The Regulation of Pol III Transcription by mTOR

by

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The research reported within this thesis is my own work except where otherwise stated, and has not been submitted for any other degree

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Abstract

Regulation of protein synthesis is an important aspect of growth control. A major determinant of this process is the availability of tRNA and 5S rRNA, which are synthesised by RNA Polymerase (pol) III. Pol III transcription is tightly linked to growth conditions, decreasing when nutrients or serum factors are low and increasing upon mitogenic stimulation. Therefore, it seems inevitable that mechanisms have evolved to couple production of the biosynthetic machinery with the needs of the cell. The target of rapamycin (TOR) signalling pathway, in conjunction with signalling through the phosphoinositide 3-kinase (PI3K) pathway, is central to this process in a diverse number of organisms.

Mammalian cell lines were investigated to assess if pol III transcription is under the control of the PI3K and mammalian TOR (mTOR) pathways. Levels of pol III transcripts were reduced *in vivo* and pol III transcription was reduced *in vitro* in response to inhibition of the pathways. Inhibition of the mTOR and PI3K pathways was not found to alter the abundance of the pol III-specific transcription factors TFIIIB and TFIIIC, or indeed of pol III itself. Moreover, the effects of inhibition of the pathways on pol III transcription were found to be independent of known regulators of pol III, such as c-Myc, Retinoblastoma protein (RB) or extracellular signal-regulated kinase (ERK) signalling, but were shown to be due, in part, to signalling through the translational effector kinase S6K1.

When the mTOR pathway is blocked by rapamycin, the interactions between TFIIIB and TFIIIC, and between TFIIIB and pol III are ablated, which correlates with the finding that mTOR activity is required for normal promoter occupancy at pol III promoters. These data may be explained by the finding that the mTOR pathway regulates phosphorylation of TFIIIB and TFIIIC subunits.

Maf1, a known negative regulator of pol III transcription in yeast, was investigated, since it has been reported to be under the control of the TOR signalling pathway. Maf1 was found to repress transcription of all class III genes in mammalian cells, and this repression can be relieved by the addition of a purified fraction of TFIIIB. Direct interaction of Maf1 with pol III and with the Brf1 subunit of TFIIIB was demonstrated, and further investigation shows that Maf1, pol III and Brf1 follow the same pattern of promoter occupancy on $tRNA^{Leu}$ genes in response to stress. *In vivo* phospho-labelling and *in vitro* kinase assays demonstrated that Maf1 is a phosphoprotein, and the phosphorylation of Maf1 was found to be inhibited by both serum-starvation and rapamycin-treatment of cells. This suggests that Maf1 may receive signals from these signalling pathways to co-ordinate pol III activity, and hence the growth capacity of the cell, with nutrient availability.

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Abbreviations

°C	degrees Celsius
µg	microgram
µM	micromolar
µl	microlitre
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
ARPP P0	acidic ribosomal phosphoprotein P0
A	adenine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
APS	ammonium persulphate
Arg	arginine
bp	base pairs
Bdp1	B double prime 1
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
BSA	bovine serum albumin
C-	carboxy-
ChIP	chromatin immunoprecipitation
cDNA	complementary DNA
Cdk	cyclin-dependent kinase
C	cytosine
Da	Dalton
dATP	2' deoxyadenosine triphosphate
dCTP	2' deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2' deoxyguanosine triphosphate
dH_2O	distilled H_2O
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2' deoxy (nucleotide) triphosphate
DSE	distal sequence element

DTT	dithiothreitol
dTTP	2' deoxythymidine triphosphate
EDTA	ethylenediamine tetra acetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
g	gram
G	guanine
GAP	GTP-ase activating proteins
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HMG	high mobility group
HAT	histone acetyltransferase
HDAC	histone deacyetyltransferase
hr	hour
HRP	horseradish peroxidase
IAA	Isoamyl alcohol
ICR	internal control region
IGS	intergenic spacer
Ig	immunoglobulin
kb	kilobases
kDa	kiloDaltons
Leu	leucine
mTOR	mammalian target of rapamycin
M	molar
mA	milliampere
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
MOPS	3-(N-morpholino)propane-sulphonic acid
MW	molecular weight
mRNA	messenger RNA
N-	amino-
NaOAc	sodium acetate
NCB	sodium bicarbonate cotransporter

ng	nanograms
nM	nanomolar
PI3K	phosphoinositide 3-kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PSE	proximal sequence element
pmol	picomole
Pol I	RNA polymerase I
Pol III	RNA polymerase III
RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase-PCR
s	second
SINE	short interspersed repeat
snRNA	small nuclear RNA
SV40	simian virus 40
SDS	sodium dodecyl sulphate
Stat3	signal transducer and activator of transcription 3
T	thymine
TAF	TBP-associated factor
TBP	TATA-box binding protein
TFIIIA	transcription factor IIIA
TFIIIC	transcription factor IIIC
tRNA	transfer RNA
TBE	tris-borate EDTA
TBS	Tris buffered saline
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer RNA
tyr	tyrosine

U	unit
UBF	upstream binding factor
UCE	upstream control element
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume

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Chapter 1

Introduction

1.1 Transcription

Cellular functions such as cell proliferation, differentiation or apoptosis, are determined by the expression of specific genes. The process of transcription, whereby an RNA product is produced from DNA, is an essential element in gene expression. Failure to produce the initial RNA transcript will render all subsequent processes redundant, and these include RNA splicing, transport to the cytoplasm, or translation into protein. The importance of transcription in the process of gene expression makes it an attractive control point for regulating the expression of genes in a particular cell type, or in response to environmental changes and metabolic requirements. Defects in transcription are responsible for a wide range of human diseases, since they play a crucial part in the development of cancer, as well as other types of disease and developmental abnormalities.

1.2 The three RNA polymerases

Transcription involves the polymerisation of ribonucleotide precursors into an RNA molecule using a DNA template. The enzymes which carry out this reaction are known as RNA polymerases. All eukaryotes contain three nuclear RNA polymerases (pols), namely RNA polymerases I, II and III. These catalyse the production of three different sets of genes, and can be distinguished by their sensitivities to the fungal toxin α-amanitin (Table 1.1; adapted from Sentenac, 1985). Each of these polymerases transcribes a different set of genes known as class I, II or III genes, respectively. RNA polymerase I (pol I) transcribes a single RNA species, the large ribosomal RNA precursor molecule (pre-RNA), from which the genes encoding the 28S, 18S and 5.8S ribosomal RNAs are processed. RNA polymerase II (pol II) transcribes the protein-coding mRNAs and the small nuclear RNAs involved in splicing. RNA polymerase III (pol III) transcribes tRNAs and 5S rRNA, which are required for protein synthesis, U6, H1 and MRP RNAs, which are involved in processing of RNA transcripts, VA1 RNAs encoded by adenovirus, which function to

subvert the translational machinery, and other transcripts with no known function, such as the short interspersed repeat (SINE) gene families, such as Alu (reviewed in Willis, 1993; White, 1994) (see Table 1.1). Although it synthesises only one product, pol I may be responsible for 70-80 % of all nuclear transcription in actively growing cells, whilst pol III contributes approximately 10-20% (White, 2001).

Each of the three pols consist of a large number of interacting subunits which form a highly complex multimeric enzyme (Acker *et al.*, 1997). Homology between pols I, II and III has been reported (Memet *et al.*, 1988). In fact, five of the subunits are common to all three polymerases and other subunits are highly related (Sentenac, 1985; Woychik *et al.*, 1990; McKune *et al.*, 1995). Such relationships indicate a basic functional similarity between the three eukaryotic pols and may also be indicative of a common evolutionary origin.

Table 1.1: **Eukaryotic RNA polymerases**

RNA Polymerase	Genes transcribed	Sensitivity to α-amanitin
I	Ribosomal RNA (45S precursor of 28S, 18S and 5.8S rRNA)	Insensitive
II	All protein-coding genes, small nuclear RNAs	Very sensitive (50% inhibition at 25ng/ml)
III	Transfer RNA, 5S ribosomal RNA, small nuclear RNA U6, SINE: Alu, B1 and B2, 7SK, 7SL, H1, MRP and VA1 RNA	Moderately sensitive (50 % inhibition at 20μg/ml)

1.3 Class III genes

Pol III is the largest and most complex of the polymerases, having 17 subunits which have been purified and characterised in yeast and in mammals (reviewed in Schramm and Hernandez, 2002). The genes transcribed by pol III encode a variety of small RNA molecules, usually less than 400bp, which are not translated (White, 2001) (see Table 1.1). Although greatly out numbered by pol II-transcribed, mRNA encoding genes, pol III genes are transcribed at very high frequencies and in fact constitute a much larger fraction of the total cellular RNA. Confocal and electron microscopy of HeLa cells revealed that pol III transcription occurs at around 2000 sites within the nucleus. Each site has a radius of around 20nm and contains, on average five molecules of active pol III (Pombo *et al.*, 1999).

1.3.1 tRNA

The initial transcripts produced from tRNA genes are precursor molecules, which are processed into mature tRNAs between 70 to 90 nucleotides in length. tRNAs are essential in translation and serve as adaptor molecules, translating the genetic information contained within mRNA into the specific order of amino acid residues of the protein it encodes. The three residue anti-codon sequence of a given tRNA is specific for a particular amino acid. Consequently, base-pairing of the tRNA anti-codon with the complementary codon of the mRNA ensures the accurate synthesis of the polypeptide chain encoded by the mRNA nucleotide sequence. Eukaryotic cells possess 50-100 distinct tRNA species (Sharp *et al.*, 1985), although the proportions of different tRNAs vary significantly between cell types (Garel, 1976). The human haploid genome contains 821 tRNA related loci, 497 of which are tRNA genes, the other 324 are tRNA-derived putative pseudogenes (International Human Genome Consortium, 2001). The considerable redundancy displayed among tRNA genes results in an average copy number of around 10 genes for each amino acid tRNA adaptor.

1.3.2 5S rRNA

At approximately 120 nucleotides long, 5S rRNA is the smallest of the ribosomal RNAs and the only one transcribed by pol III (White, 2001). Following synthesis in the nucleoplasm, 5S rRNA is transported to the nucleolus where it is processed and integrated into the large ribosomal subunit and has a critical role in translation. Eukaryotic genomes contain a wide range of numbers of 5S rRNA genes, ranging from 140 in the haploid genome of *Saccharomyces cerevisiae*, to more than 20,000 copies in *Xenopus laevis* (Elion and Warner, 1984). The majority of the latter 5S genes are required to sustain rapid growth during development of the oocyte (Wolffe and Brown, 1988). The human haploid genome contains 200-300 5S rRNA genes, many of which occur in clusters of tandem repeats (International Human Genome Consortium, 2001).

1.3.3 SINEs

The various genes families with short interspersed elements (SINEs) constitute quantitatively important classes of pol III template in higher organisms (Jelinek and Schmid, 1982; Singer, 1982). SINE DNA accounts for a substantial proportion (~ 13%) of mammalian genomes; however, a functional role for these templates has not been unequivocally demonstrated (reviewed by Howard and Sakamoto, 1990). The principal SINE in primates is the Alu family, of which there are in the region of one million copies in the haploid human genome (International Human Genome Consortium, 2001). In rodent species the most abundant SINEs are the B1 and B2 genes (Bennett *et al.*, 1984). B1 genes show approximately 80% homology with human Alu genes, whilst the B2 family is specific to rodents (Bennett *et al.*, 1984; Rodgers, 1985). B1 genes are ~130bp in length and are present at about 100, 000 copies per haploid mouse genome (Bennett *et al.*, 1984; Krayev *et al.*, 1980). B2 genes are ~180bp long, with ~80, 000 copies per haploid mouse genome (Bennett *et al.*, 1984; Rodgers, 1985). tRNA genes seem the likely evolutionary

source of SINE families such as B2 (Daniels and Deininger, 1985), whilst the Alu and B1 sequences are believed to have evolved from the 7SL gene (Ullu and Tschudi, 1984).

1.3.4 Viral class III genes

Several viruses contain short class III transcriptional units within their genomes. The best characterised of these is adenovirus, which encodes two small (~ 160 bp) pol III transcripts called VAI and VAII, that are synthesised at high levels during the late stages of viral infection (Söderlund *et al.*, 1976). The VA RNAs function to subvert the host cell's translational apparatus to allow the synthesis of VA viral proteins (Thimmappaya *et al.*, 1982). The genome of Epstein-Barr virus (EBV) also contains two small adjacent genes; EBER1 and EBER2 that are transcribed by pol III. During adenovirus infection EBERs can functionally substitute for VAI (Bhat and Thimmappaya, 1985). Like the VA RNAs, this would allow the EBERs to subvert the host cell's translational apparatus to allow the synthesis of viral proteins.

1.3.5 U6 snRNA

U6 snRNA genes also fall into the class III gene family. U6 is the smallest of five snRNA species that comprise a ribonucleoprotein (RNP) complex termed a spliceosome (Kunkel *et al.*, 1986; Moenne *et al.*, 1990). Spliceosomes function in post-transcriptional processing of pre-mRNA (Maniatis and Reed, 1987), removing introns to generate mature mRNA. The 106 nucleotide U6 transcript is the most highly conserved of the spliceosomal RNAs (Brow and Guthrie, 1988), and the only one that is not transcribed by pol II (Kunkel *et al.*, 1986; Moenne *et al.*, 1990).

1.3.6 H1 and MRP

Pol III is also responsible for the transcription of other components of ribonucleoprotein complexes including H1, MRP, 7SK and 7SL. H1 is a 369 nucleotide RNA, that forms part of RNase P, an endoribonuclease involved in processing the 5' termini of pre-tRNA (Lee and Engelke, 1989; Morrissey and Tollervey, 1995), and which exhibits some sequence homology to MRP RNA (Gold *et al.*, 1989). MRP is a 265 nucleotide RNA that forms part of RNase MRP, another endoribonuclease, which plays an important role in the endonucleolytic processing of pre-RNA (Morrissey and Tollervey, 1995; Schmitt and Clayton, 1993).

1.3.7 7SK

The 7SK gene encodes a snRNA transcript of 330 nucleotides in length. The 7SK product had no known function until recently. It was found to act as a negative regulator of the pol II elongation factor P-TEFb; a factor responsible for the phosphorylation of pol II's carboxyl-terminal domain (Nguyen *et al.*, 2001; Yang *et al.*, 2001).

1.3.8 7SL

The pol III product 7SL RNA, forms the scaffold of the signal recognition particle (SRP) that plays an essential role in intracellular localisation of proteins. This occurs through its involvement in the insertion of nascent polypeptides into the endoplasmic reticulum (Walter and Blobel, 1982). There are four 7SL genes in the human genome, encoding a highly conserved 300 nucleotide transcript (Ullu and Tschudi, 1984; Ullu and Weiner, 1984).

1.4 Class III gene promoters

A general feature of all class III genes is that they are able to form stable transcription complexes (Schaack *et al.*, 1983; Lassar *et al.*, 1983). However, genes transcribed by pol III differ substantially in the type and arrangement of individual promoter elements (presented schematically in Figure 1.1). Whilst pol III possesses the enzymatic activity necessary for transcription, it cannot function independently. Consequently, transcription involves numerous transcription factors, which must interact with the polymerase and with each other if transcription is to occur. The role of these factors is to organise a stable transcriptional complex which contains pol III and is able to undergo repeated rounds of transcription.

1.4.1 Type I Promoters

Type I promoters are uniquely found on 5S rRNA genes and in *Xenopus laevis* consist of three gene-internal sequence elements; a highly conserved 5' A block (+50 to +64), an intermediate element (+67 to +72) and a 3' C block (+80 to +97) (Pieler *et al.*, 1987; Figure 1.1). The promoter is relatively intolerant of changes in the spacing between individual elements (Pieler *et al.*, 1987). The elements contained within the minimal internal control region (ICR) (+50 to +97), are highly conserved between species, and mutations in the A- and C- blocks abolish transcription (Keller *et al.*, 1990). In contrast, the flanking sequences have limited conservation, and despite having strong modulatory effects, they are significantly more resilient to mutations (White, 2001).

1.4.2 Type II promoters

The most commonly used promoter arrangement of class III genes is found in tRNA and adenovirus VA genes and also many major SINE families. This type II promoter consists

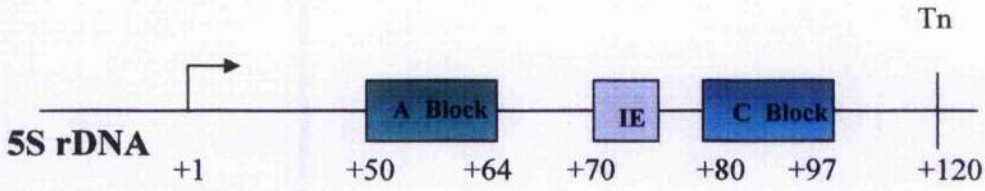
of two highly conserved sequence elements called the A and B blocks and, like the type I promoter, these sequence elements are located within the transcribed region (Figure 1.1). The A block is positioned close to the transcription start site (usually about 10-20bp) (Galli *et al.*, 1981). Since the A block is common to both type I and II promoters and is homologous, it can be interchanged in some cases, and this can occur despite the A blocks of type II promoters being much closer to the start site than type I promoters. The B block is downstream of the A block; however, the distance is extremely variable (Baker *et al.*, 1987). An A- to B-block separation of 30-60bp is usual; however, a distance of up to 365bp can still support transcription (Baker, 1987; Fabrizio, 1987).

1.4.3 Type III promoters

A typical type III core promoter, commonly represented by 7SK, MRP and U6 genes, contains a TATA box, a proximal sequence element (PSE) and a distal sequence element (DSE). The best characterised type III promoter belongs to the U6 gene (Figure 1.1). The PSE covers about 15 bp and is located around 55 bp upstream of the transcriptional start site (Kunkel and Pederson, 1988). The TATA box is located 25bp downstream of the PSE at -30 (Kunkel and Pederson, 1989; Lobo and Hernandez, 1989). There is a specific distance that separates the PSE and TATA box on type III promoters (Lescure *et al.*, 1991; Goomer and Kunkel, 1992). These elements constitute a basal promoter which is subject to activation by a variety of factors that bind to a DSE that is located between -244 and -214 (White, 2001).

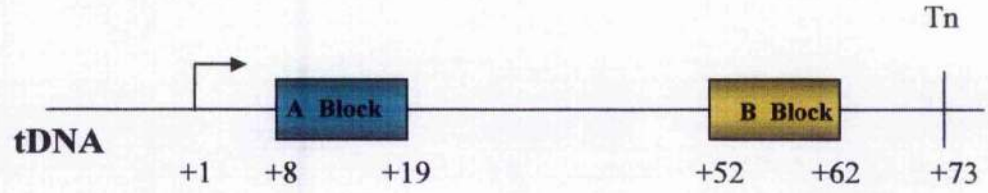
Type I promoter

e.g. *Xenopus* somatic 5S rRNA genes



Type II promoter

e.g. *Saccharomyces* SUP4 tRNA genes



Type III promoter

e.g. Human U6 snRNA gene

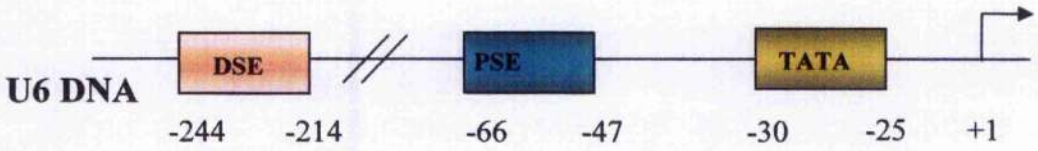


Fig. 1.1: **Promoter structure of class III genes (adapted from White, 2001)**

Schematic representation of the three general types of promoter utilised by pol III. Transcription initiation and termination sites are indicated by +1 and Tn, respectively. The relative positions of the promoter elements that characterise each promoter type are represented. Included are the intermediate elements (IE) of the type I promoter, and the proximal (PSE) and distal sequence elements (DSE) found in type III promoters.

1.4.4 Other class III promoters

Some class III genes such as 7SL do not fit into promoter types I, II or III as they use combinations of promoter elements which are different to those that have been described (Willis, 1993). The underlying theme, however, is that the promoter elements and their associated transcription factors bring the pol III factor TFIIIB to the start site of transcription and stabilise it there (Persinger *et al.*, 1999). Before transcription can commence, a pre-initiation complex must assemble at the appropriate promoter, leading to recruitment of the polymerase.

1.5 Transcription complex assembly at type II promoters

(see Figure 1.2)

Transcription factor IIIC (TFIIIC) binds directly to the intragenic promoter sequences of type II promoters (Lassar *et al.*, 1983). Type II promoters require transcription factor IIIB (TFIIIB) and TFIIIC (Segall *et al.*, 1980) for the formation of a stable complex (Lassar *et al.*, 1983). Human TFIIIC was initially resolved by ion exchange chromatography into two components, TFIIIC1 and TFIIIC2 (Yoshinaga *et al.*, 1987). TFIIIC1 has not been well characterised, but previous studies have shown that it can stabilise and extend promoter interactions of TFIIIC2 (Yoshinaga *et al.*, 1987; Wang and Roeder, 1998) and can interact with TFIIIC2 in the absence and presence of promoter DNA (Sinn *et al.*, 1995). A recent study has questioned whether TFIIIC1 is a *bona fide* component of TFIIIC and this will be further discussed (Weser *et al.*, 2004).

TFIIIC2 can be separated into five subunits of 220, 110, 102, 90 and 63 kDa according to their molecular masses (Yoshinaga *et al.*, 1989; Kovelman and Roeder, 1992). TFIIIC2 can be isolated into both active (TFIIIC2a) and inactive (TFIIIC2b) forms, which bind DNA with equal affinity and show equivalent DNase footprint patterns (Hoeffler *et al.*, 1988;

Kovelman and Roeder, 1992). Silver staining revealed that TFIIIC2a contains a 110kDa subunit (TFIIIC110) whilst the inactive form lacks this polypeptide (Sinn *et al.*, 1995). Immunodepletion with an antibody against 110kDa quantitatively removed TFIIIC1 and TFIIIC2 activity suggesting that 110kDa is an essential component of TFIIIC2a and that TFIIIC1 is tightly associated with TFIIIC2a in nuclear extracts (Sinn *et al.*, 1995).

TFIIIC functions as an assembly factor and TFIIIC2, through interactions with the promoter element and TFIIIC1, serves to recruit TFIIIB (L'Etoile *et al.*, 1994; Wang and Roeder, 1996), which is regarded as the pivotal initiation factor of the pol III system. Mammalian TFIIIB was initially found to contain TBP (TATA-binding protein; 35kDa) (White and Jackson, 1992; Lobo *et al.*, 1992) and Brf1 (TFIIB-related factor; 90 kDa) (Mittal *et al.*, 1996; Wang and Roeder, 1995), which tightly associate with each other in HeLa cell extracts (Mittal *et al.*, 1996; Wang and Roeder, 1995). A more loosely associated polypeptide B double prime (Bdp1), was identified in *S. cerevisaie* (Kassavetis *et al.*, 1995; Roberts *et al.*, 1996). However, it was not until several years later that the 160 kDa human homologue of Bdp1, was cloned, characterised and shown to be required for transcription from all pol III promoter types (Schramm *et al.*, 2000).

Pol III is only recruited after TFIIIB enters the transcription complex (Schramm and Hernandez, 2002). All three subunits of TFIIIB are required for pol III recruitment; however, the majority of direct interactions occur between Brf1 and pol III subunits (RPC32, RPC39 and RPC62) (Wang and Roeder, 1997), although TBP can also associate with RPC39 (Wang and Roeder, 1997).

Cloning and characterisation of human TFIIIC subunits established their high degree of conservation with yeast counterparts and *in vitro* data demonstrates interactions between the subunits of TFIIIB, TFIIIC and pol III (Hsieh *et al.*, 1999a and b).

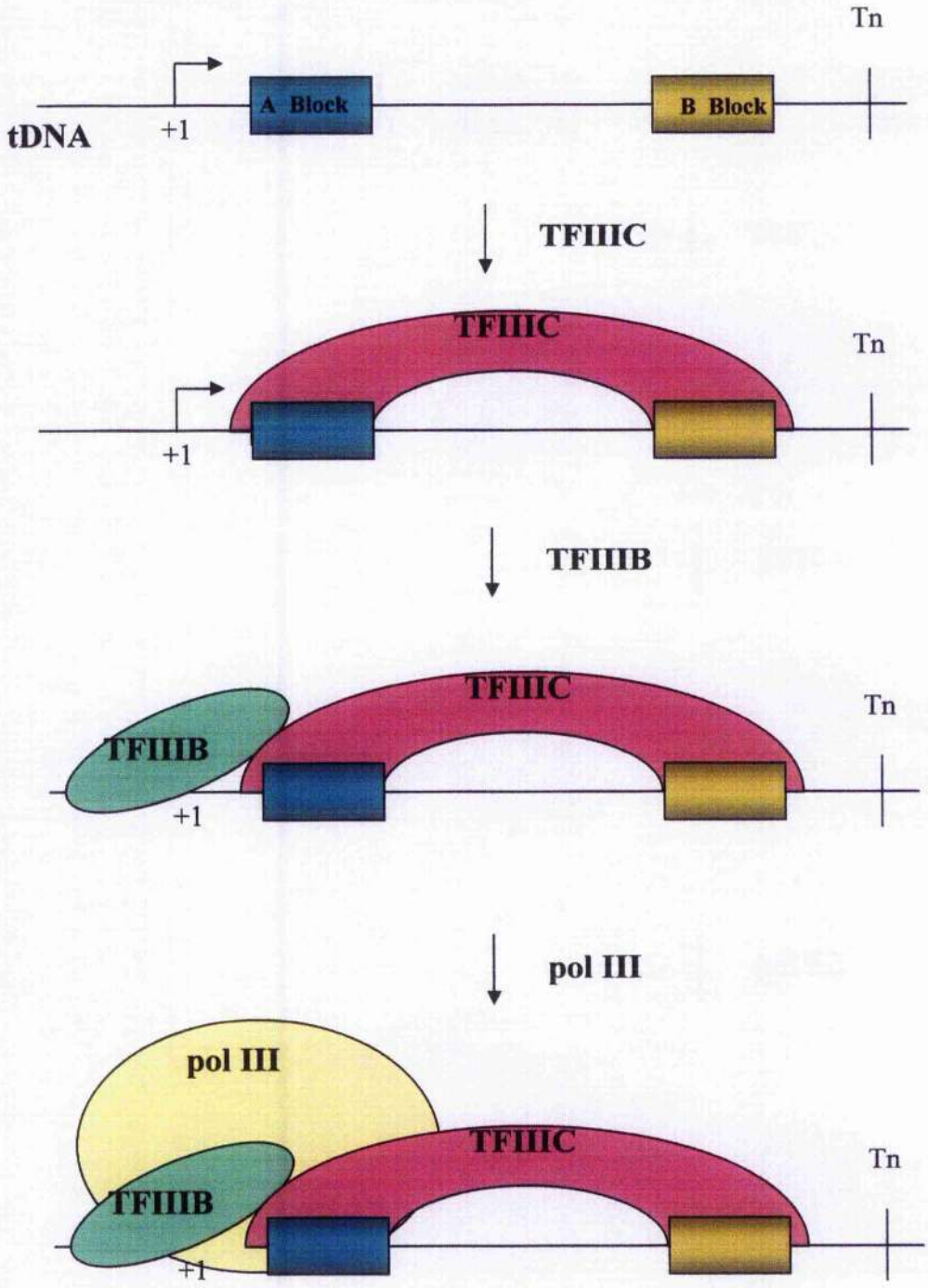


Fig. 1.2: **A model for transcription initiation at a type II promoter**
Diagram indicating the order of interaction of transcription factors and pol III with a typical type II promoter such as that of a tRNA gene. The site of transcription initiation is depicted by +1 and the site of termination is indicted by Tn.

The human TFIIIC102, TFIIIC63 and TFIIIC90 subunits bind to Brf1 (Hsieh *et al.*, 1999b; Hsieh *et al.*, 1999a) and the TFIIIC102 and TFIIIC63 subunits bind to TBP (Hsieh *et al.*, 1999b). These interactions between the TFIIIC102 and TFIIIC63 subunits, taken together with the fact that TFIIIC63 interacts with the A box (Hsieh *et al.*, 1999b); provides a link between the TFIIIB-interacting and DNA-interacting subunits of TFIIIC. In addition, TFIIIC63 was found to interact with a pol III subunit (RPC62) (Hsieh *et al.*, 1999b), therefore, contacts between TFIIIC and TFIIIB and TFIIIC-pol III may facilitate the recruitment of both TFIIIB and pol III.

1.6 Transcription complex assembly at type I promoters

(see Figure 1.3)

Competition assays using separated transcription factors derived from human cell extracts showed that a fraction containing TFIIIC is required to form stable transcription complexes with most pol III promoters (Lassar *et al.*, 1983). However, TFIIIC binding on 5S rRNA genes must be preceded by its interaction with a 5S rRNA-specific factor, transcription factor IIIA (TFIIIA) (Lassar *et al.*, 1983). Purified TFIIIA was found to have a repeated structure and be associated with between 7 and 11 atoms of zinc per molecule of purified protein (Miller *et al.*, 1985). Thus, in addition to TFIIIA's critical role in regulating transcription of 5S rRNA (Lassar *et al.*, 1983), it was the founding member of the Cys_2-His_2-containing zinc finger family (Miller *et al.*, 1985). The DNA binding ability of TFIIIA is dependent on the presence of zinc, which allows the zinc fingers to form; this highly repeated zinc finger motif is required as TFIIIA contacts a large regulatory region in the DNA. The zinc regions bind to the A-block (Clemens *et al.*, 1992), the intermediate element (Nolte *et al.*, 1998) and the C-block (Clemens *et al.*, 1992; Nolte *et al.*, 1998), the latter of these with the highest affinity; C-block binding contributes about 95% of the total binding affinity of full length TFIIIA (Clemens *et al.*, 1992; Nolte *et al.*, 1998).

