cycler at 94°C for 3 min followed by 37 cycles of (94°C for
2 min, 63°C for 3 min, 72°C for 2 min) and 72°C for
10 min.

Cloning and sequencing
The resulting fragments from the PCR were digested with
BamHI and EcoRI and ligated into M13MP19 which had
also been cut with these enzymes and the ligation mixture
transfected into competent JM109 cells. The resulting plaques were picked, bacteriophages isolated and ss DNA generated. The ss DNA containing c-myc exon 1 was then sequenced using a Pharmacia T7 DNA sequencing kit with Universal primer. The samples were electrophoresed on a 6% polyacrylamide gel which was then dried and exposed to Kodak XAR-film for 16 h.

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References