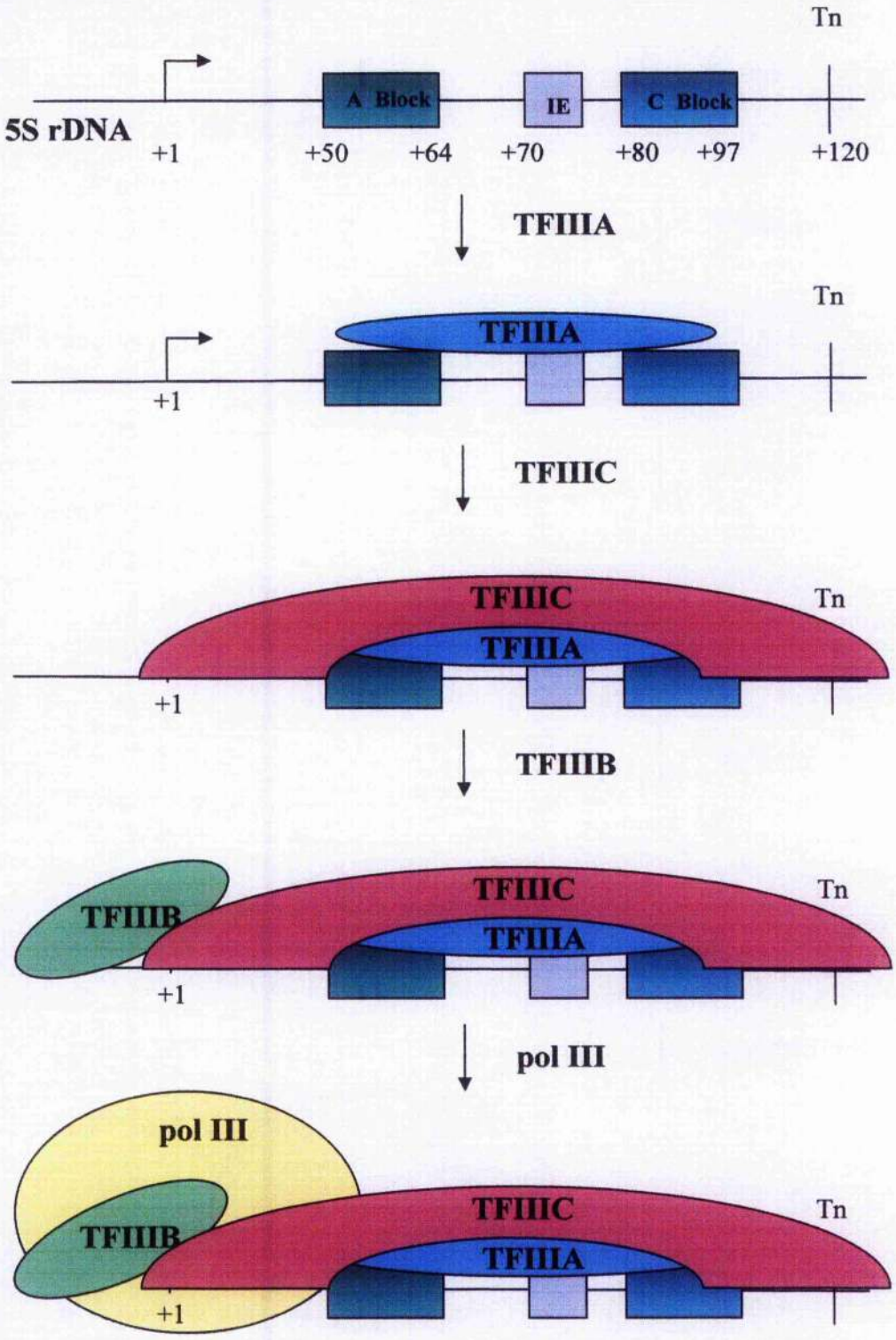


Fig. 1.3: **A model for transcription initiation at a type I promoter**

Diagram indicating the order of transcription factors binding and the recruitment of pol III to the 5S rRNA promoter. The site of transcription initiation is depicted by +1 and the site of termination is indicated by Tn. IE is the intermediate element.

Thus, TFIIIA is considered an adaptor factor which allows TFIIIC to be recruited onto genes for which it has little affinity. The way in which TFIIIA recruits TFIIIC remains largely unknown; however, both factors must be bound prior to recruitment of TFIIIB at 5S rRNA promoters (Bieker *et al.*, 1985; Carey *et al.*, 1986a; Setzer and Brown, 1985). Binding of TFIIIB just upstream of the transcription start site allows recruitment of pol III to specific genes through direct interactions between pol III and a subunit of TFIIIB (Huang and Maraia, 2001).

It can be concluded that TFIIIC performs at least three functions; it recognises promoter elements, either directly in the case of type II promoters or with the help of TFIIIA in the case of type I promoters; it recruits TFIIIB, and it contributes to the recruitment of pol III.

1.7 Transcription complex assembly at type III promoters

(see Figure 1.4)

Type III promoters are different to those found in other class III genes as they reside exclusively upstream of the coding sequence. Examples of genes which have extragenic promoter elements are U6, 7SK and RNase MRP (Das *et al.*, 1988; Kunkel and Pederson, 1989; Lobo and Hernadez, 1989). Type III promoters, unlike type I and II promoters, do not require TFIIIA or TFIIIC for promoter recognition (Lagna *et al.*, 1994; Oettel *et al.*, 1997). Instead, other factors are recruited to the upstream sequence elements and these allow initiation complexes to form so that transcription can proceed.

The DSE is typically composed of several protein binding sites, one of which is an octamer sequence (Lobo and Hernandez, 1994). The octamer sequence is recognised by the POU domain activator Oct-1 (Herr *et al.*, 1988). This factor can activate transcription of 7SK genes both *in vitro* and *in vivo* (Murphy, 1997; Murphy *et al.*, 1989).

PSE occupancy is of primary importance in assembling a stable pre-initiation complex on a type III promoter. The U6 promoter is highly similar to class II snRNA promoters,which consist of only the PSE (Henry *et al.*, 1995); therefore, the pol II and pol III PSEs are interchangeable (Boyd *et al.*, 1995) and recruit a five-subunit complex known as SNAPc (snRNA activator protein complex) or PTF (the proximal element transcription factor) (Sadowski *et al.*, 1993; Henry *et al.*, 1998; Murphy *et al.*, 1992). As a result, purified or recombinant SNAPc can function as a basal transcription factor for both types of snRNA gene (Yoon and Roeder, 1996; Henry *et al.*, 1998).

SNAPc and Oct-1 bind cooperatively to their two spatially separated PSE and DSE (Mittal *et al.*, 1996; Murphy *et al.*, 1992; Ford and Hernandez, 1997). As the PSEs are low affinity binding sites for SNAPc, the Oct-1 POU domain recruits SNAPc to the PSE (Murphy *et al.*, 1992; Mittal *et al.*, 1996). The Oct-1 binding of the DSE is then stabilised by interacting with DNA-bound SNAPc subunit, PSE occupancy is stimulated and this results in activation of transcription (Henry *et al.*, 1995; Murphy *et al.*, 1992; Mittal *et al.*, 1996; Ford *et al.*, 1998). The poor DNA binding ability of SNAPc as a single factor is due to its negative control of binding, which is achieved by masking the DNA binding domain with its C-terminal part (Mittal *et al.*, 1996). This repression can be relieved by direct protein-protein interactions with the Oct-1 POU domain, which identified SNAPc as a target for the activator Oct-1 (Ford *et al.*, 1998; Mittal *et al.*, 1999).

The type III promoters have distinct factor requirements from most pol III templates and variants of the TFIIIB complex used to transcribe U6 genes with a type III promoter were identified as different to the form used to transcribe genes with type II promoters (Lobo *et al.*, 1992; Teichmann and Seifart, 1995; Mittal *et al.*, 1996). These two TFIIIB assemblies were further investigated and PSE-bound SNAPc and the TATA box were found to recruit a different TFIIIB activity (Brf2-TFIIIB) (Schramm *et al.*, 2000; Teichmann *et al.*, 2000)

than type 1 and II promoters (Schramm *et al.*, 2000). The Brf2-TFIIIB activity which contains TBP, Brf2 and Bdp1, does not appear to form a tight complex in the absence of DNA (Schramm *et al.*, 2000). Consistent with the absence of type III promoters in *S. cerevisiae* and *S. pombe*, Brf2, Oct-1 and homologues of the SNAPc subunits are not found in these organisms (Huang and Maraia, 2001).

Because it contains TBP, TFIIIB can bind independently to a TATA box. Accordingly, the TATA box in U6 promoters recruits TBP (Lobo *et al.*, 1991). SNAPc has also been reported to recruit and stabilise TBP on the TATA box (Goomer and Kunkel 1992; Mittal and Hernandez, 1997). A nucleosome positioned between the DSE and PSE assists stable initiation complex assembly through the cooperative interactions of TFIIIB, SNAPc and Oct-1 by bringing them into spatial proximity (Zhao *et al.*, 2001). However, it is thought that together SNAPc and TBP can recruit the other pol III transcription factors and pol III and increase pol III transcription independently of the DSE and Oct-1. Brf2 can then form a complex with, and stabilise, TBP on a TATA-containing template, which extends the TBP footprint both upstream and downstream of the TATA box (Cabart and Murphy, 2001). Bdp1 is specifically recruited to the U6 promoter-bound TBP-Brf2 complex (Cabart and Murphy, 2002). TBP and Brf2 can associate strongly in this DNA-TBP-Brf2 complex, whereas Bdp1 remains loosely associated (Cabart and Murphy, 2001). Since the N-terminal region of Brf2 inhibits the direct association of Brf2 with Bdp1 in solution, it is thought that TBP bridging can relieve this auto-inhibition and promote cooperative assembly of TBP, Brf2 and Bdp1 subunits (Cabart and Murphy, 2002). Direct interaction between Bdp1 and DNA has not been reported to date.

U6 genes found in *S. cerevisiae* lack both the DSE and PSE, and instead resemble the human type II promoter since they contain A and B blocks. Interaction of TFIIIC with the B block allows it to displace nucleosomes from the U6 template, whereas TFIIIB does not

have this effect (Burnol *et al.*, 1993), so it also seems to weaken the interaction of nucleosomes with the transcribed region of at least some class III genes. This is consistent with reports that the three largest subunits of TFIIIC2 have histone acetyltransferase (HAT) activity (Hsieh *et al.*, 1999b; Kundu *et al.*, 1999).

Only TFIIIC1, but not TFIIIC2, is used by type III promoters (Oettel *et al.*, 1997; Yoon *et al.*, 1995). Little is known about TFIIIC1, but sedimentation analysis suggests it has a mass of up to 200 kDa (Yoshinaga *et al.*, 1987). The active components of TFIIIC1 have not been defined and remain under some debate; however, several candidates have been proposed (Oettel *et al.*, 1997; Wang *et al.*, 2000; Wang and Roeder, 1996; 1997). These include NF-1 polypeptides, DNA topoisomerase I and PC4, which have been reported to participate in pol III transcription as part of TFIIIC1 (Wang *et al.*, 2000; Wang and Roeder, 1998).

A recent report has found that Bdp1 is an essential component of TFIIIC1 (Weser *et al.*, 2004), since Bdp1 was found to strictly co-elute with TFIIIC1 activity and this activity could be specifically depleted with anti-Bdp1 antibodies (Weser *et al.*, 2004). Highly purified recombinant Bdp1 was able to replace TFIIIC1 activity in a reconstituted system; in fact, a splice variant of Bdp1 which contains only the N-terminal third of full length Bdp1 was sufficient to exert all essential TFIIIC1 functions in such a system (Weser *et al.*, 2004). Considering this new information, it is possible that TFIIIC1 activity may simply correspond to that of Bdp1. In support of this, Bdp1 is required for transcription from all types of pol III promoters, like TFIIIC1 (Yoon *et al.*, 1995; Yoshinaga *et al.*, 1987). There is a plausible explanation for this initial confusion, since using the phosphocellulose column which was traditionally used to separate pol III transcription factors, Bdp1 could have eluted at a slightly higher salt concentration than Brf1 and TBP. If this had occurred, Bdp1 may have ended up in the TFIIIC rather than the TFIIIB fraction. Regardless of

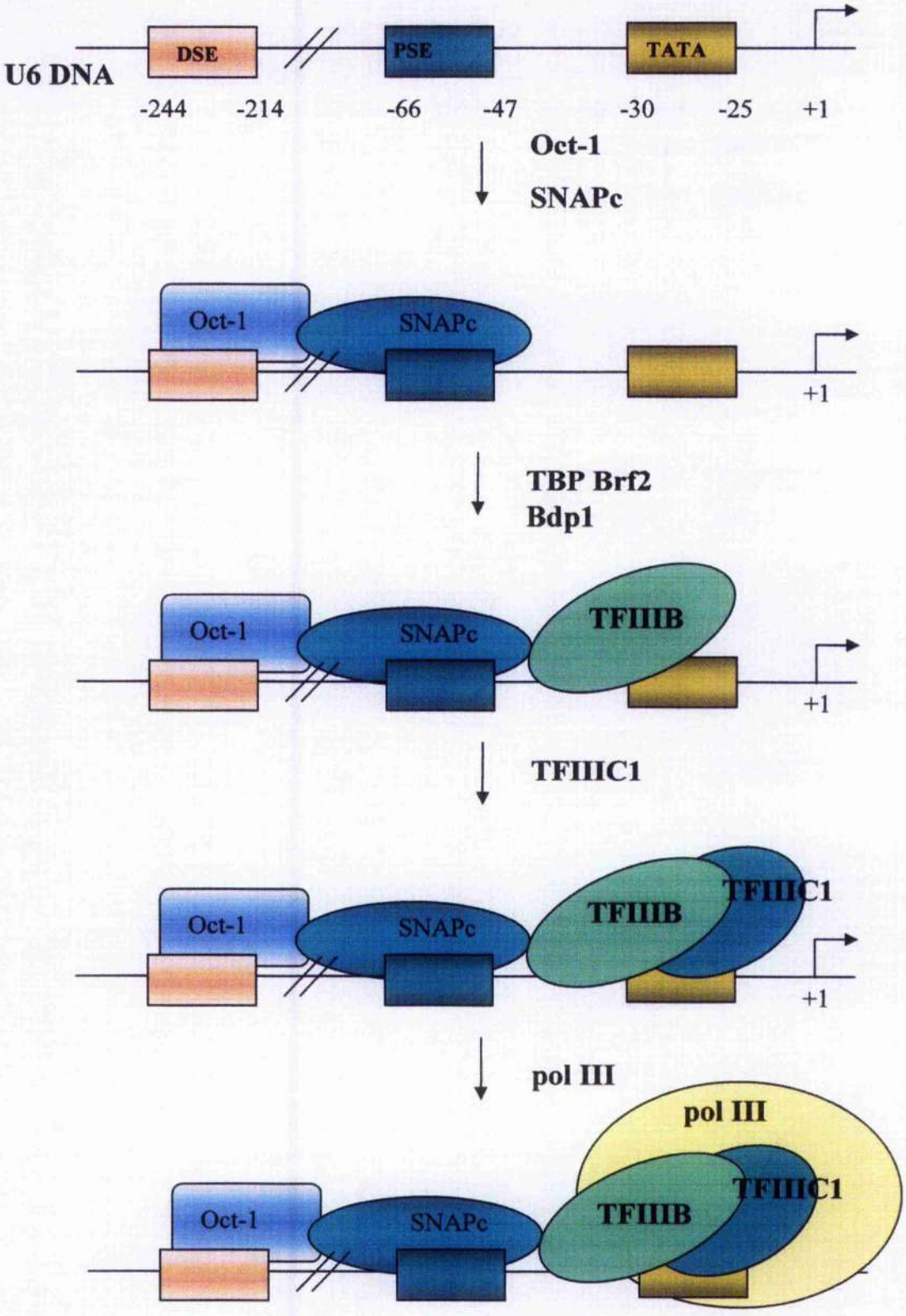


Fig. 1.4: **A model for transcription initiation at a type III promoter**

Diagram indicating the order of interaction of transcription factors and pol III with a type III promoter such as is found in a 7SK gene. The site of transcription initiation is depicted by +1.

whether it is Bdp1 or TFIIIC1 that is recruited, this occurs at a later stage in complex assembly on type III promoters. Relatively little is known about the recruitment of pol III that follows.

1.8 Stepwise assembly versus holoenzyme

The majority of our knowledge about how pol III is brought to specific promoters stems from *in vitro* studies in which transcription factors are assembled in a stepwise fashion into transcription initiation complexes. These studies provide detailed information about the protein-DNA and protein-protein interactions that facilitate recruitment of pol III. However, there remains the possibility that *in vivo* pol III is recruited along with the factors that mediate promoter recognition as part of a holoenzyme. Indeed, observation of such a holoenzyme has been reported (Wang *et al.*, 1997). When pol III was purified from a HeLa cell line that expresses a FLAG epitope-tagged subunit of pol III, it was observed that a number of polypeptides co-purify with the enzyme (Wang *et al.*, 1997). The BN51 subunit, which is specific to pol III (Wang and Roeder, 1997), was found associated with TBP, Brf1, TFIIIC220 and TFIIIC110 (Wang *et al.*, 1997). This finding raises the possibility that *in vivo*, pol III is recruited together with its accessory factors (Wang *et al.*, 1997).

1.9 Pol III transcription initiation, elongation and termination

Pol III alone has little specificity for particular DNA sequences. Its recruitment to specific genes requires the presence of TFIIIB, which sits just upstream of the transcription start site and positions pol III for initiation (Kassavetis *et al.*, 1990; Bartholomew *et al.*, 1991). In yeast, the opening of the transcription bubble occurs in an ATP-independent manner after recruitment of pol III by TFIIIB (Kassavetis *et al.*, 1990, 1992). Since the transcribing pol III never leaves the vicinity of the promoter, and DNA bending by transcription factors

further compresses the domain occupied by the transcription unit, it is probable that pol III is repeatedly recycled on the same gene without being released from the template (Geiduschek and Kassavetis, 2001). Indeed, the small size of class III genes might favour pol III retention for consecutive transcription cycles. In support of this hypothesis, pol III is retained in the original transcription complex on VA and tRNA genes after the initial round of transcription (White, 2001). The process is likely to involve a direct coupling between termination and re-initiation as demonstrated by findings that run-off termination on truncated class III genes does not allow efficient recycling and re-initiation (Deici and Sentenac, 1996), and by the recent observation that a peptide nucleic acid positioned downstream of the terminator interferes with re-initiation (Guffanti *et al.*, 2004).

Pol III can recognise a simple run of T residues as a termination signal, whereas pol I and II require accessory factors in order to terminate transcription. The termination signal has been reported to stimulate re-initiation by pol III (Wang and Roeder, 1998), whereas mutation of this signal diminishes the efficiency of single- and multiple- round transcription of VA1 in HeLa cell extract (Wang and Roeder, 1996). In higher eukaryotes, a number of factors have been implicated in efficient termination and pol III recycling; these include the La protein, and components of the holoTFIIIC; NF1 polypeptides, DNA topoisomerase I and PC4 (Schramm and Hernandez, 2002). In addition, TFIIIC has been reported to contact the terminator regions of tRNA genes (Oettel *et al.*, 1997).

Recent work in yeast has provided some insight into the pol III re-initiation mechanism by showing that facilitated recycling relies on a specific polymerase recapture pathway involving promoter-bound TFIIIB and TFIIIC (Ferrari *et al.*, 2004). A TFIIIC-less transcription system comprising reconstituted TFIIIB was sufficient to direct efficient recycling on short (~100bp) class III genes. However, on longer (>300bp) transcription units the involvement of TFIIIC was required to establish a high re-initiation rate (Ferrari

et al., 2004), probably due to its ability to cause higher order rearrangements of the DNA (Burnol *et al.*, 1993) and through its contacts with the polymerase (Hseih *et al.*, 1999a/b).

1.10 The effect of chromatin structure on transcription

DNA and its protein scaffold exist in a complex tertiary structure referred to as chromatin. The fundamental unit of chromatin is the nucleosome; a repeating motif consisting of 146bp of DNA wrapped around a histone octamer that comprises 2 molecules each of the histones H2A, H2B, H3 and H4 (Richmond *et al.*, 1984; Burlingame *et al.*, 1985). Adjacent nucleosomes are connected by linker H1 DNA, which binds at the point where DNA enters and exits the subunits (Crane-Robinson, 1997), and progressive coiling of nucleosomes leads to compact higher-order chromatin structures. DNA that is contained within this highly compacted chromatin is termed heterochromatin and is inaccessible to the transcription machinery (Wolffe, 1990). Therefore, appropriate regulation of gene expression requires the interplay of complexes that remodel chromatin structure and thereby regulate the accessibility of individual genes to sequence-specific transcription factors and the basal machinery (Struhl, 1999).

Remodelling of chromatin is the consequence of two distinct mechanisms; the first leads to covalent modification of nucleosomal histones (Reviewed in Geiman and Robertson, 2002; Peterson, 2002; Neely and Workman, 2002), including phosphorylation, acetylation, methylation and ubiquitination, which can themselves affect chromatin structure or recruit proteins that can do this. The second utilises the energy of ATP hydrolysis to alter chromatin structure (Peterson, 2002). The two processes are by no means mutually exclusive; the first has been shown to allow recruitment of factors which carry out the second (Agalioti *et al.*, 2002).

Chromatin remodelling and histone modification have been largely analysed in the context of class II genes, but chromatin structure has also been shown to play an important role in class III gene transcription (Paule and White, 2000; White, 2001). For example, the ability of nucleosomes to block assembly of pol III transcriptional initiation complexes has been reported. This can occur when a nucleosome is positioned over the C-block of the 5S rRNA promoter, which obscures the initial DNA binding recognition sites of TFIIIA. As a consequence, TFIIIA can no longer bind and 5S rRNA synthesis is repressed (Gottesfeld, 1987; Hayes *et al.*, 1991; Lee *et al.*, 1993). This repression can be relieved when the nucleosome is positioned further upstream, as the C-block is left open and accessible to TFIIIA. Consequently, transcription proceeds, albeit at a reduced rate (Tremethick *et al.*, 1990; Almouzni *et al.*, 1991; Clark and Wolffe, 1991; Hansen and Wolffe, 1994).

Class III genes that have assembled into highly condensed structures are unable to support significant levels of transcription (Hansen and Wolffe, 1992). However, the susceptibility of class III genes to nucleosomal repression is template-dependent (Reviewed in Paule and White, 2000). Most tRNA genes are resistant to chromatin-mediated repression, whereas middle repetitive genes such as B2 are highly susceptible (Carey and Singh, 1988). The differential effects of histones on pol III transcription *in vivo* can be seen using studies from *Xenopus*, where the ability of nucleosomes to block assembly of transcriptional initiation complexes at pol III promoters is enhanced by Histone H1-mediated chromatin condensation. Changes in the level of Histone H1 in developing frog embryos cause selective regulation of oocyte 5S rRNA genes (Bouvet *et al.*, 1994). Injection of mRNA encoding histone H1 causes an increase in the abundance of this linker histone and repression of oocyte 5S rRNA genes. Conversely, depletion of endogenous H1 using ribozymes causes increased expression of these genes (Bouvet *et al.*, 1994; Kandolf, 1994). These effects are specific, since transcription of genes encoding tRNA, somatic 5S rRNA, U1 or U2 snRNA is unaffected by these manipulations (Bouvet *et al.*, 1994; Kandolf,

1994). *Xenopus* 5S rRNA genes have also been used to demonstrate that histone acetylation facilitates the access of transcription factors to chromatinised promoter sequences (Lee *et al.*, 1993), thus diminishing the block of assembly of transcriptional initiation complexes.

1.11 Regulators of pol III transcription

1.11.1 Overexpression of the transcription factors TFIIIA and TFIIIC

The most obvious way to increase pol III transcription is by raising the level of one or more of the limiting transcription factors on which it depends (White, 2004a). Whilst a high rate of pol III transcription is dependent on a high level of TFIIIB, assembly factors such as TFIIIA and TFIIIC can be the target for processes which regulate the rate of transcription (White, 2001). Although these factors play an accessory role in transcription itself, they are still essential for complex assembly.

The ability to affect cellular transcriptional regulatory processes is crucial to the ability of many different viruses to transform cells. For example, the large T antigens of the small DNA tumour viruses SV40 and polyoma, and the E1A protein of adenovirus can all affect cellular gene expression and this ability is essential for the transforming ability of these viruses (Moran, 1993). The large T antigens of both SV40 and polyomavirus have been shown to increase pol III transcription (Scott *et al.*, 1983; Singh *et al.*, 1985; Carey *et al.*, 1986b; Felton-Edkins and White, 2002; Larminie *et al.*, 1999). mRNAs encoding all five subunits of TFIIIC are found at elevated levels in fibroblasts transformed with polyomavirus or SV40 (Felton-Edkins and White, 2002). TFIIIC is also specifically up-regulated in ovarian tumours and contributes to the abnormal abundance of pol III transcripts in these tumours (Winter *et al.*, 2000). The mechanisms for raising TFIIIC are,

as yet unidentified, but do not seem to reflect a response to accelerated proliferation (Winter *et al.*, 2000; Scott *et al.*, 2001).

Cells transformed by adenovirus have the ability to subvert the pol III transcription machinery to enable high level expression of the viral VAI and VAII gene products during late stages of viral infection (Weinman *et al.*, 1974; Söderlund *et al.*, 1976). Thus, cells infected with adenovirus also show a marked increase in TFIIIC activity (Hoeffler and Roeder, 1985; Yoshinaga *et al.*, 1986; Sinn *et al.*, 1995; Hoeffler *et al.*, 1998). Adenoviruses express the oncoprotein E1A, which can alter the ratio of active TFIIIC2a versus inactive TFIIIC2b, a mechanism which is also used by the SV40 large T antigen to increase TFIIIC2 activity (White *et al.*, 1990; Sinn *et al.*, 1995; Hoeffler *et al.*, 1998).

Subversion or amplification of the transcription factor TFIIIA by viruses has not been reported, however, alterations in the level of TFIIIA during *Xenopus* development control the nature of the 5S rRNA genes which are transcribed at different developmental stages (reviewed by Wolffe and Brown, 1988). Enormous stockpiles of ribosomal components are generated to sustain them through the initial stages of embryogenesis when biosynthesis is curtailed. The high expression of 5S rRNA is possible due to excessive amounts of TFIIIA molecules which results in disproportionate 5S rRNA synthesis compared to production of 18S and 28S rRNA.

Whilst overexpression and activation of TFIIIA and TFIIIC has been discussed, TFIIIB is also subject to these molecular controls and the de-repression of TFIIIB has also been reported as a mechanism for raising pol III output (reviewed in White, 2004a). The mechanisms of how TFIIIB can be targeted by tumour suppressors and transforming proteins, and the way in which it is subject to cell cycle control will be discussed in the following sections.

1.11.2 RB

The retinoblastoma protein (RB) is a nuclear protein of 105 kDa that is encoded by an important tumour suppressor gene (Weinberg, 1992; Whyte, 1995). RB functions as a negative regulator of cell-cycle progression, mediated through its ability to bind and regulate the activity of a variety of transcription factors required for entry into S phase, including the E2F family of transcription factors (Chellappan *et al.*, 1991; Hiebert *et al.*, 1992). RB is phosphorylated and dephosphorylated in a cell cycle-dependent manner and it is clear that these changes regulate its activity. Phosphorylation and deactivation of RB during G1 involves sequential phosphorylation by the cyclin D- and cyclin E-dependent kinases (Hunter and Pines, 1994; Sherr, 1994).

In addition to regulating transcription by pol II, RB family members can also repress transcription by pol I (Cavanaugh *et al.*, 1995; Voit *et al.*, 1997) and pol III (White *et al.*, 1996; Larminie *et al.*, 1997; Sutcliffe *et al*, 1999). The specific repression of pol III by RB was first demonstrated by White and co-workers. Evidence from *in vitro* transcription assays and transient transfection assays showed for the first time that high levels of RB can inhibit pol III activity (White *et al.*, 1996). Comparison of 2 osteosarcoma cell lines showed that an RB-negative (SAOS2) cell line could express a transfected pol III template five times more actively than a matched cell line (U2OS) which contained wild type RB, moreover, the level of pol III activity was elevated specifically in primary cells taken from RB-knockout mice (White *et al.*, 1996).

Several lines of evidence suggested that RB could repress pol III transcription by physically interacting with TFIIIB (Larminie *et al.*, 1997). It was shown that RB inactivation of pol III transcription can be overcome by the addition of purified TFIIIB. Also, Brf1 and TBP could be co-immunoprecipitated with RB from partially fractionated HeLa cell extracts (Larminie *et al.*, 1997). The interaction of RB with TFIIIB was

abolished in SAOS2 osteoscaroma cells which contain a truncated form of RB (Larminie *et al.*, 1997). Moreover, the level of TFIIIB activity was elevated specifically in primary cells taken from RB-knockout mice (Larminie *et al.*, 1997). TFIIIB is therefore regarded as the principal target for RB-mediated repression of pol III, although a subsidiary interaction with TFIIIC may also contribute to the effect (Chu *et al.*, 1997).

The hypophosphorylated form of RB was found to interact with TFIIIB to suppress pol III transcription (Sutcliffe *et al.*, 1999; Scott *et al.*, 2001). In fact, the hypophosphorylated form of RB and RB-related pocket proteins, p107 and p130 all bind Brf1 (Sutcliffe *et al.*, 1999). It has been demonstrated that the hypophosphorylated form of RB associates with TFIIIB during late G0 and early G1 phases, and that this interaction substantially decreases as cells approach S phase (Scott *et al.*, 2001). The hyperphosphorylated form of RB was found to release Brf1, and this correlated well with the previous demonstration that TFIIIB activity increases at the G1-S transition (White *et al.*, 1995), as this is the same time that RB is inactivated due to hyperphosphorylation. Consistent with these observations, pol III transcription is stimulated strongly when cells are transfected with vectors encoding cyclin D/cdk4 and cyclin E/cdk2 (Scott *et al.*, 2001).

RB is postulated to block pol III transcription by preventing TFIIIB from interacting with TFIIIC and by preventing pol III recruitment to the initiation complex. Since the majority of genes that are transcribed by pol II are not affected directly by the presence of RB (White *et al.*, 1996), RB is considered a gene-specific regulator of pol II, whereas it acts as a general regulator of pol III.

1.11.3 c-Myc

c-Myc was first identified as a cellular counterpart of the transforming gene from avian myelocytomatosis virus MC29 (Sheiness and Bishop, 1979). c-Myc has been shown to play an extensive role in cancer, as it can collaborate with other oncogenes to induce cellular transformation (Land *et al.*, 1983). This can be achieved as c-Myc affects cell cycle regulation, apoptosis and metabolism positively, whereas it affects cellular differentiation and cell adhesion negatively (Dang *et al.*, 1999; Eisenman, 2001). As a result, aberrant c-Myc expression is observed in many human cancers, leukaemias and lymphomas (Spencer and Groudine, 1991). In fact, it is estimated that 1/3 of all human cancers display deregulated c-Myc activity (Prendergast, 1999). Consistent with potent growth-promoting properties of this protein, c-Myc was found to stimulate pol III transcription (Gomez-Roman *et al.*, 2003). Pol III activity was induced in primary human fibroblasts by c-Myc-oestrogen receptor (Myc-ER) expressed from a retroviral vector. In contrast, when myc- knockout and matched wild type fibroblasts were compared, the knockouts show 7-fold lower expression of pol III transcripts, demonstrating that pol III activity is compromised in *c-myc* $^{-/-}$ cells. Chromatin immunoprecipitation (ChIP) analysis showed that whilst no c-Myc was present at tRNA promoters of serum-starved cells, c-Myc was present at pol III-transcribed genes in tamoxifen-treated cells carrying the Myc-ER construct (Gomez-Roman *et al.*, 2003). This recruitment was found to occur by protein-protein interactions of TFIIIB with the N-terminal transactivation domain of c-Myc, which directly activates transcription (Gomez-Roman *et al.*, 2003).

In a different study, specific depletion of endogenous c-Myc by RNAi selectively reduced tRNA and 5S rRNA gene expression in HeLa cells (Felton-Edkins *et al.*, 2003b). This report demonstrates that c-Myc can regulate the expression of several type I and II pol III-specific genes. However, type III genes such as 7SK do not appear to be regulated by c-

Myc (Schlosser *et al.*, 2003), which suggests that c-Myc regulation of pol III transcription is via a type I and II promoter-specific TFIIIB activity.

Since activation of c-Myc results in increased translation and growth, and loss of c-Myc diminishes growth and protein synthesis (Schmidt, 1999), the growth-promoting potential of c-Myc may be mediated, in part, through the regulation of pol III transcription.

1.11.4 Regulation of pol III transcription during cellular stresses

p53 is a major tumour suppressor that is lost or mutated in more than half of human cancers (Hollstein *et al.*, 1991). p53 protects cells in response to physiological stress conditions by either activating or repressing a variety of cellular promoters (Cox and Lane, 1995; Ko and Prives, 1996; Levine, 1998; Giacci and Kastan, 1998; Vogelstein *et al.*, 2000). A well characterised example is the p21/WAF/CIP gene, which is activated by p53 and plays an important role in G1 arrest (El-Deiry *et al.*, 1993). It has been shown that p53 serves as a general repressor of pol III transcription and that TFIIIB is the target (Chesnokov, 1996; Cairns and White, 1998). Endogenous p53 can interact with TFIIIB and compromise its function (Cairns and White, 1998), and this interaction appears to be mediated through TBP (Crighton *et al.*, 2003). ChIP assays show that TFIIIB is excluded from promoters when bound by p53, and coimmunoprecipitations reveal that TFIIIB can no longer bind to TFIIIC and pol III (Crighton *et al.*, 2003). As a consequence fibroblasts from p53-knockout mice display markedly elevated synthesis of tRNA and 5S rRNA (Cairns and White, 1998).

Genotoxic agents such as Ultra Violet (UV) or methane methylsulphonate (MMS) can trigger a rapid and coordinate suppression of rRNA and tRNA synthesis in *S. cerevisiae* that is mediated, in part, by CK2 (Casein Kinase II) (Ghavidel and Schultz, 2001). CK2

was found to associate with and activate the TBP subunit of TFIIIB under normal (unstressed) conditions. However, upon DNA damage, CK2-dependent transcriptional repression is induced, which coincides with the down-regulation of TBP-associated CK2 (Ghavidel and Schultz, 2001). This suggests that TBP-associated CK2 acts as the effector kinase in a genotoxic stress signalling pathway.

Recent experiments in *S. cerevisiae* have identified a structurally novel and phylogenetically conserved protein, Maf1 (Pluta *et al.*, 2001), as a negative regulator of pol III transcription in response to diverse cellular conditions (Upadhya *et al.*, 2002; Desai *et al.*, 2004). An absolute requirement of Maf1 in the repression of pol III transcription has been demonstrated in maf1-deletion strains following interruption of the secretory pathway, and in response to DNA damage, growth to stationary phase and treatments with rapamycin and the anti-fungal compound chlorpromazine (Upadhya *et al.*, 2002; Desai *et al.*, 2004). This suggests that a common mechanism functioning downstream of Maf1 acts to repress the pol III transcription machinery in yeast. Indeed, several studies have shown that this regulation is via TFIIIB (Upadhya *et al.*, 2003; Willis *et al.*, 2004; Desai *et al.*, 2004).

1.11.5 Regulation of pol III transcription during differentiation

Pol III transcription has been found to decrease dramatically during parietal endoderm formation, both in culture and in the developing mouse (Vasseur *et al.*, 1985; White *et al.*, 1989). This appears to be due to loss of TFIIIB activity (Alzuherri and White, 1998; White *et al.*, 1989). Experiments revealed that there is a significant decrease in the Brf1 subunit of TFIIIB (Alzuherri and White, 1998) and some decrease in the level of TBP (Alzhurri and White, 1998, 1999; Perletti *et al.*, 2001). Despite the fact that TBP is utilised to

transcribe all three classes of genes, there is no overall change to pol II transcriptional activity (White *et al.*, 1989). This suggests that at least one of the TFIIIB-related factors is specifically downregulated in response to differentiation.

1.12 Kinases that regulate pol III transcription

Protein phosphorylation represents a qualitative mechanism that can up-regulate or repress transcription. Transcription factors can be kinase substrates themselves or their activity can be controlled indirectly by modifying proteins that modulate their function (Hunter and Karin, 1995). In the case of pol III transcription, specific examples exist for phosphorylation as a mechanism of transcriptional regulation, one such example being the mitotic repression of pol III transcription in *Xenopus* egg extracts. This can occur by inactivation of Brf1-TFIIIB through direct phosphorylation by cdc2-kinase, as well as through an alternative pathway involving an unidentified kinase (Gottesfeld *et al.*, 1994; Leresche *et al.*, 1996). Repression in mitotic HeLa cell extracts is, however, independent of cdc2 kinase activity, and instead is by an unidentified kinase, whose activity renders Brf1 and Bdp1 functionally limiting (Fairley *et al.*, 2003).

In addition, the ERK (extracellular signal-regulated kinase), Wnt and mTOR (mammalian target of rapamycin) signalling pathways have been identified as regulators of pol III transcription in response to growth factor stimulation. Positive or negative regulation reflects whether (or not) the cell's environment is conducive for growth.

1.12.1 ERK signalling

The ERK MAPK (mitogen-activated protein kinases) are stimulated in response to mitogens through a signalling cascade involving Ras, Raf and MEK (Downward, 2002).

ERK has been shown to stimulate the assembly of pol III transcription complexes via the direct binding and phosphorylation of the Brf1 subunit of TFIIIB (Felton-Edkins *et al.*, 2003a). Consequently, a substitution in the ERK docking domain or phosphoacceptor site of Brf1 substantially reduces pol III transcription (Felton-Edkins *et al.*, 2003a). Blocking ERK signalling in cells with an inhibitor of MEK reduces the interaction of TFIIIB with both TFIIIC and pol III, as determined by coimmunoprecipitation, whilst ChIP analysis showed a reduction in the occupancy of Brf1 and pol III at tRNA and 5S rRNA promoters (Felton-Edkins *et al.*, 2003a). In direct contrast, activating mutations in upstream effectors of the ERK signalling cascade such as Ras or Raf, stimulate expression of a pol III reporter in transfected fibroblasts (Felton-Edkins *et al.*, 2003a). Mitogenic stimulation of ERK activity therefore increases cell growth by direct transcriptional activation of 5S rRNA and tRNA. However, since phosphorylation of Brf1 is only partially reduced by blocking ERK activity, it is probable that other kinases are also involved.

1.12.2 CK2

Another factor implicated in the activation of pol III transcription is CK2 (Hockman and Schultz, 1996; Ghavidel and Schultz, 1997, 2001; Johnston *et al.*, 2002). CK2 is a highly conserved enzyme which forms part of the Wnt signalling pathway in both flies and mammals (Willert *et al.*, 1997; Song *et al.*, 2000). Many studies have shown that increased abundance or activity of CK2 is associated with cell growth and proliferation, and that overexpression of CK2 results in transformation and tumorigenesis (reviewed in Johnston *et al.*, 2002; White, 2004b). One aspect of CK2's growth-promoting activity may be through its reported role in regulating pol III transcription (Hockman and Schultz, 1996; Ghavidel and Schultz, 1997; 2001; Johnston *et al.*, 2002). In yeast cells with reduced CK2 activity, pol III transcription is repressed (Hockman and Schultz, 1996). These genetic studies first demonstrated the requirement for CK2 in yeast, since shifting a strain carrying

a temperature sensitive version of CK2α to a non-permissive temperature, specifically inhibits both growth and pol III transcription (Hockman and Schultz, 1996).

The function of CK2 appears to be conserved in mammalian cells as CK2 has been reported to co-purify with TFIIIB activity in both yeast and humans (Ghavidel and Schultz, 1997; 2001; Johnston *et al.*, 2002). Experiments performed with partially purified pol III, TFIIIC and TFIIIB fractions suggested that CK2 associates with and phosphorylates TBP in yeast (Ghavidel and Schultz, 1997; 2001). In contrast, endogenous co-immunoprecipitations and phospholabelling studies have shown that CK2 associates with and phosphorylates Brf1 in mammalian cells (Johnston *et al.*, 2002). In yeast, phosphorylation of a TFIIIB fraction by CK2 is required for efficient association of TFIIIB with TFIIIC (Ghavidel and Schultz, 1997; 2001). Dephosphorylation of this fraction with a phosphatase greatly diminishes its transcriptional activity (Ghavidel and Schultz, 2001). The addition of competitive and chemical inhibitors of CK2 showed that phosphorylation of TFIIIB by CK2 is necessary for TFIIIB to interact with TFIIIC *in vitro* and in fibroblasts (Johnston *et al.*, 2002).

A recent report proposes that CK2 phosphorylation of pol III itself is required for transcription, and disputes that phosphorylation of components TFIIIB are activating for pol III transcription at a type III promoter (Hu *et al.*, 2003). Further work from this group suggests that CK2 is directed to phosphorylate different targets within the basal pol III transcription machinery at different times during the cell cycle, with opposite transcriptional effects (Hu *et al.*, 2004). CK2 treatment of the pol III complex is required for transcription, whereas treatment of Brf2-TFIIIB is inhibitory (Hu *et al.*, 2003, 2004) and occurs through the phosphorylation of Bdp1 during mitosis (Hu *et al.*, 2004). However, inhibition of CK2 in S phase extracts debilitates transcription (Hu *et al.*, 2004). These findings from Hu and co-workers may be explained by the fact that a minimal

system was used and that type I and II promoters are not regulated in the same way as type III. Furthermore, this regulation remains to be shown *in vivo*. Nevertheless, it is agreed that pol III transcription at human type I, II and III promoters requires CK2 activity (Johnston *et al.*, 2002; Hu *et al.*, 2003; Hu *et al.*, 2004). These results suggest that CK2 can activate pol III transcription from type I and II promoters by phosphorylating TBP in the yeast system and Brf1 and pol III in the human system (Ghavidel and Schultz, 1997; 2001; Johnston *et al*, 2002) and can inhibit pol III transcription at type III promoters by phosphorylating Bdp1 during mitosis (Hu *et al.*, 2004).

1.12.3 mTOR

The adaptation of growth in response to nutritional changes is essential for the proper development of all organisms. Mammalian target of rapamycin (mTOR) has been proposed as a central component of a pathway which integrates both nutrient and mitogenic signals to regulate growth (increased mass and cell size) and cell division (Reviewed in Fingar and Blenis, 2004). A key downstream target of mTOR is protein synthesis (Chou and Blenis, 1995; Lawrence and Abraham, 1997). However, studies in mammals also indicated that synthesis of rRNA is rapamycin sensitive, and thus transcription by pols I and III is controlled by mTOR (Majahan, 1994). Run-on transcription experiments using nuclei from rapamycin-treated cells indicated a dose-dependent inhibition of rRNA genes which could be relieved when rapamycin was removed from the media (Mahajan, 1994). Yeast TOR was also found to control rRNA and tRNA synthesis via pol I and pol III (Zaragoza *et al.*, 1998; Powers and Walter, 1999); extracts from cells treated for just 1 hr were 2- to 4-fold less active for pol III transcription than controls. In addition, TOR was found to regulate ribosome biogenesis in yeast, as rapamycin treatment rapidly and severely reduces pol II-mediated transcription of genes encoding ribosomal proteins (Cardenas *et al.*, 1999; Powers and Walter, 1999).

The finding that TOR positively controlled both the biosynthesis and the activity of the translation machinery underscored the central role of TOR in mediating cell growth (Schmelzle and Hall, 2000). More recent studies in both yeast (Claypool *et al.*, 2004) and mammalian cells (Hannan *et al.*, 2003; Mayer *et al.*, 2004), have gone some way toward identifying mTOR-regulated targets of the pol I system. However, relatively little is known about how mTOR regulates pol III transcription.

1.13 Coordinate regulation of pol I and pol III

Organisms adjust their translational capacity to meet but not exceed the need for protein synthesis. Consequently, the production of the eukaryotic translational apparatus involves the coordinate synthesis of the 28S, 18S and 5.8S ribosomal RNAs transcribed by pol I and 5S rRNA and tRNAs transcribed by pol III. Under balanced growth conditions, the levels of both pol I- and III-specific transcription increases proportionally with growth rate (Clarke *et al.*, 1996). Moreover, the outputs of pols I and III in mammalian cells increase in parallel following serum stimulation and fluctuate together during passage through the cell cycle (Johnson *et al.*, 1974; Mauck and Green, 1974; Klein and Grummt, 1999; White *et al.*, 1995). One method to co-ordinate transcription by pols I and III would be to target shared components of the transcription machinery. However, regulation of pols I and III by key regulators, and signalling pathways involved in growth is achieved by targeting unique rather than shared components of the pol I and III machineries.

The synthesis of rRNA and tRNA can be initiated by the phosphorylation of key transcription factors that regulate pols I and III. As an example, CK2 activity was found to be necessary for the efficient interaction of TFIIIB with TFIIIC *in vitro* and *in vivo*

(Johnston *et al.*, 2002). Thus, the phosphorylation of TFIIIB by this enzyme regulates pre-initiation complex formation and increases pol III transcription (Johnston *et al.*, 2002). Phosphorylation of the pol I transcription factor UBF, is reported to be directly dependent on mitogenic stimuli and cell cycle progression, as UBF is hypophosphorylated and transcriptionally inactive in quiescent cells (O'Mahony *et al.*, 1992; Voit *et al.*, 1992; Voit *et al.*, 1995). Since UBF phosphorylation regulates its ability to bind and recruit SL1 (Kihm *et al.*, 1998; Tuan *et al.*, 1999), it can be seen that phosphorylation controls promoter recruitment of the TBP-containing initiation factors (SL1 or TFIIIB) in both the pol I and pol III systems.

In healthy cells, pols I and III are subject to repression by p53 as well as RB (Cairns and White, 1998; Cavanaugh *et al.*, 1995; White *et al.*, 1996; Zhai and Comai, 2000). p53 has been shown to repress pol I transcription by directly binding SL1 and preventing it from binding UBF (Zhai and Comai , 2000). This binding interferes with the assembly of a protein complex that is required for transcriptional initiation on the rRNA promoter (Zhai and Comai, 2000). In the pol III system, it was found that the interaction of p53 with TFIIIB represses pol III transcription (Cairns and White, 1998; Chesnokov *et al.*, 1996). This is mediated by direct contacts with the TBP subunit (Crighton *et al.*, 2003), which prevents the association of TFIIIB complexes with TFIIIC and pol III, thus impeding the formation of functional transcription complexes (Crighton *et al.*, 2003).

In addition to regulation by c-Myc, and by ERK, Wnt and mTOR signalling, two important tumour suppressors target pol I and pol III transcription. In cancer cells which harbour inactivating mutations of p53 and RB, deregulation of growth through increased pol I and III activity might contribute to tumorigenesis. In fact, the activities of pols I and III are known to be elevated in murine tumours and by transforming agents (reviewed by White, 1997). Ribosome biogenesis is a high energy- and nutrient- consuming process, as it

accounts for a major portion (80%) of the total cellular biosynthetic output (Warner, 1999; Moss and Stefanovsky, 2002; Grummt, 2003). Pols I and III products are essential in the process of ribosome biogenesis and it is apparent that they are both entangled in a network of regulatory interactions that help coordinate their outputs and encourage the balanced production of ribosomal components.

1.14 Cell cycle progression is not required for cell growth

As a high proportion (80-90%) of the cell's dry mass is protein (Zetterberg and Killander, 1965), it follows that growth (defined as an increase in cell mass), is directly proportional to the rate of protein accumulation (Baxter and Stanners, 1978). By analysing temperature sensitive cdc mutants in *S. cerevisiae*, Hartwell and co-workers gained important insight into the relationship between cell growth and division. They found that when cell division is blocked upon the inactivation of cdc genes, cell growth continues and the yeast arrest at a large size (Johnston *et al.*, 1977). In contrast, when cells are deprived of nutrients or when biosynthetic proteins are inactivated (via conditional cdc knockouts), cell division and cell growth are co-ordinately blocked. Similarly, work in *D. melanogaster* showed that when cell cycle regulatory genes (dE2F or cdc2) are disrupted then cells arrest at a large size (Weigmann *et al.*, 1997; Neufeld *et al.*, 1998). More recently Fingar and co-workers found that inducing a G1 phase cell cycle block in mammalian fibroblasts by overexpression of the cyclin D/cdk4 inhibitor p16 induces a shift to increase cell size (Fingar *et al.*, 2002). These initial findings and subsequent studies by others demonstrated that a critical mass must be reached to allow cell cycle progression (Fingar *et al.*, 2002, Nuefeld and Edgar, 1998, Johnston *et al.*, 1977; Zetterberg and Killander 1965; Sudbery 2002). Interestingly, whilst cell growth is required for cell cycle progression, the reverse is not true, cell cycle progression is not required for cell growth (Johnston *et al.*, 1977; Fingar *et al.*, 2002). The molecular mechanisms by which cell growth is coupled to cell division

are poorly understood. However, this dissociation of cell growth and cell division is indicative that distinct pathways control these essential processes and that cross talk must occur for one to be dependent on the other.

1.15 mTOR and the regulation of growth

Increased protein synthesis is one of the major anabolic events required for the growth response (Kozma and Thomas, 2002). The availability of tRNA and rRNA is clearly an important determinant of the rate of translation. A high level of transcription by pols I and III is therefore essential to maintain rapid growth. As discussed previously, regulation of protein synthesis is an important aspect of growth control. A major determinant of this process is rate of rRNA gene transcription by pol I (Stefanovsky *et al.*, 2001), and the availability of tRNA and 5S rRNA, which are synthesised by pol III (White, 2004b). The combined activities of pol I and pol III account for more than 80 % of cellular transcription in growing cells. Abnormally high rates of transcription by pols I and III are a general feature of transformed and tumours cells (Schwartz *et al.*, 1974), highlighting their importance in the control of growth. mTOR is known to positively control both the biosynthesis and activity of the translation machinery, and therefore the rate of cellular protein synthesis. This suggests a central role of mTOR in mediating growth which may occur through the coordinate regulation of transcription by pols I and III, and translation.

1.16 Aims

The mTOR signalling pathway promotes both growth and proliferation (Thomas and Hall, 1997; Schmelzle and Hall, 2000) and increased rates of tRNA and rRNA synthesis are necessary for accelerated rates of proliferative growth (Thomas, 2000; Moss and Stefanovsky, 2002; Brown *et al.*, 2000). On the basis of these observations, we

hypothesised that mTOR, in addition to its effects on translation, might directly regulate the function of molecules that control synthesis of tRNA and rRNAs. In support of this idea, mTOR has been identified as a regulator of pol I transcription in mammalian cells. Therefore, the overall objective of this Ph.D. was to identify if pol III transcription is also under the control of the mTOR pathway and investigate possible mechanisms of this control.

The first aim was to find if the mTOR and PI3K inhibitors rapamycin and LY294002 can regulate pol III transcription. After this had been established, an analysis of whether these effects were independent of the existing known mechanisms was to be carried out, and further mechanisms of control considered. The remainder of the investigation was concerned with whether Maf1; a negative regulator of pol III in yeast, had a conserved function in mammalian cells and to elucidate the mechanisms responsible for this regulation.

Chapter 2

Materials and Methods

2.0 Materials and Methods

(for list of suppliers see Appendix 1)

2.1 Cell culture

BALB/c 3T3 (A31) and CCL39 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium), which was supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. RAT 1A cells expressing haemagglutin (HA)-tagged Brf1 were cultured in DMEM supplemented with 5% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and 50 mg/ml G418 sulphate. All cell types were grown in humidified atmosphere containing 5% CO_2 at 37°C. Cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents. Cells were routinely passaged when subconfluent, approximately every 2 to 3 days. After media was aspirated from the flask, 2 ml of buffered trypsin-EDTA (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) was added to the cells, and then aspirated immediately. A further 2 ml was added and left for approximately 2 minutes at 37°C. Following trypsinisation, fresh media was immediately added to the dissociated cells in order to neutralise the trypsin.

Cell lines were stored by cryofreezing. To do this, cells were trypsinised as described above and, following pelleting by centrifugation, cells were resuspended in a solution of 70% (v/v) DMEM, 20% (v/v) FBS (no supplements) and 10% (v/v) dimethyl sulfoxide (DMSO). Aliquots of cells were put into cryo-tubes at density of 2 x 10^6 cells/ml/tube and frozen in stages by initially being placed at –80°C overnight and subsequently being transferred to liquid nitrogen storage. Thawing of cells was performed rapidly by placing cryo-tubes in a water bath at 37°C until just thawed. Cells were then mixed with fresh growth media, centrifuged and the supernatant aspirated off to ensure removal of DMSO prior to re-suspension in 10% (v/v) FBS DMEM. For rapamycin (Calbiochem) treatment of the cells, cells were grown to 70% confluency and treated with 100 nM final concentration of rapamycin for specific times. Similarly, LY294002 (Calbiochem) treatment was carried out using 50 µM final concentration of the drug. Both Rapamycin and LY294002 stocks were diluted in DMSO.

2.1.1 Amino acid starvation of A31 cells

A31 fibroblasts cultured in normal growth media were washed 2 times with Earle's Balanced Salt Solution (EBSS) (Gibco), then incubated in EBSS containing 10% (v/v) dialysed FBS (dFBS) plus 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 4.5g/litre glucose to match that in DMEM, for the times indicated. The effect of amino acid starvation was compared to cells grown in DMEM plus 10% (v/v) dFBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.1.2 Re-addition of amino acids to A31s

A31 fibroblasts cultured in normal growth medium were serum-starved by incubating in DMEM medium without FBS for 16 hours. The cells were then washed twice with Dulbecco's phosphate–buffered saline (D-PBS) containing 4.5g/litre glucose to match that in DMEM and incubated in the same buffer for 1 hour. Re-addition of the amino acids involved changing the medium to D-PBS with 4.5g/litre glucose containing 1 x amino acids (Gibco) for 1 hour. The concentration of each amino acid designated as 1 x is as follows (in mg/L); L-Arg, 84; L-Cys, 48; L-Glu, 584; L-His, 42; L-Ile, 195; L-Leu, 105; L-Lys, 146; L-Met, 30; L-Phe, 66; L-Thr, 95; L-Trp, 16; L-Tyr, 72; L-Val, 94. A mixture of these amino acids, each at this concentration is determined "1 x amino acid mixture". In some cases, 10% (v/v) dFBS was also added with the 1 x amino acid mixture for 2 hours.

2.1.3 Cardiomyocytes

Hearts were excised from 1- to 5-day-old Sprague Dawley rats by Central Biological Services (Institute of Biomedical and Life Sciences, University of Glasgow), and primary cultures of rat neonatal cardiomyocytes were prepared by Sarah Goodfellow, Fiona Cairns and Louise Derblay. Myocytes were kindly provided plated on to 6-well tissue culture dishes that had been coated with a 1% (w/v) gelatin (Sigma) solution, in Dulbecco's modified Eagle's medium/medium 199 (DMEM/M199 at a ratio of 4:1 v/v) supplemented with 10% (v/v) horse serum (HS), 5% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (all Sigma). Cells were plated at a density of approximately 10^3 cells per mm^2. After 24 hours, confluent myocytes had begun to beat spontaneously. At this time, cells were washed in DMEM/M199 (4:1) supplemented with 4% (v/v) HS, 100 units/ml

penicillin and 100 μg/ml streptomycin (maintenance medium), then maintained in this medium until use (usually 24 hours).

For experiments, cardiomyocytes were either serum-starved in DMEM/M199 (4:1) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin or grown in the same medium with 10% (v/v) FBS for 4 or 16 hours, in the presence of vehicle (DMSO), rapamycin (100 nM) or LY294002 (50 μM).

2.2 Preparation of total cellular RNA

Total cellular RNA was isolated from cells when approximately 80% confluent using TRI reagent (Sigma), a solution of guanidine thiocyanate and phenol, in accordance with the manufacturer's instructions. Media was aspirated off cells grown in 10cm tissue culture dishes. Cells from each dish were harvested by scraping in 1ml of TRI reagent per dish and transferred to a sterile eppendorf tube. Cells were left to stand for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was then added to each tube and the samples vortexed for 15 seconds. The samples were then allowed to stand for a further 15 minutes at room temperature prior to being centrifuged at 13, 000g for 15 minutes at 4°C. This resulted in separation of the samples into three phases: a lower red organic phase (containing protein), a middle white interphase (containing precipitated DNA) and an upper colourless aqueous phase (containing RNA). The aqueous phase was carefully removed, ensuring no contamination from the remaining phases, and transferred to fresh eppendorf tubes. Isopropanol (500 μl) was added to each of these tubes containing the aqueous RNA and thoroughly mixed by repeated inverting. Following 5 – 10 minutes incubation at room temperature to allow maximal precipitation of RNA, samples were centrifuged at 13, 000g for 10 minutes at 4°C. The supernatant was then removed and the remaining RNA pellet was washed with 1ml of 75% (v/v) ethanol made up with diethylpyrocarbonate (DEPC)-treated dH_2O (0.1% (v/v) DEPC, thoroughly mixed into solution, left overnight at room temperature and then autoclaved to inactivate the remaining DEPC). The samples were vortexed briefly, subsequently microcentrifuged at 7, 500g for 5 minutes at 4°C and the supernatant aspirated off. Residual supernatant was removed with a P20 pipette following pulse microcentrifugation. Appropriate volumes of DEPC-dH_2O, in the range of 10 – 30 μl, were added to the RNA pellets and the samples were heated in a 65°C water bath for 10 – 15 minutes to facilitate resuspension of the RNA. The samples were stored at –70°C.

2.3 Quantification of nucleic acids

Nucleic acid concentrations were estimated by spectrophotometry at A_{260}/A_{280} (WPA lightwave S2000, WPA Cambridge), where an OD of 1 at 260 nm corresponds to 50 μg/ml of double-stranded DNA and 40 μg/ml of single-stranded DNA and RNA (Sambrook *et al*, 1989). RNA concentration was determined by UV spectrophotometry at A_{260}/A_{280}. Readings were zeroed with the solution in which samples had been diluted. The following formulae were used;

RNA concentration (μg/ml) = absorbance at 260nm x 40 x dilution factor.

DNA concentration (μg/ml) = absorbance at 260nm x 50 x dilution factor.

The ratio of A_{260}/A_{280} provided an estimate of nucleic acid purity. Values between 1.8 and 2.0 indicated preparations of acceptable purity.

2.4 Northern blot analysis of total cellular RNA

Typically RNA samples of 10 – 30 μg were used in analysis, made up to a total volume of 10 μl with DEPC-dH_2O. 10 μl of 2 x RNA sample buffer (1 x MOPS comprised of solutions made up with DEPC-dH_2O, (20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0), 4.4 M formaldehyde, 54% (v/v) formamide) was added to each sample prior to heating at 65°C for 15 minutes to denature the RNA secondary structure. The samples were immediately transferred to ice to prevent any renaturation, and centrifuged for 5 seconds to deposit all the fluid at the bottom of the tube. A further 2 μl of 1 mg/ml ethidium bromide and 2 μl of 10 x RNA loading buffer (50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) were added to each sample. Following a 30 minute pre-run at 40 V of a denaturing gel (1% (w/v) agarose, 2.2 M formaldehyde, 1 x MOPS) in 1 x MOPS, samples were loaded and run for approximately 5 hours at 40V in order to electrophoretically separate the different species of RNA according to size. The gel was visualised under a UV transilluminator in order to confirm separation and photographed. It was then washed for 20 minutes in 20 x SSC buffer (3 M NaCl, 0.3 M sodium citrate pH 7.0) prior to capillary transfer, as described by Maniatis *et al.* (Maniatis *et al.*, 1982).

The transfer procedure required the prepared gel to be placed, inverted, on a bridge of Whatman 3MM chromatography paper supported on a glass plate and suspended over a reservoir of 20 x SSC buffer. An appropriate size of Hybond N nylon membrane optimised for nucleic acid transfer (Amersham) was pre-soaked in 20 x SSC and positioned over the gel, followed by a further two layers of pre-soaked Whatman paper; at each stage of layering, care was taken to ensure removal of air bubbles. This arrangement was surmounted with a stack of paper towels and an appropriate weight in order to allow efficient capillary action. During transfer, the migration of the RNA from the gel to the nylon membrane is facilitated by the passive movement of the transfer solution through the gel. Plastic wrap was used to prevent direct contact between the paper towels and the Whatman bridge; this ensured movement of the buffer was only through the gel. In order to achieve high-transfer efficiency, the capillary action was allowed to proceed overnight. Following transfer, the RNA was fixed to the membrane by UV-crosslinking at 1200 μJ.

Radiolabelled DNA probes complementary in sequence to a particular RNA of interest were used to locate it on the membrane. The B2 gene probe was a 240bp *Eco*RI-*Pst*I fragment from pTB14 (White *et al.*, 1989), and the acidic ribosomal phosphoprotein P0 (ARPP P0) probe, a 1 kb *Eco*RI-*Hind*III fragment from the mouse cDNA (Hurford *et al.*, 1997). The probes were labelled using a Megaprime DNA labelling system (Amersham) according to the random oligonucleotide priming method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1984). 25 ng of purified DNA template was denatured by heating at 95°C for 5 minutes in the presence of random hexamer oligonucleotide sequences and the appropriate volume of DEPC-dH_2O. DNA synthesis is primed by the hexamer oligonucleotides which are able to anneal to the DNA during slow cooling to room temperature. 10 μl of reaction buffer (from Amersham kit; containing dATP, dGTP, dTTP in Tris-HCl pH 7.5, β-mercaptoethanol and $MgCl_2$), 2 μl (2 U) DNA polymerase I Klenow fragment and 50 μCi of [α-^{32}P] dCTP (10mCi/ml, 3000Ci/mmol) were added to initiate labelling. This reaction was allowed to proceed for 1 hour at 37°C before the labelled DNA was denatured by heating at 100°C for 5 minutes, and chilled at 4°C until the nylon membrane with bound RNA had been pre-hybridised.

While the probe was being prepared, the nitrocellulose was pre-hybridised by rotation in a hybridisation oven at 45°C for 45 minutes in 25 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) sodium dodecyl sulphate (SDS), 45% (v/v) formamide in DEPC- dH_2O). The radiolabelled probe was then added to 25ml of fresh hybridisation buffer with rotation at 45°C overnight. The nylon membrane was then washed with rotation in wash buffer (40 mM sodium phosphate buffer pH 7.2, 1

mM EDTA, 1% (w/v) SDS in DEPC-dH_2O) at room temperature for 5 minutes and then twice for 15 minutes at 65°C in order to remove non-specifically bound radioactivity before being exposed to autoradiography film overnight at –70°C. Membranes were stripped by incubating in boiling water for 5 minutes and pre-hybridised again prior to being re-probed. Quantification of the RNA was achieved using densitometry (TotalLab v1.11)

2.5 Reverse transcriptase – polymerase chain reaction (RT-PCR)

2.5.1 Preparation of cDNAs

cDNAs were prepared using 3 µg of RNA (1µg/µl). 2 µl of hexanucleotide mix (Roche) diluted 1:10 with DEPC- dH_2O was mixed with 19 µl of DEPC- dH_2O. Primer annealing was carried out at 80°C and allowed to proceed for 10 minutes before transferral to ice. 8 µl of 5 x First Strand Buffer (Invitrogen), 4 µl of 0.1 M dithiothreitol (DTT), 2 µl of 10 mM dNTP mix (prepared in DEPC- dH_2O) and 1 µl (200U) of Superscript II Reverse Transcriptase (Invitrogen) was added to initiate reverse transcription, which was performed for 1 hour at 42°C before the reaction was stopped by heating at 70°C for 15 minutes.

2.5.2 Polymerase chain reaction (PCR)

PCRs were carried out using a Uvigene FTGENE2U (UVITEC Ltd). 2 µl of cDNA was amplified with 20 pmol of relevant primers under various cycling parameters (Table 2.1). Reaction mixtures also contained 0.5U of *Taq* polymerase (Promega), 1 X Taq DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 1 µCi of [α-^{32}P] dCTP (Amersham), and 0.2 mM of each dNTP, made up with nuclease-free water to a 20 µl final volume.

Table 2.1: **Primers and cycling parameters**

Transcript	Primers	Size of Product	Cycle number	Program (Denaturation; Cycling; Final Extension)
ARPP P0	5'-GCACTGGAAGTCCAACTACTTC-3' 5'-TGAGGTCCTCCTTGGTGAACAC-3'	265 bp	18- 22	95°C for 2 min 95°C for 1 min 58°C for 30 s 72°C for 1 min 72°C for 3 min
B2	5'-GGGGCTGGAGAGATGGCT-3' 5'-CCATGTGGTTGCTGGGAT-3'	120bp	15-18	95°C for 3 min 95°C for 30 s 58°C for 30 s 72°C for 30 s 72°C for 5 min
GAPDH	5'-TCCACCACCCTGTTGCTGTA-3' 5'-ACCACAGTCCATGCCATCAC-3	452 bp	18-22	95°C for 3 min 95°C for 30 s 58°C for 30 s 72°C for 30 s 72°C for 5 min
snRNA U6	5'-GCTCGCTTCGGCAGCACATATAC-3' 5'-TATCGAACGCTTCACGAATTTGCG-3'	96 bp	18-20	95°C for 3 min 95°C for 1 min 60°C for 30 s 72°C for 1 min 72°C for 5 min
Maf1	5'-GCAGTTCTGCCAGGAGGGCCA-3' 5'-CTCCATGGTGCTGGTCTCCTC-3'	613 bp	25-30	95°C for 2 min 95°C for 30 s 60°C for 30 s 72°C for 1 min 72°C for 5 min
$tRNA^{Leu}$	5'-GTCAGGATGGCCGAGTGGTGTAAGGC GCC-3' 5'-CCACGCCTCCATACGGAGACCAGACC C-3'	88 bp	25-30	95°C for 3 min 95°C for 30 s 68°C for 30 s 72°C for 30 s 72°C for 5 min

Table 2.1 Primers and cycling paramcters continued

Transcript	Primers	Size of Product	Cycle number	Program (Denaturation; Cycling; Final Extension)
$tRNA^{Tyr}$	5'-AGGACTTGGCTTCCTCCATT-3' 5'-GACCTAAGGATGTCCGCAAA-3'	84 bp	25-30	95°C for 3 min 95°C for 1min 65°C for 30 s 72°C for 15 s 72°C for 5 min
5S rRNA	5'-GGCATACCACCCTGAACGC-3 5'-CAGCACCCGGTATTCCCAGG-3	107 bp	18-23	94°C for 3 min 95°C for 30 s 58°C for 30 s 72°C for 1 min 72°C for10 min
7SK	5'-CGATCTGGTTGCGACATCTG-3' 5'-CGTTCTCCTACAAATGGAC-3'	247bp	25-30	95°C for 3 min 95°C for 30 s 57°C for 30 s 72°C for 30 s 72°C for10 min

Reaction products were resolved on 7% (v/v) polyacrylamide sequencing gels containing 7 M urea and 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run for 30 minutes at 40W in 0.5 x TBE and 2 µl of each sample was loaded after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was carried out for a further 1 hour at 40 W and the gel subsequently vacuum-dried at 80°C for 1 hour before being exposed to autoradiography film in order to detect the radiolabelled products. Quantification of results was achieved by densitometry (TotalLab v1.11).

2.6 Preparation of whole cell extracts

Whole cell extracts were prepared from cells grown in 10cm tissue culture dishes to facilitate scraping and were harvested at approximately 80% confluency. Preparation was performed on ice as rapidly as possible and all solutions and tubes were kept ice-cold. Cells were washed twice with 5 ml of PBS before being scraped with a plastic spatula into 5 ml of ice-cold PBS. Cells were collected in 50ml Falcon tubes and pelleted by slow centrifugation at 1100g for 8 minutes at 4°C. A small volume of fresh ice-cold PBS was used to resuspend the cell pellets and allow the cells to be transferred to eppendorf tubes. These were then microcentrifuged briefly at 4°C to re-pellet the cells and the PBS removed. The volumes of cell pellets were then measured by comparison with pre-measured volumes of water. Optimal microextraction requires pellets to be between 50 – 150 µl, giving approximately 0.5 – 3 x 10^7 cells; larger pellets were subdivided. An equal volume of freshly made pre-cooled microextraction buffer (450 mM NaCl, 50 mM NaF, 20 mM Hepes pH 7.8, 25% (v/v) glycerol, 1 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.2 mM EDTA, 40 µg/ml bestatin, 1 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin) was added to the cells and, following resuspension, the cells were immediately snap-frozen on dry ice. Cells were then placed in a 30°C water bath until just thawed before being immediately returned to dry-ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis, with cells then being microcentrifuged at 7, 000g for 7 minutes at 4°C after the third thaw. The supernatant was carefully decanted into a fresh tube, leaving behind the cell debris, and then promptly aliquotted and snap frozen. These whole cell extracts were then stored at –80°C.

2.7 Measuring protein concentration

The protein concentration of samples was determined using Bradford's reagent (Biorad). Quantification of the colour reaction produced upon mixing 1ml of diluted reagent (1:4 in distilled water) with a volume of sample containing protein in the range of ~1 –12 µg gave an accurate indication of protein concentration. A Bradford assay standard curve was constructed by measuring the absorbance of bovine serum albumin (BSA) standards at 595 nm in a UV spectrophotometer, as absorbance in response to increasing amounts of protein under these conditions is approximately linear. Three sample dilutions were measured and averaged, so as to provide a more accurate measurement of protein concentration.

2.8 Immunoprecipitation

Antibodies for immunoprecipitation experiments were coupled to protein A-sepharose beads (Pharmacia) or HA-tagged proteins were bound directly to monoclonal anti-HA-agarose conjugate (Sigma). 30 µl of packed beads was used per sample. Beads were washed twice with 200 µl TBS (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl), centrifuged at 6, 000g, and the supernatant removed prior to incubation with the appropriate antibody on a shaker for 1 hr at 4°C. The beads were then washed twice with 300 µl of TBS to ensure removal of unbound antibody.

For co-immunoprecipitation reactions, typically 500 µg of cell extract (made up to a total volume of 500 µl with TBS) and/or 5 -10 µl of *in vitro* translated protein was incubated with the prepared protein A-Sepharose beads covalently linked to the antibody of interest at 4°C for 3 hours on a rotating wheel with 0.25 µM phenymethylsulfonylfluoride (PMSF). The beads were gently pelleted by pulse microcentrifugation and the supernatants carefully removed. The beads were then washed with TBS and pulse- centrifuged so that the supernatant could be removed. This was repeated 4 times before the bound material was released by the addition of an equal volume of 2 x protein sample buffer (125 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0.25% (w/v) bromophenol blue), and analysed by SDS-PAGE and either Western Blotting or autoradiography.

2.9 SDS-polyacrylamide gel electrophoresis (PAGE) analysis

2.9.1. Separation of proteins by SDS-PAGE

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis. Typically, 7.8% (v/v) polyacrylamide resolving minigels (375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS) were used with a stacking layer comprised of 4% (v/v) polyacrylamide gel (125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS) based on the discontinuous buffer system described by Laemmli (Laemmli, 1970). Samples were boiled for 2 minutes in 1 x protein sample buffer (62.5 mM Tris-HCl pH 6.8, 0.5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.125% (w/v) bromophenol blue) prior to

loading. Electrophoresis was performed in 1 x SDS running buffer (0.1% (w/v) SDS, 76.8 mM glycine, 10 mM Tris-HCl, pH 8.3) at an initial voltage of 80 V while the bromophenol dye front moved through the stacking gel and a subsequent voltage of 120 V after reaching the resolving gel. Electrophoresis was allowed to proceed until the dye front had reached the bottom of the gel, approximately 1 – 1.5 hours.

2.9.2 Western blot analysis

Electrophoretic transfer of proteins resolved by SDS-PAGE to 0.2 μM pore nitrocellulose membrane (Biorad) was achieved using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8 mM glycine, 10 mM Tris-HCl pH 8.3, 16.5% (v/v) methanol) at 50 V for 2 hours. Following transfer, the membrane was blocked in milk buffer (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl, 0.2% (v/v) Tween-20, 4% (w/v) skimmed milk powder) (Marvel) for 1 hour at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer) overnight at 4°C (see Table 2.2 for list of antibodies). Excess primary antibody was removed by washing the blot 3 times for 5 minutes in fresh milk buffer before incubating for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in milk buffer) (DAKO). To ensure removal of excess secondary antibody, the blot was washed in fresh milk buffer, 3 times for 10 minutes. After one further 5 minute wash using 1 x TBS (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl), the blot was developed using the enhanced chemiluminescence method (ECL™, Amersham Pharmacia).

2.9.3 Western signal detection

HRP-conjugated (horseradish peroxidase) secondary antibodies were used to detect signals on Western blots. Horseradish peroxidase activity can be detected using either chemiluminescence or DAB substrates. Chemiluminescence detection, using the ECL™ Western Blotting analysis system (Amersham Pharmacia), was performed by adding equal volumes of reagent 1 and reagent 2 to the filter, incubating at room temperature for 1 minute, and then exposing the blot, covered in Saran Wrap, to ECL film (Amersham Pharmacia) for different lengths of time before developing using the X-OMAT film processor.

2.9.4 Coomassie staining of SDS-PAGE gels

When required, after SDS-PAGE, the gels were stained using Coomassie Brilliant Blue (5 % (v/v) glacial acetic acid, 45 % (v/v) methanol and 0.25 g/l Coomassie Brilliant Blue R which was filtered through Whatman No.1 filter paper). The gel was submerged in stain for approximately 1 hour before rinsing and destaining in the same acetic acid/methanol/H_20 mixture, without Coomassie dye. The destaining solution was changed frequently until the bands on the protein gel appeared sharp and the background on the gel clear, this typically took 3 hours.

2.9.5 Peptide design and Maf1 antibody synthesis

The rabbit polyclonal anti-Maf1 antibodies were directed against peptides corresponding to amino acids 136-148 (YDFSTARSHEFSREPS) and 265-281 (GGEGRAEETSTMEEDR). which were conserved between the human and mouse sequences. Peptides were synthesised and antibodies raised by Eurogentec. They were supplied after purification by peptide affinity chromatography.

2.9.6 Primary antibodies

Table 2.2: **Primary antibodies**

Protein	Antibody	Type	Company
Actin	C11	Polyclonal	Santa Cruz Biotechnology
Brf1	1284	Serum	In house
Cyclin D1	SC-450	Monoclonal	Santa Cruz Biotechnology
Cyclin E	SC-20684	Polyclonal	Upstate
c-MYC	9E10	Polyclonal	Santa Cruz Biotechnology
RB	C15	Monoclonal	Santa Cruz Biotechnology
phospho-RB	44-576	Polyclonal	Biosource International
S6K1	C-18	Polyclonal	Santa Cruz Biotechnology
phospho-S6K1	9202	Polyclonal	Cell signalling Technology
4EBP-1	R-113	Polyclonal	Santa Cruz Biotechnology
TFIIIC 63	N-15	Polyclonal	Santa Cruz Biotechnology
TFIIIC 90	1898	Polyclonal	In house
TFIIIC 102	3238	Polyclonal	In house
TFIIIC 110	4286-4	Polyclonal	In house
TFIIIC 220	Ab-7	Polyclonal	In house
p16	C-20	Polyclonal	Santa Cruz Biotechnology
TBP	mTBP-6	Monoclonal	In house
TBP	58C9	Monoclonal	Santa Cruz Biotechnology
BDP 1	2663	Polyclonal	In house
TAF I48	C19	Polyclonal	Santa Cruz Biotechnology
p44/42 ERK	9102	Polyclonal	Cell Signalling Technology
Phospho- ERK	E10 9101	Monoclonal	Cell Signalling Technology
Maf1	SK-2667	Polyclonal	Eurogentec
BN51	113	Serum	Gift from G. Ittman
RPC155	1900	Polyclonal	In House
TFIIB	C-18	Polyclonal	Santa Cruz Biotechnology

2.10 Storage, propagation and preparation of plasmid DNA

2.10.1 Plasmid selection

The plasmids used in this study contained the ampicillin resistance gene encoding β-lactamase and so were selected for by the presence of 100 μg/ml ampicillin when grown on L-Agar or in L-Broth. This antibiotic was made as a 100 mg/ml stock solution (w/v) in 50% (v/v) H_2O, 50% (v/v) ethanol and stored at –20°C.

2.10.2 Storage of bacterial cultures

1 ml of bacterial culture was added to 40% (v/v) glycerol solution (in H_2O) before being frozen in liquid nitrogen. Frozen stocks were stored at -80°C.

2.10.3 Transformation of competent cells

E.coli XL-1 Blue supercompetent cells (Stratagene) were transformed for plasmid storage and propagation. Cells, which were stored at –80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation ability. 0.4 μl of β-mercaptoethanol, which enhances transformation efficiency, was added to the 50 μl of cells that were required per transformation reaction to give a final concentration of 25 mM. Typically 10 – 20 ng of plasmid DNA was then gently mixed into the chilled cells. The contents were gently tapped occasionally during a 30 minute incubation on ice, before being heat shocked at 42°C for exactly 45 seconds and then transferred to ice for a further 2 minutes. Cells were incubated at 37°C for 1 hour on an orbital shaker (225 – 250 rpm) following the addition of 450 μl of preheated (42°C) SOC medium (LB broth, 0.04% (w/v) glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$). Typically 10 μl and 100 μl of the transformation mixture was then plated on LB agar (2% (w/v) LB, 2% (w/v) agar) plates containing 50 μg/ml ampicillin (Amp) and the plates were incubated at 37°C overnight to allow growth and colony-formation of the transformed cells.

2.10.4 Preparation of plasmid DNA

For large scale plasmid DNA preparation, a single isolated bacterial colony was selected from a freshly-streaked plate and used to inoculate 5ml of LB medium containing the selective antibiotic (100 μg/ml ampicillin). This was allowed to incubate with vigorous shaking at 37°C for ~6 hours to form a mini-culture and was subsequently used to inoculate 250ml of LB medium containing 100 μg/ml ampicillin. Following an overnight incubation at 37°C on an orbital shaker (~300rpm), cells were harvested by centrifugation at 6, 000g for 15 minutes at 4°C and plasmid DNA retrieved using the Qiagen Plasmid Maxi Kit.

The bacterial pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A) and then gently but thoroughly mixed with 10 ml of Buffer P2 (200 mM NaOH, 1% (w/v) SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5), which subsequently resulted in formation of a precipitate of potassium dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA were co-precipitated with the detergent, whilst the plasmid DNA remained in solution due to a lack of close protein associations. Precipitation was enhanced by a 20 minute incubation on ice and the precipitate pelleted by centrifugation at 20, 000g for 30 minutes at 4°C. The supernatant containing plasmid DNA was promptly removed and applied to a Qiagen-tip 500 column, pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to bind tightly. The resin was then washed twice with 30 ml of Buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol) and the purified plasmid DNA was subsequently eluted with 15 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol) and precipitated with 10.5 ml (0.7 volume) of room-temperature isopropanol. This was immediately followed with a 15, 000g centrifugation at 4°C for 30 minutes. The plasmid DNA pellet was then washed with 70% (v/v) ethanol, dried at room-temperature for 5 – 10 minutes and resuspended in an appropriate volume of sterile water or TE.

2.11 Restriction digests and DNA analysis

2.11.1 Restriction digests

Restriction digests were typically performed in 20µl volume and contained 1 µg DNA, 1 µl enzyme, 2 µl enzyme buffer appropriate to the restriction enzyme being used, and were made up to the final volume with H_2O. All enzymes and buffers were supplied by Promega. DNAs were digested with enzymes for 1 hour at 37°C. When double digestion was required the reaction was initiated with the enzyme with the lower strength buffer and after heat inactivation (at 65°C for 20 minutes) an appropriate amount of salt was added before addition of the second enzyme. When this was not feasible due to very different buffers required for each enzyme, purification of the first digestion product was performed using the Qiagen PCR Purification Kit and according to the manufacturers' instructions. Amounts of DNA in a restriction digest varied between 1-3 µg for plasmid DNA and 3-5 µg for genomic DNA.

Digests were mixed with 6 x Agarose Gel DNA Loading Buffer (0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene Cyanol, 30% (v/v) glycerol), analysed on 1% (w/v) agarose gels containing ethidium bromide (Sigma) and visualised on an ultraviolet light box. The concentrations of nucleic acid solutions were determined spectrophotometrically using a quartz cuvette.

A_{260} of 1 = 50 µg/ml dsDNA

2.11.2 Ethanol precipitation of DNA

When it was necessary to concentrate or clean DNA, ethanol precipitation was carried out. 0.1 volume 3 M sodium acetate, pH 5.3, was added to samples followed by 2.5 volumes of ethanol. The sample was kept at -20°C for at least 30 min to allow precipitation to occur. The DNA was then pelleted by centrifugation at 4°C for 30 min at 15, 000 *g*. The pellet was washed with 70% (v/v) ethanol and allowed to dry thoroughly before being resuspended in an appropriate volume of dH_2O (Sambrook *et al,* 1989).

2.11.3 Agarose gel electrophoresis of DNA

DNAs were separated in 1 % (w/v) agarose in 1x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) containing 0.1 μg/ml EtBr as described previously (Sambrook and Russell, 2001), using 1x TAE as the electrophoresis buffer. Sizes were compared to a 1kb ladder (Gibco-BRL). Prior to loading, 6 x loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in water) was added to the samples to a final 1x concentration of loading dye in the sample.

2.11.4 Purification of DNA from gels

DNA bands were excised from the gel using a clean scalpel blade and the DNA extracted using the Qiagen Gel Extraction Kit according to the manufacturers' instructions. DNA was typically eluted in 30 μl of pre-warmed buffer EB (Qiagen) or H_2O.

2.11.5 DNA ligations

For cloning inserts into vectors, plasmid DNA containing the vector and the insert were digested with appropriate restriction enzymes, as described in Section 2.11.1. For directional cloning of inserts, two different enzymes were used to restrict the vector and the insert, which permits cloning of the insert into the vector in a predicted and directional manner. After the restriction digest, both vector and insert were electrophoresed on a 1% (w/v) agarose gel and the DNA bands excised from the gel and gel-purified (see Section 2.11.4).

For the ligation reaction, a molecular ratio of 3:1 insert:vector was used and typically 50-100 ng of vector. Ligation reactions were carried out with 2 μl 10x T4 ligase buffer (Gibco BRL), with 1 μl (1 U) of T4 ligase (Gibco BRL) in a final volume of 20 μl with sterile water. The ligation was carried out overnight at 14°C. For transformation of XL1-Blue, 1 μl and 4 μl of the ligase reaction was used.

2.12 Subcloning of Maf1 into pCDNA3HA

A cDNA encoding *Mus Musculus* Maf1 (MmMaf1.His) was a gift from Olivier Lefebvre (CEA/Saclay, France). Primers were designed to add an *Xba*I site on to the 3' end of the gene (5'-GCGATCTAGACAAATACAGATCACTGGGAC-3'). An additional primer

which incorporated the *Bam*HI site already present on the gene was also designed (5'-TTCCACGGATCCATGAAGCTATTGGAGAAC-3'). The mammalian expression vector pCDNA3HA was digested with the restriction enzymes *Bam*HI and *Xba*I and MmMaf1.His was ligated into the polylinker of this vector.

2.13 Production of recombinant Maf1

Recombinant Maf1 was prepared by Danuta Oficjalska (CEA/Saclay, France). The *Homo sapiens* (Hs) Maf1 gene was cloned into the pFastBac vector from the Bac-to-Bac Baculovirus Expression System (Invitrogen) using *Bam*HI and *Sal*I restriction sites. The recombinant protein has 6 His tags at its C-terminal and was obtained by expression in insect cells infected by baculovirus. HsMaf1.His was then affinity purified using a Poros MC20 (Boeringer Mannheim) Cobalt-chelate affinity column, and was eluted using 50 mM Imidazole. The HsMaf1.His was 95% pure and was used at a concentration of 2 µg/µl. A fraction that was obtained from control purification of HsMaf1 with no His tag was used as control buffer for the HsMaf1.His experiments.

2.14 Chromatin Immunoprecipitation (ChIP)

Formaldehyde was added directly to plates of cells containing media to a final concentration of 1% (v/v). They were then incubated at 37°C for 10 minutes. Glycine was added to a final concentration of 0.125 M to stop the cross-linking. The plates were then transferred to ice, and scraped in the media, transferred to Falcon tubes, and centrifuged at 1500 rpm for 5 minutes at 4°C. The cells were then washed twice by resuspension in 40 ml chilled PBS and pelleted by centrifugation at 1, 500 g. The supernatant was then decanted off and the cells were incubated on ice for 30 minutes in 40 ml of high salt buffer (0.5% (v/v) NP-40/PBS, 1 M NaCl). Cells were then re-centrifuged and washed with 0.5% (v/v) NP-40/PBS. This was followed by hypotonic disruption for 30 minutes on ice with 40 ml of low salt buffer (0.1% (v/v) NP-40, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 M NaCl). The samples were then centrifuged at 1, 500 g at 4°C, the supernatant decanted off, and the pellets resuspended in 1 ml of low salt buffer. Nuclei were obtained by passing the sample through a 26 gauge needle three times. The samples were re-centrifuged and the nuclei resuspended in 2.7 ml of low salt buffer and lysed with 300µl sarkosyl. The samples were then transferred to a sucrose solution containing 40 ml low salt buffer/100 mM sucrose,

and centrifuged at 4000g for 10 minutes at 4°C. The supernantant was then discarded and the pellet resuspended in 3 ml of 1 x TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The process was then repeated. The pellet containing the genomic DNA was finally resuspended in 2 ml of 1 x TE and sheared by sonication (Branson sonifier, 10 x 10 second intervals at 40% duty cycle).

Sonicated material was adjusted with 1/10 volume of 11 x NET (1.65 M NaCl, 5.5 mM EDTA, 5.5% (v/v) NP-40, 550 mM Tris-HCl pH 7.4). Samples were centrifuged at 13, 000 g for 5 minutes at room temperature and aliquots of the supernantant were put into eppendorfs. The volume of the sample used in the subsequent immunoprecipitation depended on the number of antibodies used. 10 % of each sample was stored at 4°C to use as input control. 4 µg (~25µl) of antibody was added to each tube and they were incubated overnight at 4°C. Protein A-Sepharose beads (Pharmacia) were prepared by washing in 1 x NET buffer and centrifuging at 6, 000 g for 30 seconds at room temperature. This was repeated and after the supernatant was removed a further 500 µl of 1 x NET buffer was used to resuspend the protein-A beads. 100 µl of the protein A/ NET suspension was added to each tube and these were left rotating for 1-2 hours at 4°C. The negative control was incubated with beads alone. The samples were re-suspended in 1 ml of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 % (w/v) SDS, 0.5% (w/v) deoxycholate, 1% (v/v) NP-40) and loaded onto a Polyprep column (Biorad). The columns were washed twice in 10 ml of RIPA buffer, twice in 10 ml of LiCl buffer (10 mM Tris-HCl, 250 mM LiCl, 0.5% (v/v) NP-40, 0.5% (w/v) deoxycholate, 1 mM EDTA pH 8.0) and twice in 10 ml of TE. Once all the remaining TE had eluted the columns were stoppered and 200 µl of 1 x TE/1% (w/v) SDS was used to re-suspend the beads in the columns. The eluant was collected in a 1.5 ml tube and then rotated for 10 minutes at room temperature before being centrifuged at 8, 000 g for 1 minute at room temperature. The supernatant was transferred to a fresh tube, and the process was repeated until a further supernatant was collected, and unified with the first supernantant. 1/10 volume of the initial immunoprecipitated sample was pipetted into a fresh eppendorf and made up to 400 µl with 1 x TE/1% (w/v) SDS, before adding 5 µl of proteinase K (10mg/ml). Inputs taken prior to immunoprecipitation were made up in the same way as the samples and all tubes were then incubated overnight at 42°C to degrade proteins and antibodies. DNA was extracted twice using PhOH/$CHCl_3$/IAA, and ethanol precipitated. The immunoprecipitated DNA was then re-suspended in 40 µl of 1 x TE and quantified by PCR.

2.15 Pol III *in vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 20 μg of HeLa nuclear extracts (Computer Cell Culture Center, Mons, Belgium) to provide the basal pol III transcription components. This was supplemented with the addition of 250 ng of plasmid DNA to supply a specific pol III template and reactions were carried out in a 25 μl volume with a final concentration of 12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM $MgCl_2$, 0.28 mM EDTA, 1.2 mM DTT, 10% (v/v) glycerol, 1 mM creatine phosphate, 0.5 mM each of rATP, rCTP and rGTP and 10 μCi [α-^{32}P] UTP (400mCi/mmol) (Amersham). Transcription components were assembled on ice and the reaction was performed at 30°C for 1 hour. Transcription was terminated by the addition of 250 μl of 1 M ammonium acetate/0.1% (w/v) SDS containing 20 μg of yeast tRNA which acts as a carrier for the synthesised RNA. Phenol-chloroform extraction of the samples was performed to remove protein and DNA by adding 250 μl of a 25:24:1 ratio solution of PhOH/$CHCl_3$/IAA. The samples were vortexed, microcentrifuged at 13, 000g for 5 minutes and 200 μl of the upper aqueous layer was then transferred to a fresh eppendorf tube containing 750 μl of 96% ethanol in order to precipitate the RNA. The samples were thoroughly mixed by repeated inversion, left at –20°C overnight before being microcentrifuged at 13,000g for 30 minutes to pellet the precipitated RNA. The supernatant was carefully removed and 750 μl of 70% (v/v) ethanol was added to each sample to wash the pellet. This was also carefully removed to avoid dislodging the pellet and the samples were heated at 50°C for 5 – 10 minutes to dry. 4 μl of formamide loading buffer (98% (v/v) formamide, 10 mM EDTA pH 8.0, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF) was added to each sample, which was then vortexed for 1 hour to ensure the RNA was fully re-dissolved. 1.5 μl of each sample was loaded on a pre-run 7% (v/v) polyacrylamide sequencing gel containing 7 M urea and 0.5 x TBE after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed at 40 W for 1 hour in 0.5 x TBE before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts. Quantification of results was achieved by densitometry (TotalLab v1.11).

2.15.1 Plasmid templates used in *in vitro* transcription assays

The plasmid templates used for *in vitro* transcription assays were as follows: pVA_1 is a 221bp *Sal*I-*Bal*I fragment of adenovirus 2 DNA containing the VA_1 gene subcloned into pUC18 (Dean and Berk, 1988); pLeu is a 240bp *Eco*RI-*Hind*III fragment of human genomic DNA carrying a $tRNA^{Leu}$ gene, subcloned into pAT153 (McLaren and Goddard, 1986); PHu5S3.1 is a 638bp *Bam*HI-*Sac*I fragment of human genomic DNA containing a 5S rRNA gene, subcloned into pBluescript SK+; pTB14 is a 200bp *Bgl*II fragment containing a B2 gene from upstream of the mouse (BALB/c) D^d class I MHC gene subcloned into pUC18; the pol I template was pHrP2 (Lescure *et al.*, 1994), which has an 800 bp *Sau*3A fragment of the human rRNA promoter, from -411 to +378, subcloned into *Bam*H1-linearised pUC9; p7L30.1 contains a *Hind*III-*Eco*RI fragment carrying a human 7SL gene subcloned into pUC13 (Ullu and Weiner, 1985); 7SK-PAT/153 contains a *Hind*III-*Asp*718 fragment containing human 7SK sequences from -250 to +1, subcloned into pUC8 (Kleinert *et al.*, 1990).

2.15.2 Peptide substrate inhibitors used in *in vitro* transcription assays

The peptide substrate inhibitors used for *in vitro* transcription were PKB substrate peptide inhibitor GRPRTSSFAEG (Biomol) or PKA phospho-acceptor peptide LRRASLG (Upstate).

2.15.3 Preparation of fractions used in *in vitro* transcription assays

PC-B is the 0.1 to 0.35 M KCl step fraction from a phosphocellulose column, and contains both TFIIIB and pol III (Segall *et al.*, 1980). PC-C is the 0.35 to 0.6M KCl step fraction from a phosphocellulose column and contains both TFIIIC and pol III (Segall *et al.*, 1980). The CHep-1.0 fraction was generated as described previously (White *et al.*, 1995b) by loading PC-C onto a heparin-sepharose CL-6B in BC buffer (25 mM Tris-HCl, pH 7.9, 10% (v/v) glycerol, 10 mM β-mercaptoethanol) plus 100 mM KCl (BC-100). The column was washed with BC-280 and eluted with BC-1000 to produce the CHep-1.0 fraction

containing TFIIIC. The A25 (0.15) fraction containing TFIIIB was generated as described previously (White *et al.*, 1995b) by applying PC-B to an A25 DEAE-Sephadex column in buffer A (20 mM HEPES-KOH pH 7.9, 20% (v/v) glycerol, 5 mM $MgCl_2$, 3 mM DTT, 0.2 mM PMSF) plus 50 mM $(NH_4)_2SO_4$. After extensive washing with this buffer, the A25 (0.15) fraction containing TFIIIB was eluted in buffer A plus 150 mM $(NH_4)_2SO_4$. These fractions were provided by Prof. Robert J. White.

2.16 Electrophoretic Mobility Shift Assay (EMSA)

2.16.1 Preparation of the Probe

TFIIIC2 DNA-binding activity was determined by EMSAs which were carried out using a γ-^{32}P labelled oligonucleotide containing a B-block consensus (5'-AGAGGTCCTGAGTT-CAAATCCCAG-3' (RJW1; 10 ng/µl) annealed to the complementary strand (RJW2; 10 ng/µl). For use in EMSAs, oligonucleotides were 5' end-labelled using T4 polynucelotide kinase (PNK). 4 µl (40 ng) of RJW1 was assembled on ice with 1 µl (10 U) of PNK (Promega) in 1 x PNK buffer (Promega). 20 µCi of [γ-^{32}P] dATP (10 mCi/ml) and the appropriate volume of nuclease-free water were then added to give a total volume of 10 µl. The reaction was allowed to proceed for 1 hour at 37°C, before being stopped by heating at 65°C for 10 minutes. The PNK enzyme was then phenol chloroform-extracted by adding 50 µl of PhOH/$CHCl_3$/IAA (25:24:1), followed by vortexing and microcentrifugation at 13, 000 g for 5 minutes. The aqueous layer was transferred to a fresh eppendorf tube and 5 µl of 3 M sodium acetate and 125 µl of 96% ethanol added. After a 30 minute incubation on dry ice, the precipitated oligonucleotide was pelleted by microcentrifugation at 13, 000 g for 10 minutes. The supernatant was removed and the pellet washed by sequential addition and removal of 100 µl of 70% (v/v) ethanol to ensure removal of unincorporated label. The pellet was then dried by heating at 47°C for 10 minutes before being re-dissolved by incubation at 30°C for 30 minutes in 20 µl of TE buffer (final concentration 2 ng/ µl). This was followed by heating in a hot block at 90°C for 2 minutes in the presence of unlabelled complementary oligonucleotide (RJW2), which was added in 2.5-fold excess to ensure that all the labelled oligonucleotide was annealed. The hot block was then turned off and the sample cooled slowly overnight. It was then stored at 4°C until ready for use, at which point it was diluted to 0.5ng/ µl with TE.

2.16.2 EMSA Assay

Each binding reaction was performed in a total volume of 10 µl, with an optimal salt concentration of 60 mM KCl, and contained 1 µg of poly(dI.dC) (2 µl), 100 ng of non-specific or specific competitor oligonucleotide (2 µl), typically 2-4 µl of cell extract and 1ng of labelled probe (2 µl). A pre-incubation of 15 minutes at 30°C was carried out prior to addition of the probe, followed by a further 15 minutes at 30°C. Analysis of the formation of protein-DNA complexes was achieved by electrophoresis of samples on a 4% (v/v) non-denaturing polyacrylamide gel. The gel was pre-run in 1 x TAE buffer at 150 V for 1 hour at 4°C, and samples were run for 1.5 hours in the same conditions. The gel was then dried and exposed to autoradiography film overnight at -80°C.

2.17 Primer Extension

Expression levels of the transfected pol III template VA_1 (0.25 µg) and the GFP gene (0.25 µg), which was co-transfected as an internal control for transfection efficiency, were analysed by primer extension. VA_1 (5'-CACGCGGGCGGTAACCGCATG-3') or GFP (5'-CGTCGCCGTCCAGCTCGACCAG-3') oligonucleotides were γ-^{32}P end-labelled using T4 polynucleotide kinase (PNK). For each primer extension reaction, 10 µg of total RNA (made up to 10 µl with DEPC- dH_2O) were incubated at 80°C for 10 minutes with 9 µl of First Strand Buffer (Invitrogen) and 1 µl of the relevant labelled probe to act as a primer. Samples were immediately transferred to a hot block for further 2 hours incubation. 30 µl of an elongation mix (23 µl DEPC-dH_2O, 0.5 µl DEPC- dH_2O, 1 M DTT, 5 µl 5 mM dNTP mix (5 mM in DEPC- dH_2O), 0.5 µl 4 mg/ml actinomycin D, 0.5 µl (100 U) of Superscript II Reverse Transcriptase (Invitrogen)), was then added to the samples to initiate reverse transcription and the reaction was allowed to proceed for 1 hour at 42°C. Reaction products were ethanol precipitated overnight, as described in section 2.11.2. Pellets were resuspended in 4 µl of formamide loading buffer (98% (v/v) formamide, 10 mM EDTA pH 8.0, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF) was added to each sample, which was then vortexed for 1 hour to ensure the RNA was fully re-dissolved. 1.5 µl of each sample was loaded on a pre-run 7% (v/v) polyacrylamide sequencing gel containing 7 M urea and 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid, 0.625 mM EDTA pH 8.0), after being boiled at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed at 40 W for 1 hour in 0.5 x TBE, before being dried and exposed to autoradiography film in order to detect the radiolabelled transcripts. Quantification of results was achieved by densitometry (TotalLab v1.11).

2.18 Random polymerase assay

Random polymerisation assays were based on a method described by Roeder (Roeder, 1974). Reactions were performed in a total volume of 50 µl with a final concentration of 6 mM HEPES pH 7.9, 30 mM KCl, 3.6 mM $MgCl_2$, 6 mM Tris-HCl pH7.9, 200 µM EDTA, 7.5 mM ammonium sulphate, 800 µM mangenese chloride, 600 µM rATP, rCTP and rGTP, 50 µM UTP, 0.6 mM DTT and 5% (v/v) glycerol. This was supplemented with 5 µg of poly(dA-dT) to provide a non-specific template, 20 µg BSA, 10 µCi [α-^{32}P] UTP (400Ci/mmol) and 10 µg of whole cell extract prepared from cells treated with and without rapamycin (100nM) for 4 hours. For inhibition of pol II activity, α-amanitin (diluted in DEPC- dH_2O) was added to give a final concentration of 1 µg/ml or of 100 µg/ml for inhibition of both pol II and pol III activities. Reactions were performed at 30°C for 20 minutes and were stopped by transferral to 2 cm^2 Whatman DE5 paper discs, which were then subjected to serial 5 minute washes: 6 washes in 0.5 M Na_2HPO_4, twice in dH_2O, twice in 96% ethanol and finally once in ether. Discs were then dried at room temperature for a few minutes and transferred to scintillation vials containing 5 ml of Optiflow scintillation fluid (Fisons chemicals). Levels of incorporated radioactivity were then measured in a scintillation counter counting ^{32}P for 5 minutes. Pol III activity was calculated by subtracting the polymerisation obtained in the presence of 100 µg of α-amanitin per ml (due to pol I) from that obtained in the presence of 1 µg of α-amanitin per ml (due to pol III and pol I).

2.19 *In vitro* kinase assay

Recombinant Maf1 (HsMaf1.His) (2µg) was bound to Ni^{2+} beads (20 µl) which had been washed twice in 300 µl TBS. These were incubated for 10 minutes at 30°C in the presence of control buffer or with 50 µl of whole cell extract taken from cells treated with either vehicle (DMSO), or rapamycin (100nM) for 4 hours. The samples were incubated for 15 minutes at 30°C in a master mix containing 20 mM Tris-HCl pH7.4, 20 µM ATP, 10 mM $MgCl_2$, 1 mM DTT and 5 µCi of [γ-^{32}P] ATP. Immunoprecipitates were then washed once with 300 µl TBS/0.1 % (v/v) Triton X-100 and then washed a further twice in 300 µl TBS with microfuging at 13, 000 g for 1 minute between each wash. The bead-bound samples were then re-suspended in 2 x protein sample buffer (125 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0.25% (w/v) bromophenol blue), and analysed by SDS-PAGE. The amount of ^{32}P incorporated into HsMaf1.His was assessed by autoradiography.

2.20 Targeting of endogenous Maf1 using siRNA

2.20.1 siRNA design

Four Maf1 target sequences were selected. Beginning with the AUG start codon, the length of the Maf1 gene was scanned for sequences beginning with AA and the adjacent 19 nucleotides 3' of AA were noted. These 33 potential siRNA target sites were compared to the mouse genome database (using the BLAST alignment tool) and targets with significant homology to other genes were eliminated in this way. Since the length of the target was 21mer, a complete homology match score was 42 (21 X 2). Four sequences that appeared to be specific to the gene of interest were chosen for siRNA design.

Table 2.3: **siRNA oligonucleotides**

Target Sequence (Gene Position)	**Antisense siRNA oligonucleotide template**	**Sense siRNA oligonucleotide template**
5'-AAGATGGCGGG AGATGATAAA-3' **(103)**	5'-AAGATGGCGGGAGA TGATAAACCTGTCTC-3'	5'-AATTTATCATCTCC CGCCATCCCTGTCTC-3'
5'-AAGTCATGAAT TCAGCCGAGA-3' **(342)**	5'-AAGTCATGAATTCAG CCGAGACCCTGTCTC-3'	5'-AATCTCGGCTGAATT CATGACCCTGTCTC-3'
5'-AAATGCAGTCA ACTGCAGCCT -3' **(384)**	5'-AAATGCAGTCAACTG CAGCCTCCTGTCTC-3'	5'-AAAGGCTGCAGTTG ACTGCATCCTGTCTC-3'
5'-AACTGCAGCCT GTTTTCAGCT -3' **(394)**	5'-AACTGCAGCCTGTTT TCAGCTCCTGTCTC-3'	5'-AAAGCTGAAAACAG GCTGCACCTGTCTC-3'

DNA oligonucleotide sequences for each target were designed using the "siRNA Template Design Tool for the Silencer siRNA Construction Kit". This was achieved by entering the Maf1 mRNA target sequence beginning with AA into http://ambion.com/techlib/misc/silencer_siRNA_template.html. This tool determines the final 29mer DNA sequences of the sense and antisense siRNA oligonucleotide templates by adding a CCTGTCCTC sequence which is complementary to the T7 promoter primer, to the 3'end of each oligonucleotide. Transcription of the antisense strand oligonucleotide generates RNA that is fully complementary to the mRNA target sequence. Transcription of

the sense strand template generates a 3' terminal UU that is not complementary to the antisense strand of the siRNA. It is unknown whether this non-complementary dinucleotide is important for the activity of siRNAs.

2.20.2 siRNA preparation

Two 29-mer DNA oligonucleotides for each of the four selected Maf1 siRNAs were synthesised using the sense and antisense siRNA oligonucleotide template sequences (Genosys), and made up to 200 μM in nuclease–free water. In separate reactions, 2 μl of either the sense or antisense siRNA oligonucleotide templates were hybridised to 2 μl of T7 promoter primer in the presence of 6 μl of DNA hybridisation buffer (all reagents are from Ambion). The mixture was heated to 70°C for 5 minutes and left at room temperature for 5 minutes. The hybridised oligonucleotides were then added to a mixture containing 2 μl of 10 x Klenow Reaction Buffer, 2 μl of 10 x dNTP Mix, 4 μl of Nuclease-Free water and 2 μl of Exo-Klenow, and gently mixed, followed by slow vortexing and brief centrifuging in order to collect the mixture at the bottom of the tube. The mixture was then incubated at 37°C for 30 minutes to allow the 3' ends of the hybridised oligonucleotides to be extended by Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates.

The sense and antisense siRNA templates were transcribed by T7 RNA polymerase in a reaction containing 2μl of either sense or antisense template, 4 μl Nuclease-Free water, 10 μl 2 x NTP Mix, 2 μl 10 x T7 Reaction Buffer and 2 μl T7 Enzyme Mix. These transcription reactions were incubated for 2 hours at 37°C after brief vortexing, and centrifuging to allow collection of the reaction mixture at the bottom of the tube. The resulting RNA transcripts were hybridised to create dsRNA by combining the sense and antisense transcription reactions at 37°C in an overnight incubation. The leader sequences and the DNA template were removed by digesting the dsRNA with a single-strand specific ribonuclease and a deoxyribonuclease. The dsRNA (made by *in vitro* transcription) was gently mixed with 6 μl Digestion Buffer, 48.5 μl Nuclease-Free water, 3 μl RNase, 2.5 μl DNase and incubated for 2 hrs at 37°C. 400 μl of siRNA Binding Buffer was added to the nuclease digestion reaction prior to incubation for 2-5 minutes at room temperature. Filter Cartridges were prepared by pre-wetting with 100 μl of siRNA Wash Buffer and 2 ml collection tubes were attached. To bind the siRNA, the premixed siRNA nuclease digestion reaction and the siRNA Binding Buffer were added to the Filter Cartridge and spun at 10, 000g in a microcentrifuge tube for 1 minute. The flow-through was discarded and the filter

was washed twice with 500 µl of siRNA Wash Buffer to remove excess nucleotides, short oligomers, proteins and salts from the reaction. A new 2 ml tube was attached for each wash. 100 µl of Nuclease-free water (preheated to 75°C) was added to each Filter Cartridge and incubated for 2 minutes at room temperature. The Filter Cartridge was then spun at 12, 000g for 2 minutes to collect the purified siRNA. The end product is a double stranded 21-mer siRNA with 3' terminal uridine dimers which was stored at -80°C prior to transfection into A31 cells.

The RNA concentrations (µg/ml) of the siRNAs were quantified by measuring the absorbance at 260nm (see section 2.3), and the molar concentration determined using the formula:

$$\mu M = \frac{\text{Concentration in } \mu g/ml}{14}$$

2.21 Transient transfection

2.21.1 Transient transfection of Brf1 and Maf1

A31 and CCL39 cells were transfected using the Lipofectamine reagent (Life Technologies Inc.) The transient transfection with lipofectamine required cells to plated out at 1 x 10^5 cells/well on 6 well plates or 10 cm dishes, 24 hours prior to transfection, resulting in a confluency of ~75% at the time of transfection. Two wells of a 6 well plate were transfected per treatment. Master-mixes for each set of wells were made. The first of these comprised the appropriate plasmid DNA (2 µg) and 0.12 ml of OptiMEM (Gibco) (volumes per well). In a separate tube, 0.12 ml OptiMEM and 8 µl lipofectamine were mixed (volumes per well), and these were transferred into the tube containing the plasmid with OptiMEM. The two were mixed by pipetting up and down and the tube was incubated for 45 minutes in the dark at room temperature. During the incubation period, each 6 well plate to be transfected was washed with 1 ml of OptiMEM per well, and then 0.76 ml of OptiMEM was added per well and the plates incubated at 37°C. After the incubation period, 1 ml of the Lipofectamine-DNA-OptiMEM mix was overlaid dropwise into each well. The plates were gently rocked back and forth to evenly distribute the complexes and then were left for 3 hours at 37°C in an incubator. The Lipofectamine-DNA mix was then removed by aspiration from each well and replaced with 3 mls of fresh medium. Cells were incubated for a further 48 hours to allow expression of the transfected DNA, with media

being renewed again after 24 hours. Cells were then harvested and total RNA or protein extracted for analysis.

10 cm dishes were transfected using the same method as the 6-well plates, although, volumes were adjusted. Cells were transfected with 10 μg of plasmid DNA and master-mixes for each plate comprised the appropriate plasmid DNA and 0.828 ml of OptiMEM per dish, which was mixed with 0.828 mls OptiMEM and 30 μl Lipofectamine per dish. The DNA-OptiMEM and OptiMEM-Lipofectamine mixes were incubated as before for 45 minutes in the dark at room temperature. During this time 10 cm plates were washed with 6 mls of OptiMEM and 5.6 mls of OptiMEM was added until the end of the 45 minute incubation time. At this point, 1.6 mls of the Lipofectamine-DNA-OptiMEM mix was added to each plate. Media on plates containing transfected cells was replaced after 3 hours, and again after 24 hours. RNA or protein was extracted 48 hours post-transfection.

2.21.2 Transfection of siRNAs

A31 cells were plated at 1 x 10^5 cells/well on 6 well plates and grown for 24 hours in media containing DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were transfected when 70% confluent, with the cocktail of siRNAs described in section 2.20 at a final concentration of 50 nM. *Silencer*TM scrambled GAPDH siRNA and *Silencer*TM GAPDH siRNA were also transfected, all at 50 nM final concentration. Two wells per 6 well plate were used per treatment. The same volumes for transient transfections were used as described in section 2.21.1, except that 50 nM of siRNA was used where DNA had previously been transfected and 4 μl of the transfection reagent siPORT (Ambion) was used per well. The siPORT-RNA-OptiMEM mix was removed after 4 hours and replaced with fresh media. RNA was extracted 48 hours post-transfection.

2.21.3 Transfection of plasmid templates for primer extensions

Asynchronously growing A31 fibroblasts cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin were transfected for 48 hours with a total of 2 μg of DNA per well of a 6 well plate. This was made up of 0.5 μg of the plasmids pVA1 and pGFP (Promega), each at 0.25μg, plus one of the

following plasmid templates; pCDNA3-Brf1, which encodes wt Brf1 (Sutcliffe *et al.*, 2000), pCDNA3-PKB (Burgering and Coffer, 1995), pCDNA3-PKB kd (Burgering and Coffer, 1995), pCDNA3-Δp85 (Hara *et al.*, 1994), pCDNA3-mTOR D2338A which encodes kinase-dead mTOR (Brunn *et al.*, 1997), pCDNA3-mTOR which encodes wild type mTOR (Brunn *et al.*, 1997) or pCDNA3-S6KED3E, which is constitutively active and partially rapamycin-resistant, containing the point mutations T389E, S411D, S418D, T421E and S424D (Pearson *et al.*, 1995). During the final 16 hours of transfection, the cells were either maintained in the same medium, or in a medium containing 0.5% (v/v) serum. For the final 4 hours, either vehicle (DMSO) or rapamycin (100nm) was added. VA1 and GFP levels were assayed by primer extension.

2.22 Phosphate labelling *in vivo*

Labelling was carried out with 0.5 mCi/ml [^{32}P] orthophosphate for 3 hours in phosphate-free DMEM (Gibco). After incubation, cells were washed twice in 5 mls of ice-cold PBS and then solubilised in 0.25 ml of lysis buffer (50mM HEPES pH 7.5, 5 mM EDTA, 10 mM NaF, 150 mM NaCl, 25% (v/v) glycerol, 0.5% (v/v) Triton X-100, 0.5 mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 μg/ml aprotinin, 40 μg/ml bestatin, 1 mM sodium vanadate and 50 mM β-glycerophosphate) and left on ice for 15 minutes. Samples were then passed through a 26G needle three times before centrifuging at 13, 000g for 10 minutes at 4°C to pellet the cell debris. An aliquot of the supernatant was then used in a Bradford Protein Assay to determine protein concentration and 500 μg of protein was used in subsequent immunoprecipitations.

2.23 *In vitro* transcription-translation

Proteins were synthesised *in vitro* using the Single Tube Protein System 3 (STP3)-T7 kit (Novagen) following the manufacturer's protocol. Reactions were assembled on ice. 8 μl of STP3 T7 Transcription Mix was added to 1 μg plasmid DNA to a final reaction volume of 10 μl made up with nuclease-free water. These were incubated at 30°C for 20 minutes. Translation was carried out by adding 30 μl of STP3 Translation Mix and 4 μl (40 μCi) ^{35}S-labelled Cys/Met or 2 μl of unlabelled Methionine (625 μM), made up to a final reaction volume of 50 μl with nuclease-free water. Reactions were gently mixed and incubated at 30°C for 1 hour. 5 - 10 μl of each sample was analysed via separation by SDS-

PAGE. The gel was then incubated with 20 mls of amplify (Amersham) for 2 hours, coomassie stained and destained and was dried and visualised by autoradiography.The plasmids used for *in vitro* transcription-translation were: pCDNA3HA.Brf1; pCDNA3HA.Maf1 and pCITESTP3 (luciferase), which all contained the T7 promoter.

2.24 Recombinant Maf1 immunoprecipitations

10 µl of [^{35}S]-Methionine-labelled Maf1, luciferase, or Brf1 were prepared as described in section 2.23, using *in vitro* transcription and translation with reticulocyte lysate. Each *in vitro* translated protein was incubated with Ni^{2+} for 1 hour at 4°C in 500 µl of TBS. These pre-cleared samples were then microfuged at 13, 000g for 1 minute and the supernatant removed. The supernatant containing the *in vitro* translated protein was then incubated with 2 µg recombinant Maf1 (pre-bound to Ni^{2+} for 1 hour at 4°C) for 30 minutes in incubation buffer (TBS, 1 mM DTT, 0.25 µM PMSF, 40 µg/ml bestatin, 1 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin). The bound proteins were then washed once with TBS and 0.1 % (v/v) Triton X-100 and the supernatants were removed after microfugation at 13, 000g for 1 minute. This was followed by a further three washes with TBS. Bound material was separated by SDS-PAGE and incubated with Amplify (Amersham) for 2 hours at room temperature before coomassie staining and de-staining and being dried and exposed to autoradiography.

2.25 Pull-down assays

2.25.1 Maf1 pull-downs

10 µl of Methionine-labelled Brf1 was prepared as described in section 2.23, using *in vitro* transcription and translation with reticulocyte lysate and unlabelled methionine. 500 µl of TBS and 30 µl of anti-HA-agarose beads (Sigma) were added to Brf1 protein and this HA immunoprecipitation was carried out for 1 hour at 4°C. The supernatant was removed after microfugation at 13, 000g for 1 minute. 10 µl of [^{35}S]-Methionine-labelled Maf1, luciferase, or Brf1 prepared as described in section 2.23, was added to the HA-beads with 500 µl incubation buffer (TBS, 1 mM DTT, 0.25 µM PMSF, 40 µg/ml bestatin, 1 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin), and this

was left to incubate for 30 minutes at 4°C. The bound proteins were then washed once with TBS and 0.1 % (v/v) Triton X-100 and the supernatants were removed after microfugation at 13, 000g for 1 minute. This was followed by a further three washes with TBS. Bound material was separated by SDS-PAGE and incubated with Amplify (Amersham) for 2 hours at room temperature before coomassie staining and de-staining and being dried and exposed to autoradiography.

2.25.2 GST pull- downs

Glutathione *S*-transferase (GST) fusion proteins were expressed in bacteria and purified on glutathione-agarose beads (Sigma) by Jennifer Fairley, Niall Kenneth and Louise Derblay. GST fusion proteins were stored at -80°C for use in pull-down experiments. GST –Zn contains residues 1-93 of Brf1, GST-H1 contains residues 281-397 of Brf1 and GST-H2 contains residues 393-513 of Brf1. Equal amounts of immobilised GST (previously prepared according to manufacturers' instructions) and GST-Zn, GST-H1 and GST-H2 (estimated by Coomassie staining) were used in the reactions. 25 µl of packed glutathione beads bearing GST-Zn, GST-H1 and GST-H2 or GST alone were incubated with 5-10 µl of ^{35}S-labelled recombinant Maf1 (which had been pre-cleared for 2 hours with Glutathione agarose) in 500 µl of TBS. Samples were incubated on a rotating wheel for 30 minutes at 4°C. Beads were then washed in 300 µl of TBS, 0.25mM NaCl, 0.5 % (v/v) Triton X-100 followed by microfuging at 12, 000g for 1 minute. This was followed by a further 4 washes with TBS. Bound material was separated by SDS-PAGE and incubated with Amplify (Amersham) for 2 hours at room temperature before coomassie staining and de-staining and being dried and exposed to autoradiography.

2.26 Statistical analysis

Data are presented as the mean plus or minus the standard error. Statistical significance was determined by the Student's *t*-test (paired two samples for means; two-tails) using Microsoft Excel. *P* values of <0.05 were considered significant.

Chapter 3

The mTOR and PI3K Pathways Regulate Pol III Transcription

3.1 Introduction

3.1.1 The mTOR signalling pathway

The proteins TOR1, TOR2 and FPR1 were first identified in *Saccharomyces cerevisiae* in a screen for mutants that could confer resistance to the anti-proliferative effects of the macrolide antibiotic rapamycin. FKBP12 (FK506 –binding protein of 12kDa; encoded by the FPR1 gene) is a direct cellular receptor of the drug rapamycin, and is an abundant, and ubiquitously expressed, petidyl-prolyl cis/trans isomerase that may function in protein folding (Schreiber, 1991; Harding *et al.*, 1989). Whilst the *FPR1* mutant is recessive, the *TOR* mutants are dominantly acting point mutations, which map to a region of TOR, namely the FKBP12-rapamycin binding (FRB) domain. Upon exposure to rapamycin these *TOR* mutants cannot bind, nor be inhibited by, the rapamycin-FKBP12 complex, resulting in proliferation in the presence of rapamycin (Stan *et al.*, 1994; Chen *et al.*, 1995). The presence of FKBP12, not its activity, is required for the toxic, anti-proliferative action of rapamycin in yeast. This is demonstrated by the fact that deletion of all four FKBP12 genes in *S. cerevisiae* is not lethal, the yeast remain viable and exhibit resistance to the toxic effects of rapamycin (Heitman *et al.*, 1991; Koltin *et al.*, 1991).

The mammalian TOR (mTOR) protein is a 289 kDa Ser/Thr kinase orthologue of yeast Tor 1 and Tor 2. TOR is highly conserved from yeast to mammals; human, mouse and rat TOR proteins share 95% identity at the amino acid level (Heitman *et al.*, 1991; Oldham *et al.*, 2000; Long *et al.*, 2002). TOR homologues have also been identified in plants (*Arabidopsis thaliana*), worms (*Caenorhabiditis elegans*) and flies (*Drosophila melanogaster*) (Menand *et al.*, 2002; Long *et al.*, 2002; Oldham *et al.*, 2000; Zhang *et al.*, 2000). mTOR is a member of the phosphoinositide 3-kinase related kinases (PIKKs), which comprise a family of high molecular mass signalling proteins that play central roles in the control of cell growth, gene expression and genome surveillance and repair in

eukaryotic cells. Mammalian cells express six PIKK family members, including; ataxia-telangiectasia mutated (ATM) and ATM- and Rad3- related (ATR) kinases, DNA – dependent protein kinase (DNA-PK), suppressor of morphogenesis in genitalia-1 (SMG-1), and transformation/ transcription domain-associated protein (TRAAP). A bioinformatics-based analysis suggests that the amino-terminal domains of mTOR, ATM and ATR are composed almost entirely of Huntington, elongation factor 3, A subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats. Remarkably, the PIKK amino termini appear to contain 40-54 HEAT repeats (Perry and Kleckner, 2003) and this terminal half appears to be the site of critical protein-protein interactions.

The structural motif that defines the PIKKs as members of a common kinase superfamily is the catalytic domain. As the family name indicates, this domain shows significant homology to the catalytic domains of the phosphoinositide-3-kinases (PI3K). The sequence homology is particularly high with respect to the amino acid residues involved in the ATP-binding site of the PIKK catalytic domain (Hunter, 1995). However, in spite of this homology to the PI3K catalytic domain, all of the PIKKs for which biochemical information is available have been shown to use proteins, rather than lipids, as phosphoacceptors for the γ-phosphate of ATP (Brunn *et al.*, 1997; Burnett *et al.*, 1998; Isotani *et al.*, 1999). The catalytic domains of the PIKKs are flanked by two loosely conserved stretches of amino acids termed FAT and FATC domains (Bosotti *et al.*, 2000). The exact function of the FAT region is unclear, but mutagenesis and deletion studies performed with mTOR and ATR indicate that the integrity of the FATC domain is critical for the kinase activity of these otherwise intact polypeptides (Abraham, 2004).

Four of the five PIKKs that express kinase activities show strong preference for the phosphorylation of Ser/Thr followed by a glutamine (Gln) residue. Consequently, ATM, ATR, DNA-PK and hSMG-1 are known as " S/T-Q-directed" PIKKs and are particularly

common in proteins involved in checkpoint signalling and DNA repair, relevant examples being BRCA1, MDC1, hChk1 (Abraham, 2004). In contrast, mTOR, shows no preference for the Ser/Thr-Gln motif, and studies to date have no clear consensus site for Ser-Thr phosphorylation by the mTOR kinase domain. It has been suggested that mTOR's lack of preference for S/T-Q sites, may prevent mTOR inappropriately phosphorylating downstream targets of the genome surveillance/repair pathways (Abraham, 2004).

There is much controversy over the sub-cellular location of mTOR. Whilst an early report indicated much of the protein was associated with microsomes (Withers *et al.*, 1998), subsequent work suggested most of it was associated with the mitochondria (Desai *et al.*, 2002). Other reports have found much of the mTOR to be nuclear (Zang *et al.*, 2002). It is not clear whether these differences arise from the fact that different techniques were employed or whether its location does vary under different conditions.

3.1.2 PI3K

PI3K function is important for a wide variety of cellular processes including cell growth, cell cycle progression and proliferation, cell survival and cell migration (Manning *et al.*, 2002). Many of the downstream changes in cell physiology leading from activation of PI3K are the result of so far unknown signalling events. In response to a wide variety of extracellular stimuli, PI3K phosphorylates the lipid phosphatidylinositol-4, 5-bis-phosphate (PIP2) at the 3'-OH position to generate the second messenger phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) (see Figure 3.1). PIP3 then recruits proteins containing pleckstrin homology (PH) domains, such as PDK1 and the Ser/Thr kinase PKB, to the plasma membrane where they can be fully activated (Rameh and Cantley, 1999; Katso *et al.*, 2001; Cantley, 2002).

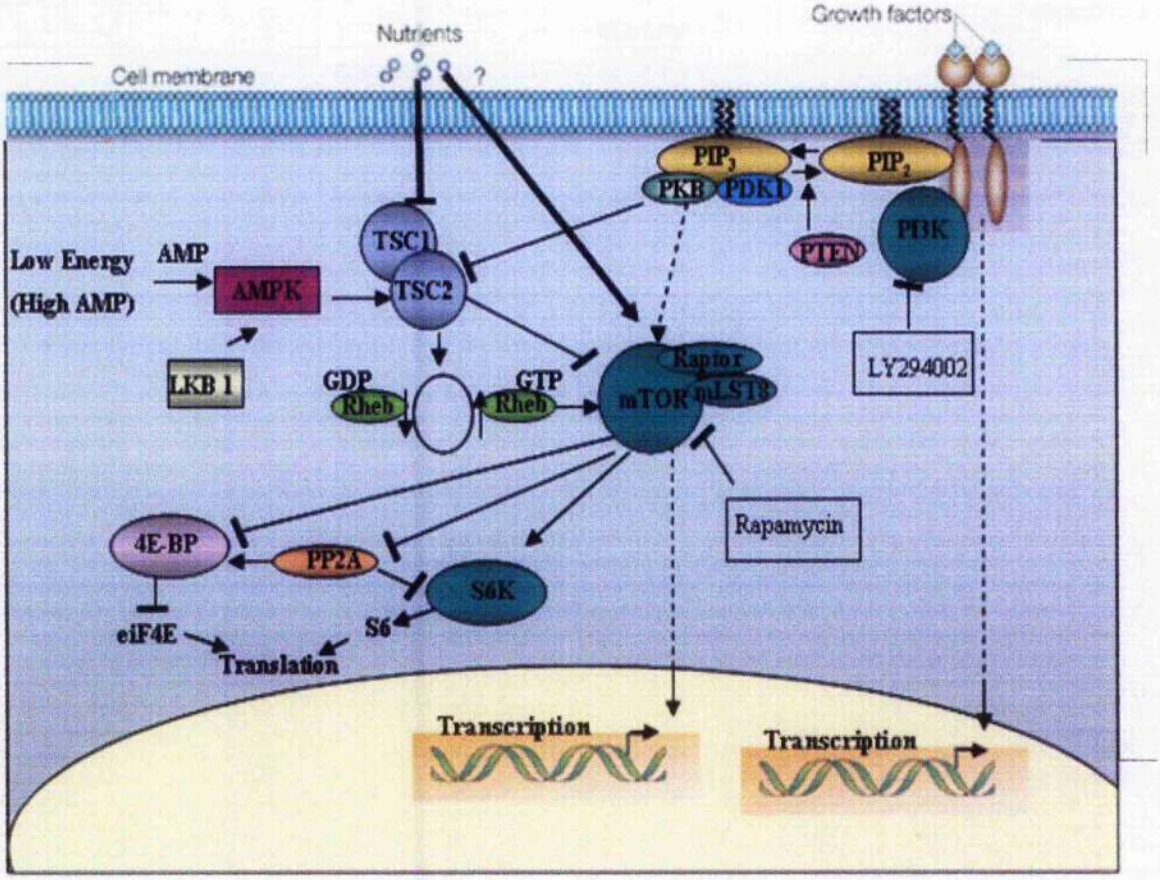


Fig. 3.1 : **mTOR integrates signals from nutrients and growth factors leading to cell growth (adapted from Jacinto and Hall, 2003)**

Fig. 3.1 : **mTOR integrates signals from nutrients and growth factors leading to cell growth (adapted from Jacinto and Hall, 2003)**

Growth factor stimulation activates phosphatidylinositol 3-kinase (PI3K) which phosphorylates phosphatidylinositol-4-5-bisphosphate (PtdIns(4,5)P2) (PIP_2) at position 3 to generate PtdIns(3,4,5)P3 (PIP_3). The lipid phosphatase PTEN antagonises PI3K action by converting PIP_3 back to PIP_2. The protein kinases PKB and 3-phosphoinositide-dependent protein kinase-1 (PDK1) are recruited to the membrane and bind PIP_3 through their pleckstrin homology (PH) domains. Membrane-bound PKB is activated, leading to the phosphorylation of TSC2 and inactivation of the TSC complex- formed by TSC1 and TSC2. This thereby relieves the inhibitory constraint on the mammalian target of rapamycin (mTOR) and allows activation the mTOR complex, which consists of mTOR bound to raptor and mLST8. mTOR promotes hierarchical phosphorylation of the translational activator S6K and the translation inhibitor 4E-BP either directly or indirectly by inhibition of protein phosphatase A (PP2A). Phosphorylation of S6K and 4E-BP promotes translation, via the ribosomal protein S6 and the eukaryotic initiation factor 4 E (eIF4E), respectively. Nutrients phosphorylate and inactivate TSC2 which acts as a negative regulator of the small GTP-binding protein Rheb. It is thought that TSC2 functions as a Rheb-GAP, shifting the equilibrium to inactive Rheb-GDP. Under low energy conditions, mTOR is inhibited as the AMP/ATP ratio rises. LKB1 is a tumour suppressor which phosphorylates and activates AMPK under such conditions. AMPK then phosphorylates TSC2 to enhance the tumour suppressor function resulting in inhibition of mTOR.

Both mTOR and PI3K-dependent signals co-ordinately control the translational effectors S6K1 and 4E-BP1. Initial studies showing phosphorylation of S6K1 and 4E-BP1 were sensitive to inhibition by both rapamycin and wortmannin, placed PI3K and mTOR in a linear signalling pathway (Gingras, 2001; Martin, 2002). However, this simplistic view was challenged when a rapamycin-resistant mutant of S6K1 remained sensitive to inhibition by wortmannin, indicating that PI3K controls S6K1 activation via an mTOR-independent mechanism (Cheatham *et al* 1995; Weng *et al.*, 1995; Dennis *et al.*, 1996). More recent developments revealing upstream regulators of mTOR suggest a model whereby PI3K lies both upstream of and parallel to mTOR with both branches converging on common downstream targets (Manning and Cantley, 2003).

Overexpression of an activated form of PKB in HEK293 cells promotes 4E-BP1 phosphorylation in the absence of growth factors and in a wortmannin-resistant and rapamycin-sensitive manner (Gingras *et al.*, 1998). Furthermore, overexpression of a dominant negative form of PKB impairs insulin-mediated phosphorylation of 4E-BP1 (Gingras *et al.*, 1998). These findings unequivocally place PKB upstream of mTOR and are also consistent with several studies in *Drosophila*, where overexpression of *Drosophila* PKB (dPKB) increases organ and cell size (Verdu *et al.*, 1999) and a non-phosphorylatable form of *Drosophila* 4E-BP suppresses this phenotype (Miron *et al.*, 2001), whereas loss of dPKB reduces cell and body size (Scanga *et al.*, 2001). Recent knockdown experiments using RNAi in *Drosophila* S2 tissue culture cells also provide evidence that PKB is a positive regulator of mTOR (Lizcano *et al.*, 2003; Miron *et al.*, 2003). Studies in mice lacking two of the three PKB family members provide the first genetic evidence that PKB functions upstream of mTOR in mammalian cells (Peng *et al*, 2003). However, the upstream positive regulatory role of PKB in mTOR activation has been questioned, as S6K1 phosphorylation does not always correlate with PKB activity in both mammalian cells and in *Drosophila* (Dufner *et al.*, 1999; Radimerski *et al.*, 2002). A

major breakthrough in the understanding of how growth factors and PKB regulate mTOR activity was the discovery that TSC1 (Tuberous sclerosis complex 1, also known as hamartin) and TSC2 (Tuberous sclerosis complex 2, also known as tuberin) are upstream regulators of mTOR (Gao and Pan, 2001; Potter *et al.*, 2001; Tapon *et al.*, 2001; Radimerski *et al.*, 2002; Inoki *et al.*, 2002; Gao *et al.*, 2002; Tee *et al.*, 2002; Tee *et al.*, 2003a). Several pieces of information suggested that the TSC1/2 complex was somehow required for mTOR to sense nutritional sufficiency. This was preceded by genetic and biochemical studies which found that the small G protein, Rheb (Ras homolog enriched in brain), was a direct target of TSC2, and that TSC2 functions as a GAP (GTPase-activating protein) to inhibit Rheb activity and thus inhibit its ability to stimulate the mTOR signalling pathway (Castro *et al.*, 2003; Garami *et al.*, 2003; Inoki *et al*, 2003; Saucedo *et al.*, 2003; Tee *et al.*, 2003b; Zhang *et al.*, 2003). Thus, it appears that the TSC complex is a negative regulator of mTOR. When membrane-bound PKB is activated this leads to the phosphorylation and inactivation of the TSC complex, which thereby relieves the inhibitory constraint on mTOR.

3.1.3 Nutrient sensing by mTOR

In *S. cerevisiae*, TOR is sensitive to changes in amino acid, nitrogen, and glucose levels. Furthermore, inhibition of TOR with rapamycin or by TOR gene deletion triggers a stress response program that strongly resembles the nutrient starvation phenotype (Barbet *et al.*, 1996; Thomas and Hall, 1997). These observations provided the first indication that TOR signalling coordinates nutrient availability with cell growth and proliferation (Schmelzle and Hall, 2000). It is apparent that the nutrient sensing function of TOR is evolutionarily conserved since the TOR signalling pathway has been found to couple nutrient availability with cell growth in *S. cerevisiae*, *D. melanogaster* and mammalian cells (Raught *et al.*, 2001; Rohde *et al.*, 2001; Crespo and Hall, 2002; Schmelzle and Hall, 2000). A major role

of the mTOR pathway in mammalian cells is to co-ordinate the synthesis of ribosomal proteins with the levels of available amino acids. This is accomplished by controlling the translational regulator S6K1. The S6 protein is a component of the small (40S) subunit of eukaryotic ribosomes, and is phosphorylated by S6K1 in response to a wide variety of extracellular signals (Fingar and Blenis, 2004). This drives translation of 5'TOP (terminal oligopyrimidine) mRNA-encoding proteins, such as c-myc, early response genes, cyclins and ribosomal elongation factors (Jefferies *et al.*, 1994, 1997; Terada *et al.*, 1994; Sonenberg and Gingras, 1998). Up-regulation of 5'-TOP mRNA translation will enhance the cellular levels of ribosomes and other components required for translation, thus favouring higher rates of protein synthesis and cell growth and /or proliferation. Striking data from targeted genetic knock-outs of S6K in fruit flies (where there is only one S6K gene) demonstrated a key role for this enzyme in both cell and organismal growth (Montagne *et al.*, 1999). Knockout flies were much smaller than wild-type flies, an effect that was due to reduced cell size rather than diminished cell number. Similar but more modest effects were observed when one of the S6K genes was knocked out in mice (Shima *et al.*, 1998). These data show that the S6 kinase, and thus mTOR signalling, plays a key role in growth control.

Given that both ribosome biogenesis and general protein synthesis require ample amino acids, it makes physiological sense for mTOR signalling and S6K activity to be positively regulated by amino acid supply. Consistent with this hypothesis, activity and phosphorylation of the mTOR substrates S6K1 and 4E-BP1 decreases in amino acid- and/or glucose-free medium and is stimulated upon their re-addition (Hara *et al.*, 1998; Fox *et al.*, 1998; Gingras *et al.*, 2001; Inoki *et al.*, 2003a; Kim *et al.*, 2002). Furthermore, a rapamycin-resistant allele of S6K1 caused cells to be unresponsive to amino acid depletion (Hara *et al.*, 1998), suggesting that mTOR is key to this sensing mechanism.

Recent studies have elucidated some of the molecular mechanisms of mTOR regulation in response to nutrients and growth factors, in mammalian cells (summarised in Fig. 3.1). Reports that mTOR was found in complexes of around 2MDa, indicate that these complexes contain a number of mTOR-interacting proteins. Two such proteins have recently been identified: these are the scaffold protein Raptor (regulatory-associated protein of mTOR) (150kDa; homologous to the TOR partner KOG1 in yeast) (Kim *et al.*, 2002; Hara *et al.*, 2002; Loewith *et al.*, 2002) and the protein GβL (36kDa; homologous to the TOR-binding protein LST8 in yeast) (Loewith *et al.*, 2002, Kim *et al.*, 2002). Since reduction of Raptor or GβL expression with siRNA reduces the phosphorylation of S6K1 on a rapamycin-sensitive site, it seems clear that these proteins play an important role in mTOR action (Kim *et al.*, 2003). In fact, TOR was reported to interact with Raptor to transduce nutrient signals to downstream translational machinery in mammals (Kim *et al.*, 2002; Hara *et al.*, 2002). Raptor associates in a near stochiometric ratio with mTOR to form a complex that functions as a nutrient sensor (Kim *et al.*, 2002) and it has been proposed that Raptor acts as a scaffold to bridge mTOR with its putative phosphorylation targets (Abraham, 2002; Kim *et al.*, 2002; Hara *et al.*, 2002).

5' Cap–dependent translation, which accounts for 85% of total translation, requires mTOR activation. Nevertheless , mTOR activity requires not only TSC inactivation by PI3K/PKB (Inoki *et al.*, 2003a; Manning *et al*, 2002; Potter *et al*, 2002), but also appropriate ATP and nutrient levels (Dennis *et al.*, 2001; Fang *et al.*, 2001). As protein synthesis not only consumes amino acids but also requires large amounts of metabolic energy, it was perhaps not surprising to discover that energy supply modulates the activity of components required for protein synthesis. One way in which mTOR may sense cellular energy levels is through AMP-activated protein kinase (AMPK); a kinase which is activated during states of low cellular energy (high AMP:ATP ratio). The result of AMPK activation is two fold; to reduce ATP expenditure by inhibiting key enzymes of biosynthetic pathways and

to increase the ATP supply by activating enzymes that produce ATP (reviewed in Hardie and Hawley, 2001). AMPK can modulate mTOR function by phosphorylating TSC2, which negatively regulates mTOR (Inoki *et al.*, 2003b). TSC2- knockout studies have shown that upon ATP depletion, the mTOR reporter proteins S6K1 and 4E-BP1 are not dephosphorylated, and that these cells are more susceptible to apoptosis due to glucose deprivation (Inoki *et al.*, 2003b). Hence, it is likely that direct sensing of ATP levels by mTOR, as suggested in earlier studies (Dennis *et al.*, 2001), does not occur but rather that the AMP/ATP ratio regulates mTOR indirectly through activation of AMPK. It has been concluded that the energy- and stress-sensing mechanism (via AMPK) and the peptide growth factors and mitogen-stimulated pathway (through PI3K) bifurcate on TSC2 above mTOR (Fingar and Blenis, 2004).

3.1.4 Transcriptional control by mTOR

In addition to TOR's well established role in the control of translation initiation, TOR proteins play a dynamic role in controlling transcription in response to nutrient signals. Transcriptional profiling of cells treated with rapamycin or starved of various nutrients have revealed that TOR controls expression of broad groups of genes with roles in protein, lipid, and nucleic acid metabolism (Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Peng *et al.*, 2002; Shamji *et al.*, 2000). In yeast, TOR regulates expression of specific classes of starvation response genes by sequestration of transcription factors such as GLN3/GAT1 (regulated by nitrogen), MSN2/MSN4 (carbon) and RTG1/RTG3 (glutamine) (Beck and Hall, 1999; Komelli *et al.*, 2000).

Inhibition of TOR also results in the rapid repression of genes involved in ribosome biogenesis, including tRNAs and rRNAs transcribed by pol I and pol III as well as ribosomal proteins expressed by pol II (Cardenas *et al.*, 1999; Mahajan, 1994; Powers and

Walter, 1999; Zaragoza *et al.*, 1998). Studies in *S. cerevisiae* found that when active yeast extracts with a conditional *tor 2* mutant strain were shifted to the non-permissive temperature *in vitro*, pol III transcription was blocked (Zaragoza *et al.*, 1998), suggesting a direct effect of TOR on the pol III system. These studies implicated the TFIIIB factor Brf1 as a target for control; however, the mechanism by which mTOR regulates pol III transcription in mammals remains to be determined.

As discussed previously, the high specificity of rapamycin for mTOR allows use of this drug to delineate the down-stream effects of the mTOR signalling cascade. Therefore, rapamycin was used to investigate if the mTOR pathway was controlling pol III transcription. The PI3K inhibitor LY294002 was also used in this mammalian cell system to verify if (any) effects on pol III transcription were due to pathways upstream of mTOR.

3.2 Results

3.2.1 The mTOR signalling pathway regulates pol III transcription

Initial experiments were carried out to show how quickly pol III transcript levels change when quiesced fibroblasts were stimulated with serum. An immediate rise in pol III transcription occurs within minutes of serum addition to growth arrested fibroblasts (Figure 3.2A, upper panel), where pol III transcripts derived from the B2 middle repetitive gene family were compared to levels of a pol II transcript ARPP P0. In fact there is a 3.7-fold increase in levels of the B2 transcripts within 10 minutes of serum addition (Fig. 3.2B). This effect is specific, since levels of the pol II transcript ARPP P0 do not respond to serum (Figure 3.2A lower panel). A useful property of the pol III transcript B2 is its short half-life so that it provides a reliable indication of transcriptional output. It was found that B2 expression increases proportionally with time of serum stimulation, until the 720th

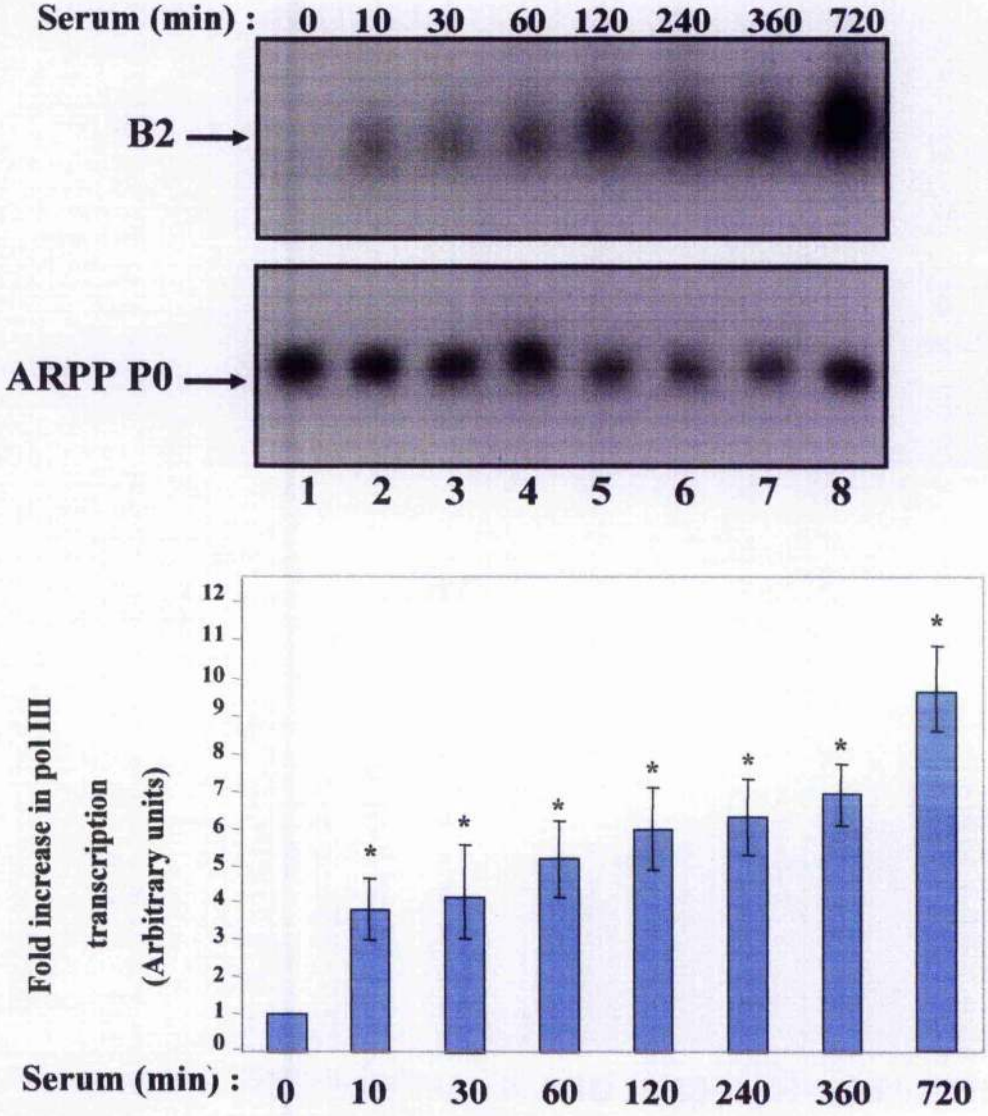


Figure 3.2: **Serum induces pol III transcription.**

(A) Northern blot of total RNA (20μg) extracted from A31 mouse fibroblast cells cultured in 0.5% serum for 24 hrs (lane 1) and then stimulated with 10 % serum for the times indicated (lanes 2-8). The upper panel shows the blot probed with a B2 gene; the lower panel shows the same blot that has been stripped and re-probed with the ARPP P0 gene.

(B) The B2 signals from (A) were quantified by densitometry and normalised against the ARPP P0 signal. The graph shows the mean and standard deviations from three independent experiments; values obtained for cells grown in medium containing 0.5% serum for 12 hrs (lane 1) were set as 1 and other values were calculated as a fold increase.

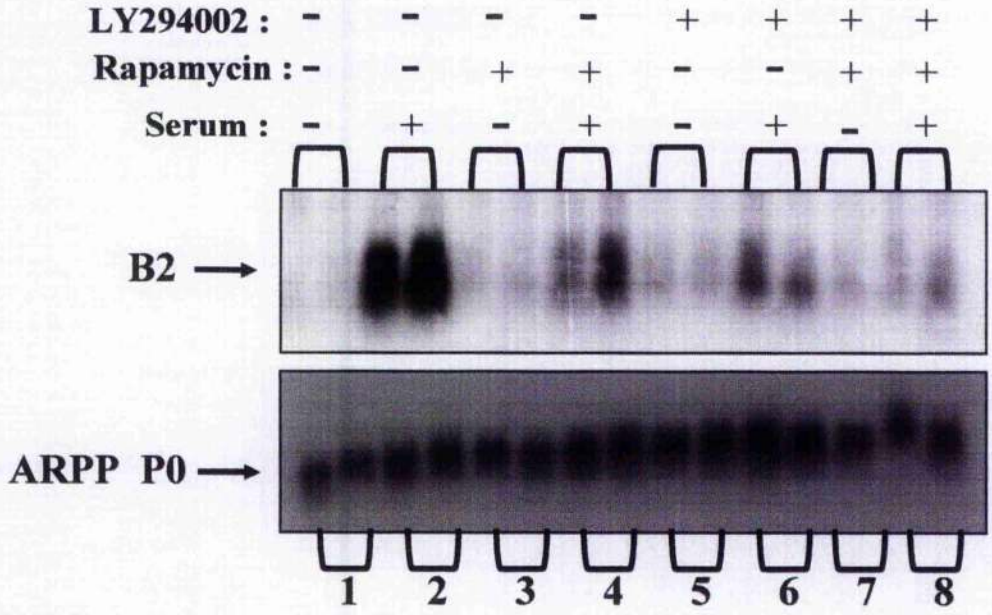
* denotes statistically significant difference from 0 min, at $p < 0.05$

minute (12hr) time point, at which point B2 expression increases dramatically. This significant increase is in agreement with a finding that near maximal B2 expression is reached by 12 hrs in these cells, shortly before S phase entry, as determined by flow cytometry (Scott *et al.*, 2001).

In mammalian cells, growth factors and cytokines not only regulate nutrient uptake but also activate signalling pathways that act in parallel or in concert with nutrients (Hay and Sonenberg, 2004). Given the complex mix of mitogens that are present in serum, the full response of pol III activation is likely to involve multiple signalling pathways that may feed into several components of the pol III machinery. However, initial studies in *S.cerevisiae* implicated the TOR signalling pathway as a regulator of pol III transcription in response to nutrient availability (Zaragoza *et al.*, 1998). In light of this evidence, we investigated whether the mTOR pathway could regulate pol III transcription in mammalian cells.

When quiescent fibroblasts were stimulated with serum for 16hrs there was an 8-fold increase in B2 transcript abundance (Fig 3.3, upper panel). Serum-stimulated levels of pol III transcription could be markedly reduced by the addition of either the mTOR inhibitor rapamycin or the PI3K inhibitor LY294002. In contrast, neither compound had any significant effect on basal B2 expression in serum-deprived cells. Interestingly, addition of both rapamycin and LY294002 together in the presence of serum repressed pol III transcription more than either drug alone, although the effect on the levels of the B2 transcript was not additive. The effect is specific since neither drug affected levels of the pol II transcript ARPP P0. Quantification of these results showed that rapamycin treatment reduced the pol III response to serum by approximately 35% +/- 13%, whilst LY294002 treatment reduced it by 45% +/- 13%. Moreover, the combination of both rapamycin and

A


LY294002 : - - - - + + + +
Rapamycin : - - + + - - + +
Serum : - + - + - + - +
B2
ARPP P0
1 2 3 4 5 6 7 8

B

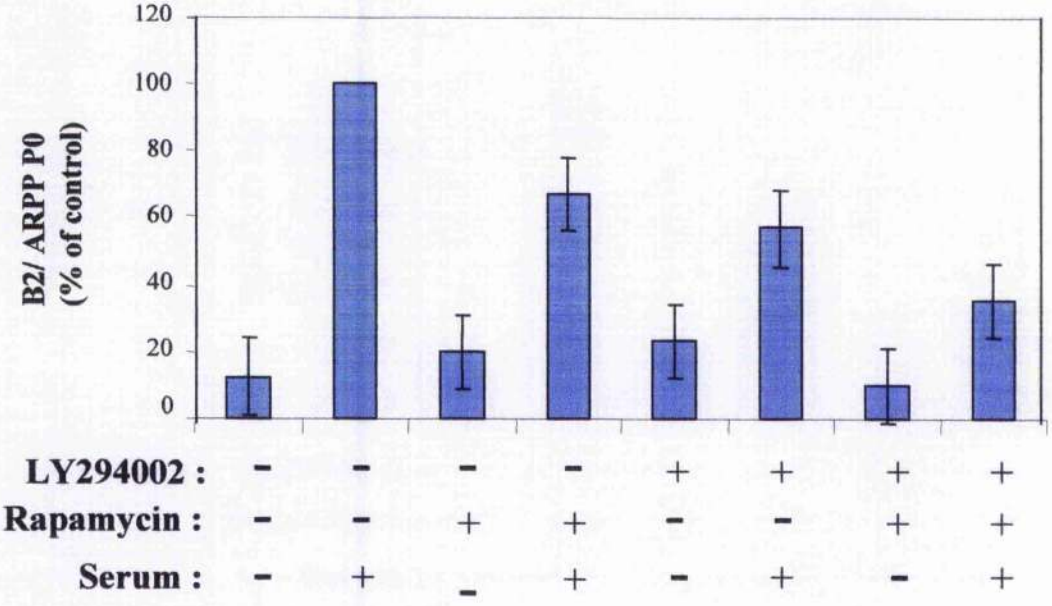

B2/ ARPP P0
(% of control)
120
100
80
60
40
20
0
LY294002 : - - - - + + + +
Rapamycin : - - + + - - + +
Serum : - + - + - + - +

Figure 3.3: **Blocking the mTOR and PI3K signalling cascade reduces serum-stimulated pol III transcription in mammalian cells**

(A) Northern blot of total RNA (20μg) extracted from A31 mouse fibroblast cells cultured in 0.5% serum for 24hrs (lanes 1, 3, 5, 7) or in medium containing 10% serum (lanes 2, 4, 6, 8) and treated for 16hrs with vehicle (lanes 1 and 2), rapamycin (100nM; lanes 3, 4, 7, 8) or LY294002 (50μM; lanes 5, 6, 7, 8). The upper panel shows the blot probed with a B2 gene; the lower panel shows the same blot that has been stripped and re-probed with the ARPP P0 gene (blot shows two replicates numbered as one lane).

(B) The B2 signals from (A) were quantified by densitometry and normalised against the ARPP P0 signal. The graph shows the means +/- the standard error of the mean for two replicates; values obtained for cells serum-stimulated for 16 hrs (lane 2) were set as 100 and other values were calculated as a percentage of this.

LY294002 resulted in more than a 65% reduction in pol III transcription, as compared to serum-stimulated fibroblasts.

A further way to determine the effects of rapamycin on pol III transcription was to use primer extension analysis. For this pCDNA$_3$ was transfected into cells along with a VA1 pol III reporter construct and GFP as a control. Following 32hrs transfection, rapamycin or vehicle was added for a further 16hrs in the presence and absence of serum. VA1 transcription is stimulated upon addition of serum for 16hrs (Fig. 3.4A, lane 3) and this effect can be blocked in the presence of rapamycin (Fig. 3.4A, lane 4). Levels of the pol II reporter GFP remained constant in each condition (Fig 3.4A, lower panel).

Whilst it was clear that blocking the mTOR and PI3K pathways using inhibitors decreased pol III transcription, it was interesting to further determine if pol III transcription could be specifically altered by using constitutively active or dominant negative components of these pathways. For this purpose, vectors encoding active and kinase dead PKB (Burgering and Coffer, 1995), dominant-negative p85 subunit of PI3K (Hara *et al.*, 1994), or wild-type and a catalytically inactive mTOR Asp2338Ala mutant that abrogates phospho-transferase activity (Brunn *et al.*, 1997), were transiently transfected into fibroblast cells, along with the adenoviral VA1 gene and a pol II control GFP gene. VA1 and GFP RNA levels were assayed by primer extension. Brf1 has previously been found to be limiting for pol III transcription in these cells; this was used as a positive control to induce pol III activity (Felton-Edkins *et al.*, 2003a). Transfection of Brf1 into cells indeed causes a significant induction of VA1 transcription by pol III, as previously reported (Felton-Edkins *et al.*, 2003a). Transfection of these cells with either the wild-type mTOR or PKB stimulated the transcription of the pol III reporter VA1, as compared to those cells which were transfected with vector alone (Fig 3.4, upper panel). In contrast, transfection of kinase dead PKB or catalytically inactive mTOR resulted in similar levels of pol III transcription as the control

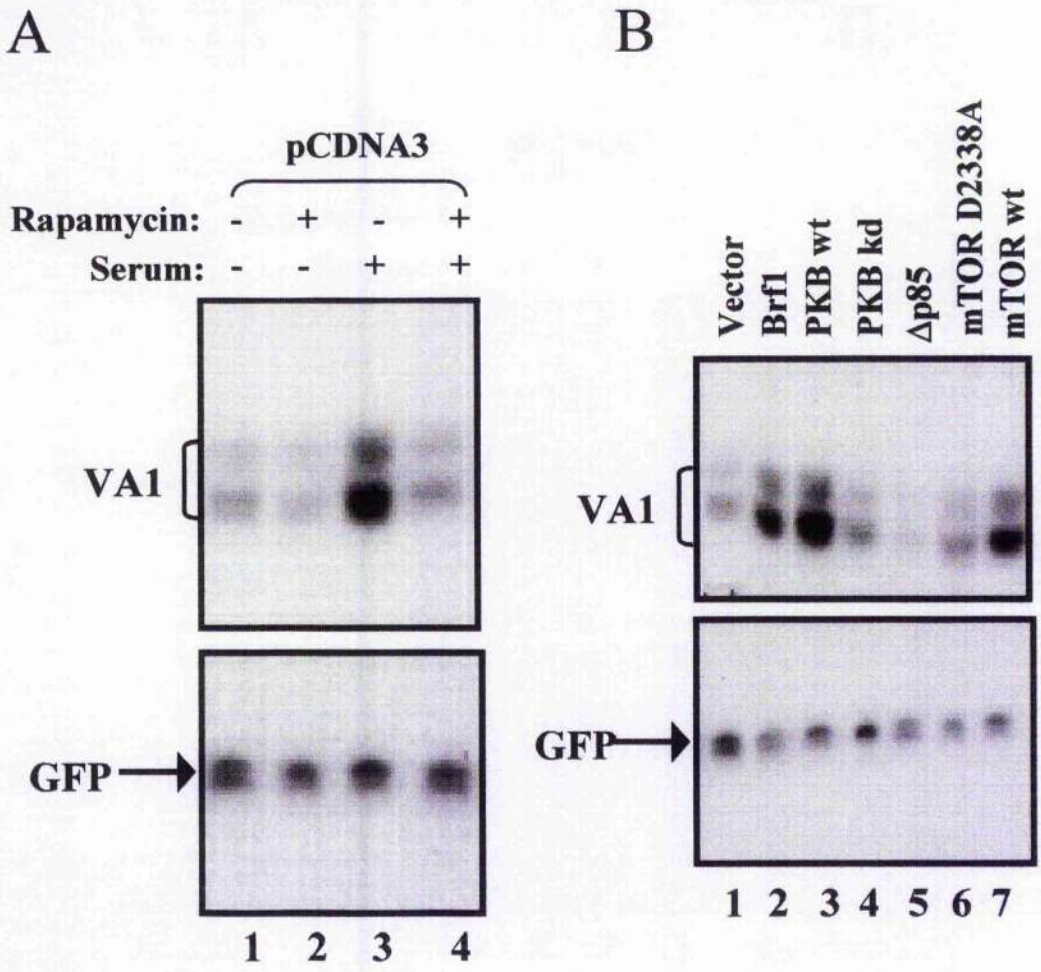


Figure 3.4: **Manipulation of the mTOR and PI3K pathway affects pol III activity**
(A) Asynchronously growing A31 fibroblasts were transfected with pVA1 (0.25μg; all lanes), pGFP (0.25μg; all lanes), pCDNA3 (1.5μg; all lanes). Lanes 3 and 4 were serum-stimulated (10% FBS) and lanes 2 and 4 were rapamycin-treated (100nM) for 4 hrs. VA1 (upper panel) and GFP levels (lower panel) were assayed by primer extension as shown. This result is representative of three independent experiments.
(B) Asynchronously growing A31 fibroblasts were transfected with pVA1 (0.25μg; all lanes), pGFP (0.25μg; all lanes), pCDNA3 (1.5μg; lane 1), pCDNA3-Brf1 (1.5μg; lane 2), pCDNA3-PKB (1.5μg; lane 3), pCDNA3-PKB kd (1.5μg; lane 4), pCDNA3-Δp85 (1.5μg; lane 5), pCDNA3-mTOR D2338A (1.5μg; lane 6), and pCDNA3-mTOR(1.5μg; lane 7). VA1 (upper panel) and GFP levels (lower panel) were assayed by primer extension as shown. This result is representative of two independent experiments.

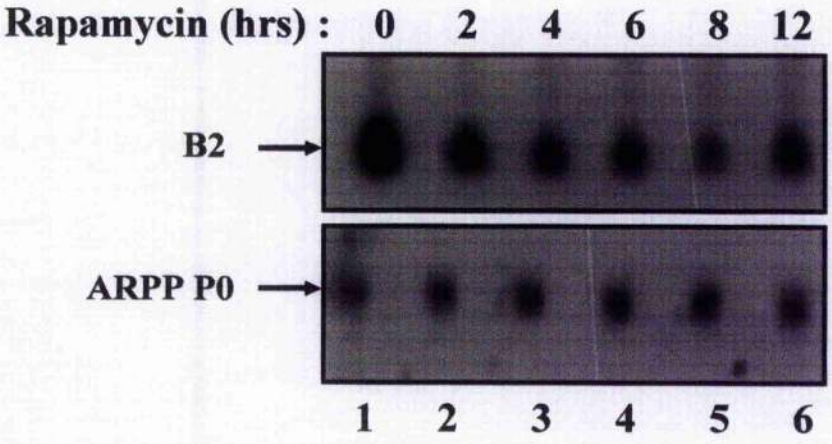
vector, whereas dominant- negative PI3K reduced pol III transcription below control levels. The pol II reporter GFP was used to check transfection efficiency and this remains virtually unchanged (Fig. 3.4).

Having established that long term exposure to either rapamycin or LY294002 reduces serum-induced activity, and that components of the mTOR and PI3K pathway could specifically activate pol III transcription, it was important to assess the time frame of inhibition. Since blocking this pathway with rapamycin or LY294002 has previously been found to cause a G1 arrest (Wiederrecht *et al.*, 1995; Zhang *et al.*, 2000), I determined whether the effects of these compounds were due to blocking entry to S phase.

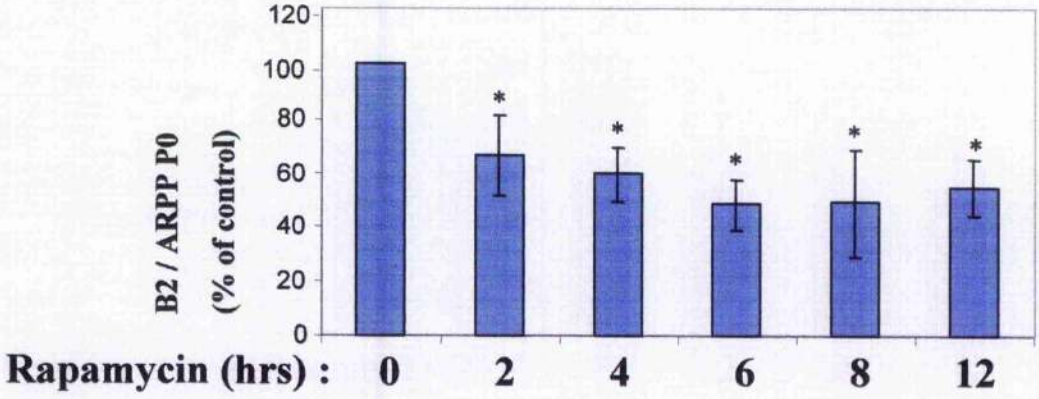
Total RNA was extracted from cells in culture at various time points following rapamycin treatment, and Northern analysis was performed. As shown in Figure 3.5A, there is a pronounced decrease in the level of the pol III transcript B2 within 2 hrs of rapamycin treatment as compared to untreated cells, this decrease was quantified as being a 35% reduction after normalisation to the ARPP P0 control transcript (Fig 3.5B). Pol III inhibition is optimal between 4 and 6 hrs of rapamycin treatment, at which point levels of B2 have been reduced to around 50% of cells treated with vehicle only. These inhibitory effects of pol III transcription are specific, as the pol II transcript ARPP P0 remained unchanged throughout (Fig. 3.5A, lower panel).

Having established the apparent involvement of the mTOR and PI3K pathways in the serum response of pol III transcription, it was important to check that the downstream effectors of mTOR were blocked during the rapamycin and LY294002 drug treatments. This was accomplished by checking the phosphorylation status of a well known translational effector of the mTOR pathway, namely S6K1, which belongs to the ribosomal protein S6 kinases. One of the ways mTOR has previously been found to regulate protein

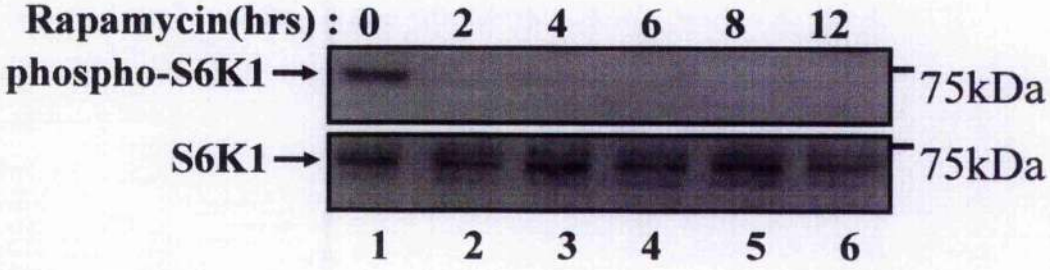
A


Rapamycin (hrs) : 0 2 4 6 8 12
B2
ARPP P0
1 2 3 4 5 6

B


B2 / ARPP P0 (% of control)
120
100
80
60
40
20
0
*
Rapamycin (hrs) : 0 2 4 6 8 12

C


Rapamycin(hrs) : 0 2 4 6 8 12
phospho-S6K1
S6K1
75kDa
75kDa
1 2 3 4 5 6

D

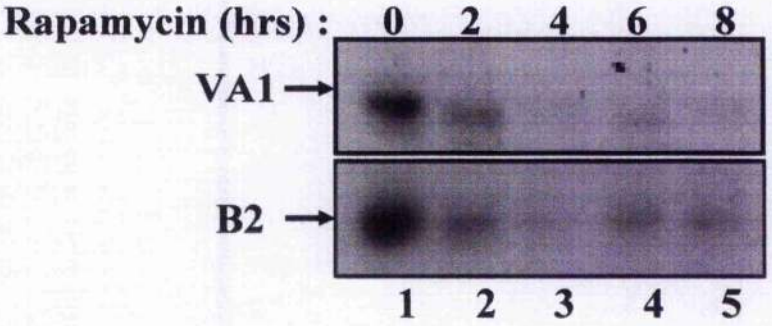


Figure 3.5: **Blocking the mTOR signalling cascade reduces pol III transcription**

(A) Northern blot of total RNA (20μg) from A31 mouse fibroblast cells cultured in 10% serum and vehicle treated (lane 1) or treated with rapamycin (100nM) for the times indicated (lanes 2-6). The upper panel shows the blot probed with a B2 gene; the lower panel shows the same blot that has been stripped and re-probed with the ARPP P0 gene.

(B) The B2 signals from (A) were quantified by densitometry and normalised against the ARPP P0 signal. The graph shows the mean and standard deviations from three independent experiments; values obtained for cells grown in serum plus vehicle for 12 hrs (lane 1) were set as 100 and other values were calculated as a percentage of this.

(C) Protein (50μg) extracted in parallel to the experiment described in (A) was resolved by SDS-PAGE and then analysed by western blotting with antibodies against S6K1 phosphorylated at Thr389 (upper panel), and total S6K1 (lower panel). These blots are representative of at least three independent experiments.

(D) 15μg of whole cell extract was prepared from A31 fibroblast cells grown in either 10% serum and vehicle (lane 1) or 10% serum and rapamycin (100nM) for the times indicated (lanes 2-5). Samples were transcribed in an *in vitro* transcription assay using the templates (250ng) of VA1 (upper panel) or B2 (lower panel). These results are representative of three independent experiments.

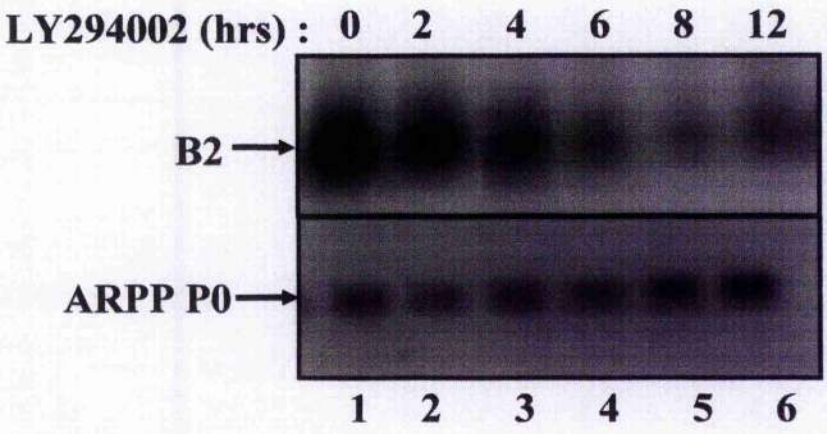
* denotes statistically significant difference from control, at $p < 0.05$

synthesis is by the phosphorylation and activation of S6K1. Phosphorylation of S6K1 is prevented by treating cells with rapamycin *in vivo* (Brunn *et al.*, 1997; Burnett *et al.*, 1998). Western analysis show that the phosphorylation of S6K1 was also ablated at all time points in the present study during exposure of cells to rapamycin, as compared with vehicle-treated cells (Fig 3.5C, upper panel) and that this effect was not due to a reduction in levels of total S6K1 (Fig 3.5C, lower panel). In order to further investigate whether the mTOR pathway controls pol III transcription, protein cell extracts were made at the same time as the RNA extractions and were used in an *in vitro* transcription assay. Extracts of fibroblasts treated for just 2hrs with rapamycin transcribe B2 and VA1 genes significantly less than vehicle alone (Fig 3.5D). This is in agreement with Northern analysis of pol III transcript levels (Fig 3.5A).

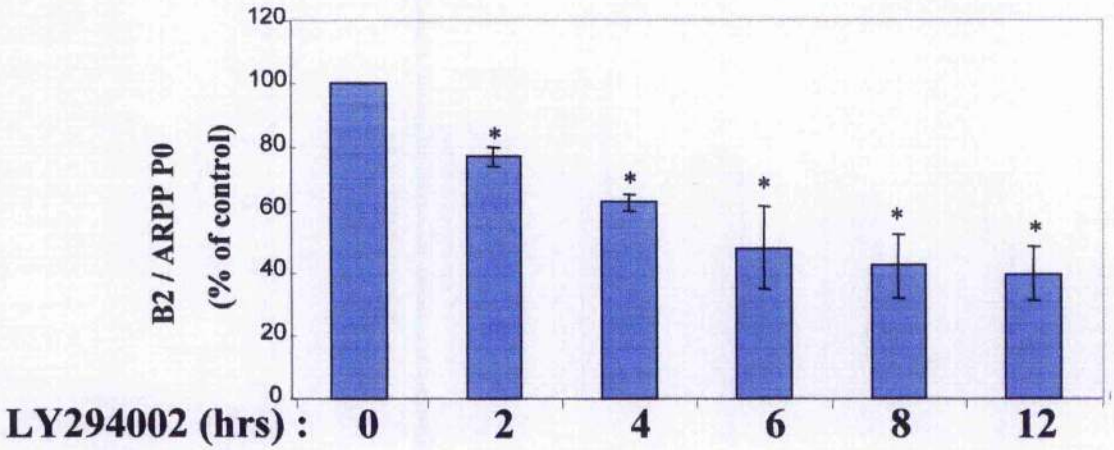
The effect of LY294002 on pol III transcription was also determined over time. Total RNA was extracted from cells in culture at various time points during LY294002 treatment and Northern analysis performed. Within 2hrs there is a significant reduction in the level of the pol III transcript B2 (Fig. 3.6A/B), which is further reduced by 6hrs to 40% for the remainder of the time course. Since PI3K lies upstream of mTOR, then blocking this enzyme is also known to reduce S6K1 phosphorylation (Wiederrecht *et al.*, 1995). The effect on the phosphorylation status of S6K1 was confirmed in this study when the PI3K pathway was blocked with LY294002. Once again, the S6K1 protein phosphorylated at Thr 389, was undetectable in those cell extracts which had been treated with LY294002, whereas total levels of S6K1 remained unchanged throughout the experiment (Fig. 3.6C). Western analyses shown in Fig. 3.5 and 3.6 show clearly that both the mTOR and PI3K pathways are inhibited under these conditions.

To verify if blocking the mTOR and PI3K pathways affected the RNA transcript levels of different pol III promoter types, RT-PCR was performed on total RNA extracted from both

A


LY294002 (hrs) : 0 2 4 6 8 12
B2
ARPP P0
1 2 3 4 5 6

B


B2 / ARPP P0 (% of control)
120
100
80
60
40
20
0
*
LY294002 (hrs) : 0 2 4 6 8 12

C

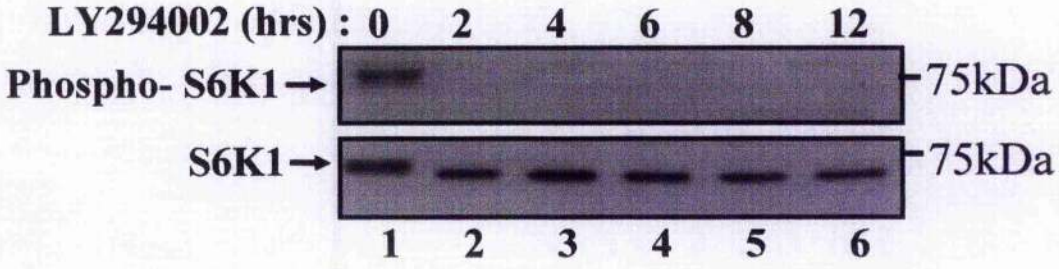

LY294002 (hrs) : 0 2 4 6 8 12
Phospho- S6K1
75kDa
S6K1
75kDa
1 2 3 4 5 6

Figure 3.6: **Blocking the PI3K pathways reduces pol III transcription**

(A) Northern blot of total RNA (20μg) from A31 mouse fibroblast cells cultured in 10% serum and vehicle treated (lane 1) or treated with LY294002 (50μM) for the times indicated (lanes 2-6). The upper panel shows the blot probed with a B2 gene; the lower panel shows the same blot that has been stripped and re-probed with the ARPP P0 gene.

(B) The B2 signals from (A) were quantified by densitometry and normalised against the ARPP P0 signal. The graph shows the mean and standard deviations from three independent experiments; values obtained for cells grown in serum and vehicle for 12 hrs (lane 1) were set as 100 and other values were calculated as a percentage of this. These blots are representative of at least three independent experiments.

(C) Protein (50μg) extracted in parallel to the experiment described in (A) was resolved by SDS-PAGE and then immunoblotted and analysed by western blotting with antibodies against and S6K1phosphorylated at Thr389 (upper panel) and total S6K1 (lower panel). This blot is representative of at least three independent experiments.

* denotes statistically significant difference from control, at $p < 0.05$

rapamycin and LY294002-treated cells. A substantial decrease was observed for both $tRNA^{Leu}$ and $tRNA^{Tyr}$ RT-PCR products following treatment with rapamycin or LY294002 for 6 and 12 hrs (Fig. 3.7). Similarly, levels of 5S rRNA were significantly inhibited by both rapamycin and LY294002 during the same time course. In contrast to the type I (5S rRNA) and type II (tRNA) promoter products, the level of the type III promoter product U6 was not altered when cells were treated with rapamycin or LY294002 (Fig. 3.7). These effects were specific as there was no change in the level of the class II gene ARPP P0 mRNA (Fig. 3.7). This data suggest that the production of most pol III transcripts *in vivo* is sensitive to the inhibition of the mTOR and PI3K pathways.

To confirm that this pathway influences pol III transcription, the effect of blocking the cascade was determined *in vitro*. Since a peptide substrate inhibitor of PKB is commercially available, I looked at the effect of this in an *in vitro* pol III transcription assay. It was found that VA1 transcription was reduced in a dose-dependent manner up to 90% by the substrate peptide containing the PKB consensus phospho-acceptor site (Fig. 3.8, upper panel). The inhibitory response was specific, since a protein kinase A (PKA) substrate peptide had minimal effect (Fig 3.8, lower panel). This data suggests that the signalling pathway leading to mTOR can contribute significantly to the level of pol III transcription *in vitro*.

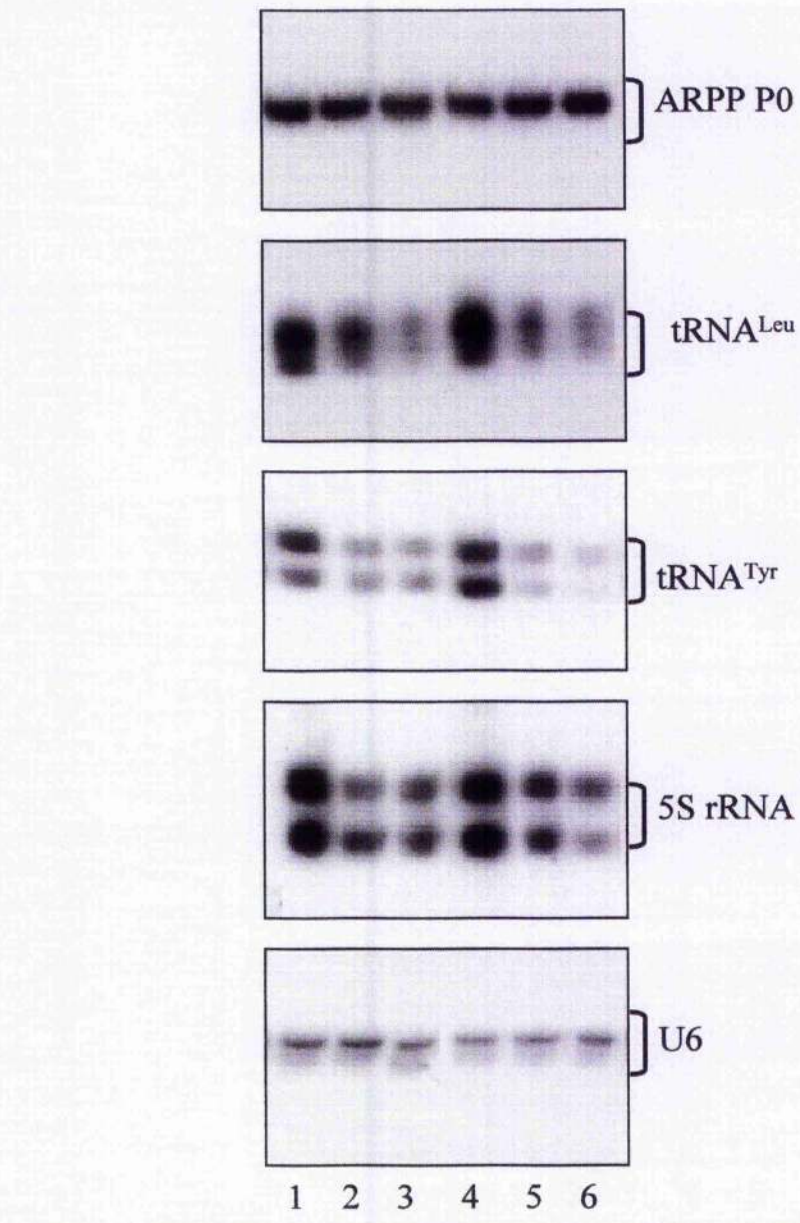


Figure 3.7: **Blocking the mTOR and PI3K pathways reduces pol III transcript levels of type I and type II promoters.**

A31 fibroblast cells growing in 10% serum were treated with vehicle (lanes 1 and 4), rapamycin (100nM; lanes 2 and 3) or LY294002 (50μM; lanes 5 and 6), for the times indicated. RNA was extracted and cDNAs were generated using reverse transcription. These cDNAs were PCR amplified by using primers specific for ARPP P0 (upper panel), $tRNA^{Leu}$ (second panel), $tRNA^{Tyr}$ (third panel), 5S rRNA (fourth panel), and U6 (lower panel). These results are representative of two independent experiments.

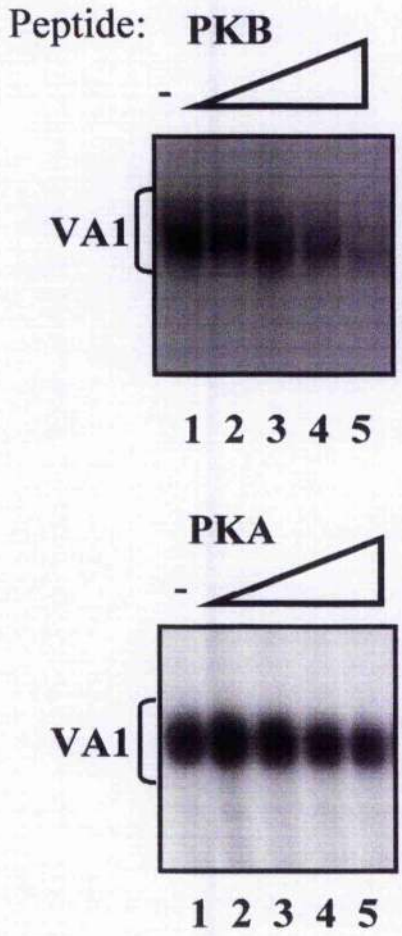


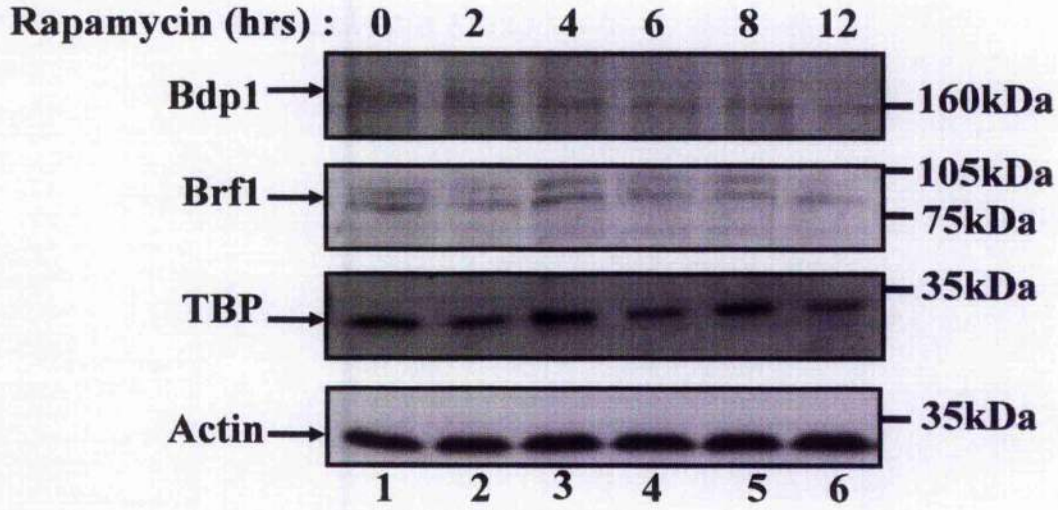
Figure 3.8: **Pol III transcription is blocked specifically by a peptide substrate inhibitor of PKB**

In vitro transcription was carried out using 20μg of HeLa nuclear extract and a VA1 template (250ng) after pre-incubation with buffer (lane 1), or with 10, 20, 30, 40μg of PKB substrate peptide inhibitor (upper panel, lanes 2-5, respectively) or with 10, 20, 30, 40μg of PKA phospho-acceptor peptide (lower panel, lanes 2-5, respectively). This result is representative of three independent experiments.

3.2.2 Blocking the mTOR signalling cascade does not affect the levels of TFIIIB or TFIIIC and doesn't affect the abundance or activity of pol III.

Several studies have shown that one of the molecular mechanisms for raising pol III output in transformed cells occurs via the overexpression of pol III transcription factors (White, 2004a). A potential mechanism by which the inhibition of the mTOR or PI3K pathways reduced pol III activity was via decreased abundance of the pol III transcription factors. To assess this hypothesis, levels of the pol III transcription factors TFIIIB and TFIIIC were monitored by Western blotting. Extracts from cells treated over time with rapamycin were subjected to SDS-PAGE analysis and immunoblotting with antibodies specific to components of TFIIIB (Fig. 3.9A) or TFIIIC (Fig. 3.9B); actin was used as a loading control in each case. Protein levels remain virtually unchanged; suggesting that changes in the abundance of these proteins was not the mechanism by which pol III transcription was downregulated in response to mTOR inhibition. Protein levels of two of the subunits of pol III; RPC155 and RPC53 were also measured during rapamycin treatment and were found to be unchanged (Fig. 3.10A). Therefore, a change in the abundance of pol III-specific transcription factors is unlikely to be responsible for de-regulating pol III output during rapamycin treatment. To determine the activity of the pol III enzyme, we measured its ability to catalyse randomly initiated RNA synthesis independently of transcription factors, using a poly (dA-dT) template. Two matched pairs of extracts were tested. No consistent difference in the level of random polymerisation by pol III was observed between extracts prepared from cells incubated in vehicle-treated or rapamycin-treated conditions (Fig 3.10B).

A


Rapamycin (hrs) : 0 2 4 6 8 12
Bdp1
160kDa
Brf1
105kDa
75kDa
TBP
35kDa
Actin
35kDa
1 2 3 4 5 6

B

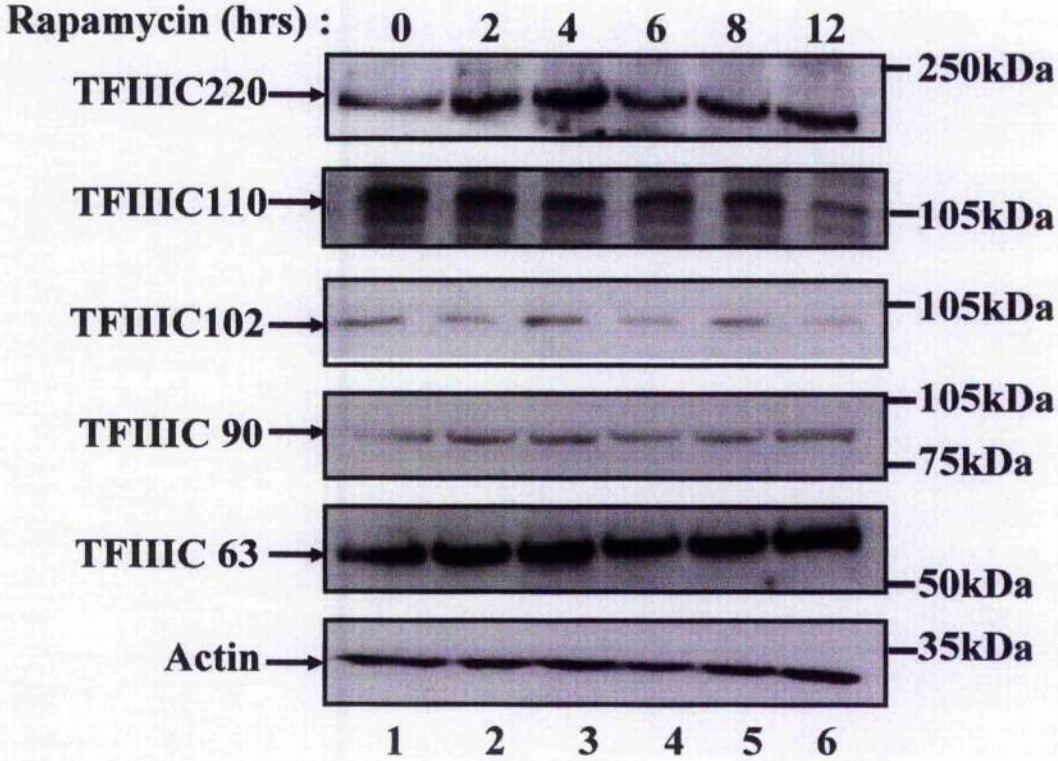
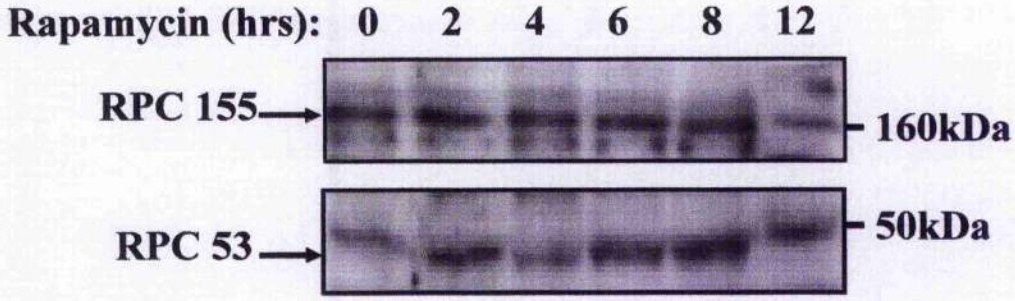

Rapamycin (hrs) : 0 2 4 6 8 12
TFIIIC220
250kDa
TFIIIC110
105kDa
TFIIIC102
105kDa
TFIIIC 90
105kDa
75kDa
TFIIIC 63
50kDa
Actin
35kDa
1 2 3 4 5 6

Figure 3.9 : **Decrease in Pol III transcription in rapamycin-treated cells is not due to reduced TFIIIB or TFIIIC levels.**

(A) Extracts of cells (50μg) treated with (100nM) rapamycin for the times indicated were resolved by SDS-PAGE and analysed by western blotting with antibodies against the TFIIIB subunits Bdp1 (upper panel), Brf1 (second panel) and TBP (third panel), or in (B) with antibodies against the TFIIIC subunits TFIIIC220 (upper panel), TFIIIC110 (second panel), TFIIIC102 (third panel), TFIIIC90 (fourth panel) and TFIIIC63 (fifth panel). Actin was used as loading control in each case (A; fourth panel and B; sixth panel). These blots are representative of at least three independent experiments.

A



B

	Treatment	Pol III Activity (cpm: % of control values)
Set 1	Vehicle	**100**
	Rap 4hr	**94.4**
	Rap 12hr	**98.3**
Set 2	Vehicle	**100**
	Rap 4hr	**94.1**
	Rap 12hr	**95.5**

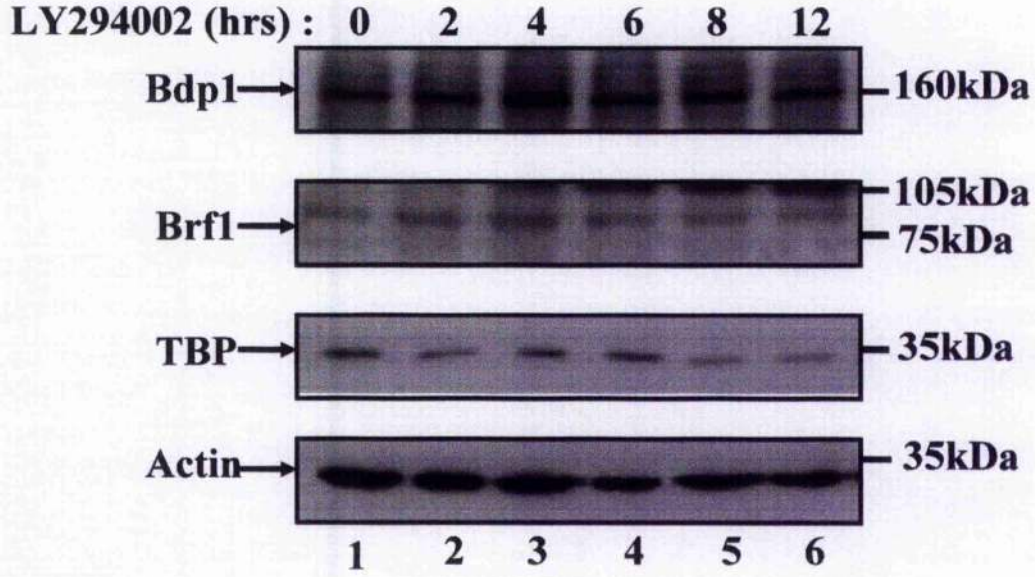
Figure 3.10 : **Rapamycin treatment does not alter pol III abundance or activity.**

(A) Extracts of cells cultured in 10% serum were incubated with vehicle for 12 hrs (lane 1) or rapamycin (100nM; lanes 2-6) over the times indicated. Samples were subjected to SDS-PAGE analysis and immunoblotted with antibodies against RPC-155 (upper panel) , or RPC 53 (lower panel). These blots are representative of two independent experiments.

(B) Extracts of cells cultured in 10% serum plus vehicle for 12 hrs or rapamycin (100nM) for 4hrs or 12hrs, were used to measure their ability to catalyse randomly initiated RNA synthesis, using a poly (dA-dT) template. Results from two independent experiments are shown with the vehicle treated value set to 100 in each case.

To determine whether the PI3K and mTOR pathways converge and/or act on different downstream targets, the protein levels of the multi-subunit complexes TFIIIB (Fig 3.11A), TFIIIC (Fig. 3.11B) and pol III (Fig. 3.12A) were investigated during a time course of LY294002 treatment in fibroblast cells. No changes in abundance were noted at any time point, verifying that protein levels of the transcription factors TFIIIB and TFIIIC and the enzyme pol III remain unaffected when either the mTOR or PI3K pathways are blocked. The activity of pol III was assessed during exposure to LY294002 and was found to have the same levels of activity in both vehicle-treated and LY294002-treated extracts (Fig. 3.12B).

A


LY294002 (hrs) : 0 2 4 6 8 12
Bdp1
160kDa
Brf1
105kDa
75kDa
TBP
35kDa
Actin
35kDa
1 2 3 4 5 6

B

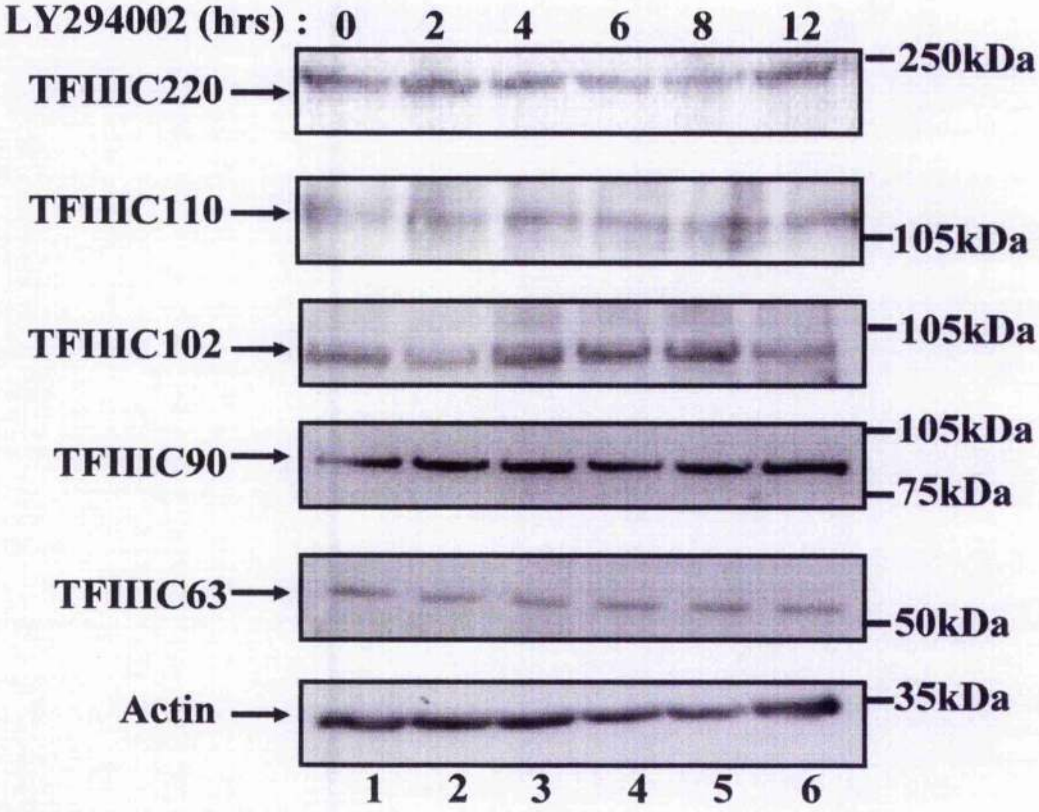
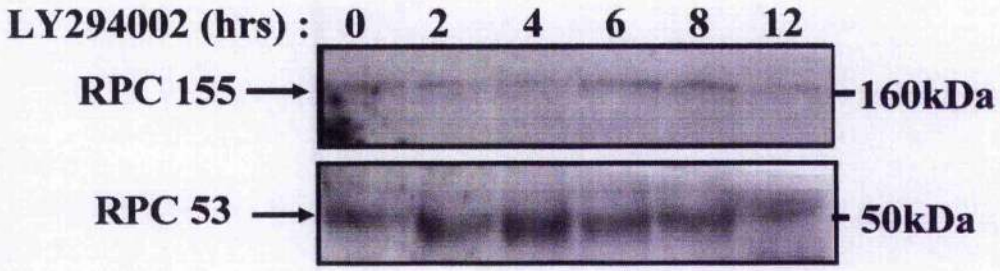

LY294002 (hrs) : 0 2 4 6 8 12
TFIIIC220
250kDa
TFIIIC110
105kDa
TFIIIC102
105kDa
TFIIIC90
105kDa
75kDa
TFIIIC63
50kDa
Actin
35kDa
1 2 3 4 5 6

Figure 3.11 : **Decrease in pol III transcription in LY294002-treated cells is not due to reduced TFIIIB or TFIIIC levels**

(A) Cell extracts (50μg) treated with (50 μM) LY294002 for the times indicated were resolved by SDS-PAGE and analysed by western blotting with antibodies against the TFIIIB subunits Bdp1 (upper panel), Brf1(second panel) and TBP (third panel), or in (B) with antibodies against the TFIIIC subunits TFIIIC220 (upper panel) TFIIIC110 (second panel), TFIIIC102 (third panel) TFIIIC90 (fourth panel) and TFIIIC63 (fifth panel). Actin was used as loading control in each case (A; fourth panel and B; sixth panel). Blots are representative of at least three independent experiments.

A



B

	Treatment	Pol III Activity (cpm: % of control values)
Set 1	Vehicle	**100**
	LY 4hr	**100.8**
	LY 12hr	**98.3**
Set 2	Vehicle	**100**
	LY 4hr	**94.6**
	LY 12hr	**104.6**

Figure 3.12; **LY294002 treatment does not alter pol III abundance or activity**

(A) Extracts of cells cultured in 10% serum were incubated with vehicle for 12hrs (lane 1) or LY294002 (50μM; lanes 2-6) over the times indicated. Samples were subjected to SDS-PAGE analysis and immunoblotting with antibodies to RPC 155 (upper panel) or RPC 53 (lower panel). Blots are representative of two independent experiments.

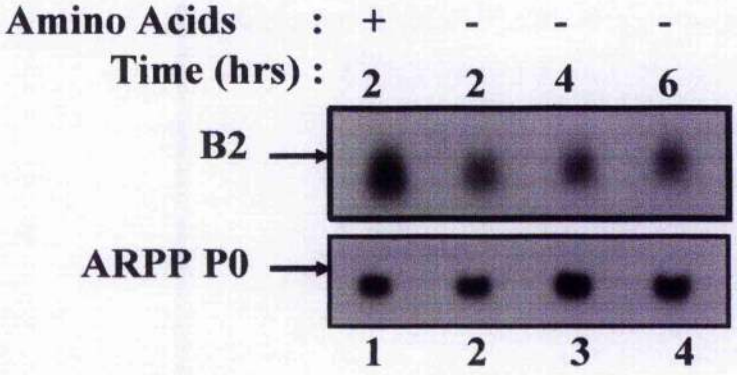
(B) Extracts of cells cultured in 10% serum plus vehicle for 12 hrs or LY294002 (50μM) for 4hrs or 12hrs, were used to measure their ability to catalyse randomly initiated RNA synthesis, using a poly (dA-dT) template. Results from two independent experiments are shown with the vehicle treated value set to 100 in each case.

3.2.3 Pol III transcription is sensitive to amino acid availability

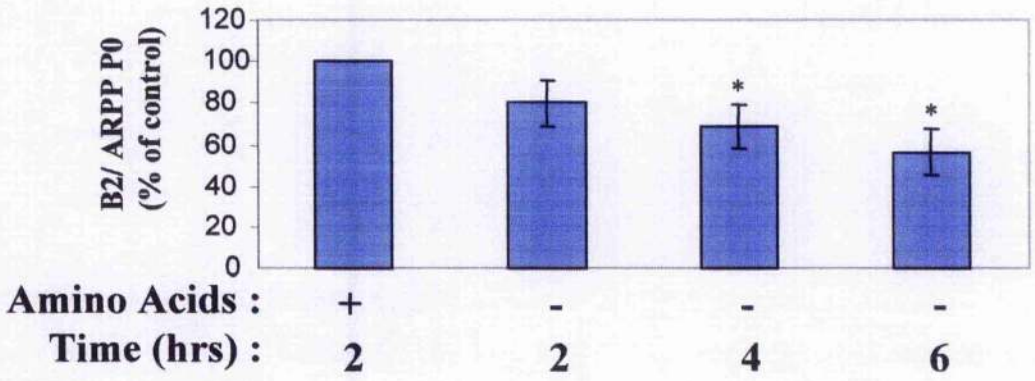
It has previously been shown that transfer of cultured mammalian cells from standard growth medium into amino acid-free medium leads to rapid dephosphorylation of both S6K1 and 4E-BP1 (Hara *et al.*, 1998). This indicates that mTOR is involved in an amino acid sensory pathway which co-ordinates protein synthesis with amino acid availability (Proud, 2002). The knowledge that the mTOR pathway regulates protein synthesis with amino acid availability, together with the experimental data so far, which show that mTOR regulates pol III activity (indirectly controlling the cell's protein biosynthetic capacity), led to the hypothesis that the mTOR pathway could be involved in the regulation of pol III transcription in response to amino acid availability.

Total RNA was extracted from cells grown in culture for various times in the presence or absence of amino acids, and northern analysis was performed. Within 2hrs of amino acid deprivation there is a 20 % reduction in the level of the pol III transcript B2 (Fig. 3.13A/B), which is further reduced by 6hrs to 60% of control cells. The effect is specific since levels of the pol II transcript ARPP P0 remained constant (Fig. 3.13A). Protein cell extracts were also prepared from fibroblasts deprived of amino acids for various times. Subsequent SDS-PAGE analysis and immunoblotting shows that the phosphorylation of mTOR effectors S6K1 and 4E-BP1, are blocked at each time point (Fig. 3.11C), confirming previous studies (Hara *et al.*, 1998). 4E-BP1 exists as a number of species with different mobilities on SDS-PAGE due to differences in phosphorylation, with the fastest mobility form being the hypo-phosphorylated (α) form. It can be seen that the under-phosphorylated form is only present in extracts from cells which had been cultured in the amino acid-free medium (Fig. 3.13C, lower panel).

A


Amino Acids : + - - -
Time (hrs) : 2 2 4 6
B2
ARPP P0
1 2 3 4

B


B2/ ARPP P0 (% of control)
120
100
80
60
40
20
0
*
*
Amino Acids : + - - -
Time (hrs) : 2 2 4 6

C

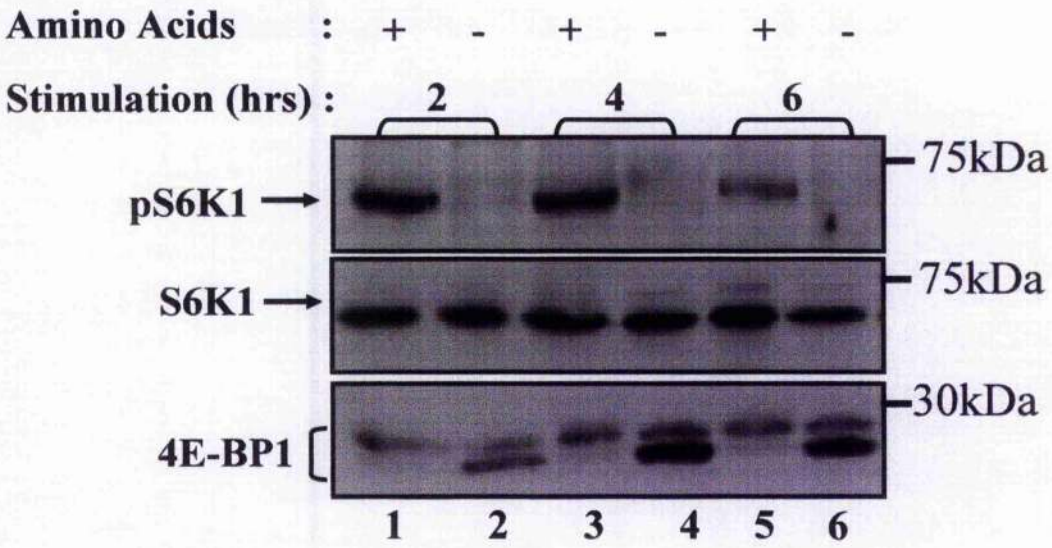

Amino Acids : + - + - + -
Stimulation (hrs) : 2 4 6
pS6K1
S6K1
4E-BP1
75kDa
75kDa
30kDa
1 2 3 4 5 6

Figure 3.13 : **Signalling through mTOR is blocked in the absence of amino acids**

(A) Total RNA (20μg) was extracted from fibroblasts cultured in DMEM containing 10% dFBS (lane 1), or in EBSS containing 10% dFBS (lanes 2-4) for the times indicated. Northern analysis was performed and the upper panel shows the blot probed with a B2 gene; the lower panel shows the same blot that has been stripped and re-probed with the ARPP P0 gene.

(B) Levels of the B2 transcript were quantified by densitometry and normalised against the ARPP P0 signal. The graph shows the mean and standard deviations from three independent experiments; values obtained for cells grown in DMEM + 10% dFBS were set as 100 and other values were calculated as a percentage of this.

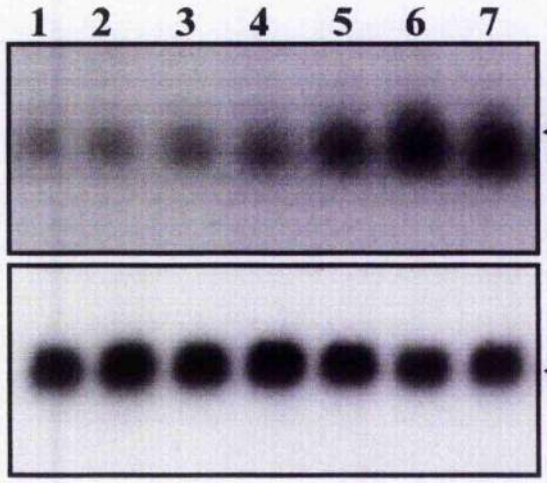
(C) Extracts (50μg) were prepared from cells grown in culture as in (A) and western analysis performed with antibodies against S6K1 phosphorylated at Thr389 (upper-panel), S6K1(middle panel) and 4E-BP1 (lower panel) over the time points indicated. Blots are representative of at least three independent experiments.

* denotes statistically significant difference from control, at $p < 0.05$

The effect of addition of amino acids to nutrient-deprived fibroblasts was also investigated. Overnight serum starvation was followed by amino acid starvation (except lane1) for 1 hr and then different media treatments for 2hrs. Withdrawal of amino acids from serum-starved cells did not effect pol III transcription in A31 cells (Fig. 3.14, compare lanes 1 and 2), yet addition of essential amino acids to amino acid starved cells produced a stimulation of B2 levels (1.5-fold; Fig. 3.14B, lanes 3 and 4) either by addition of DMEM or 1 x amino acids. Exposure of amino acid-starved cells to dialysed serum yielded an increase in pol III transcription, albeit incomplete (2-fold; Fig. 3.14B, lane 5), suggesting autophagy may play a role in salvaging amino acids in cells. However, the addition of amino acids together with dialysed serum produces the greatest pol III response (3-fold; Fig. 3.14B, lanes 6 and 7).

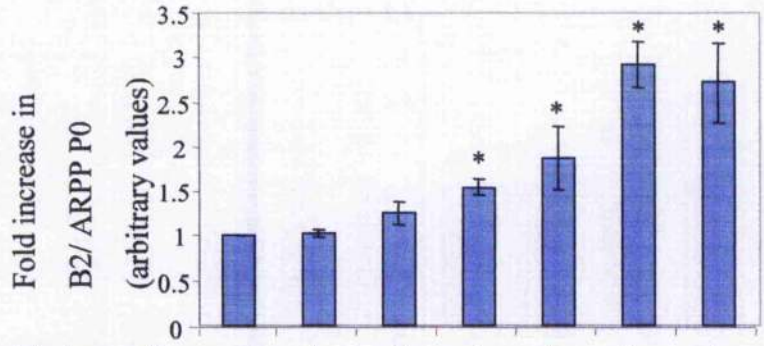
To determine whether changes occurred to the key components of the pol III transcriptional apparatus, in response to amino acid deprivation, the effect of this on levels of TFIIIB and TFIIIC subunits was investigated. Western blot analysis shows no change in the levels of Bdp1, Brf1 and TBP up to 6hrs of amino acid deprivation (Fig. 3.15A). Similarly, no change in the abundance of the TFIIIC subunits; TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90 or TFIIIC63 is detected (Fig. 3.15B) when these cells were deprived of amino acids, suggesting that this repression of pol III transcription is not due to limited availability of these transcription factors. These preliminary experiments show that pol III transcription is sensitive to the availability of amino acids.

A



Amino acid starvation	-	+	+	+	+	+	+
Fresh DMEM	-	-	+	-	-	-	+
Amino acids	-	-	-	+	-	+	-
Dialysed serum	-	-	-	-	+	+	+

B



Amino acid starvation	-	+	+	+	+	+	+
Fresh DMEM	-	-	+	-	-	-	+
Amino acids	-	-	-	+	-	+	-
Dialysed serum	-	-	-	-	+	+	+

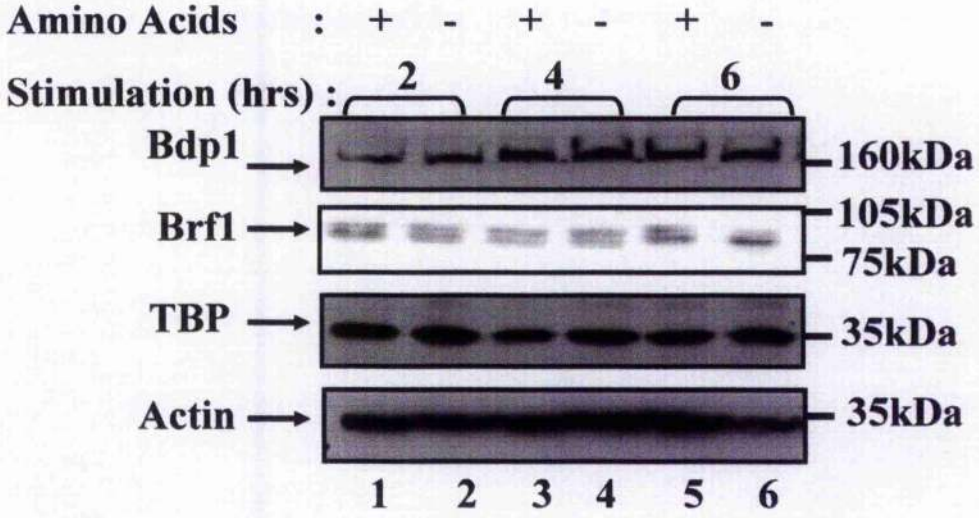
Fig 3.14 : **Pol III transcription is up regulated in response to amino acids**

(A) Serum starved A31 fibroblasts were amino acid starved for 1 hr (lanes 2-7), then DMEM (lanes 3 and 7), a 1 X stock of amino acids (Lanes 4 and 6), or no amino acid stock (lanes 1, 2, 3 , 5 and 7) was added and the cells were incubated with (lanes 5,6 and 7) or without (lanes 1 -4) 10% dFBS for 2 hrs. Total RNA was extracted and Northern analysis performed using the B2 and ARPP P0 probes.

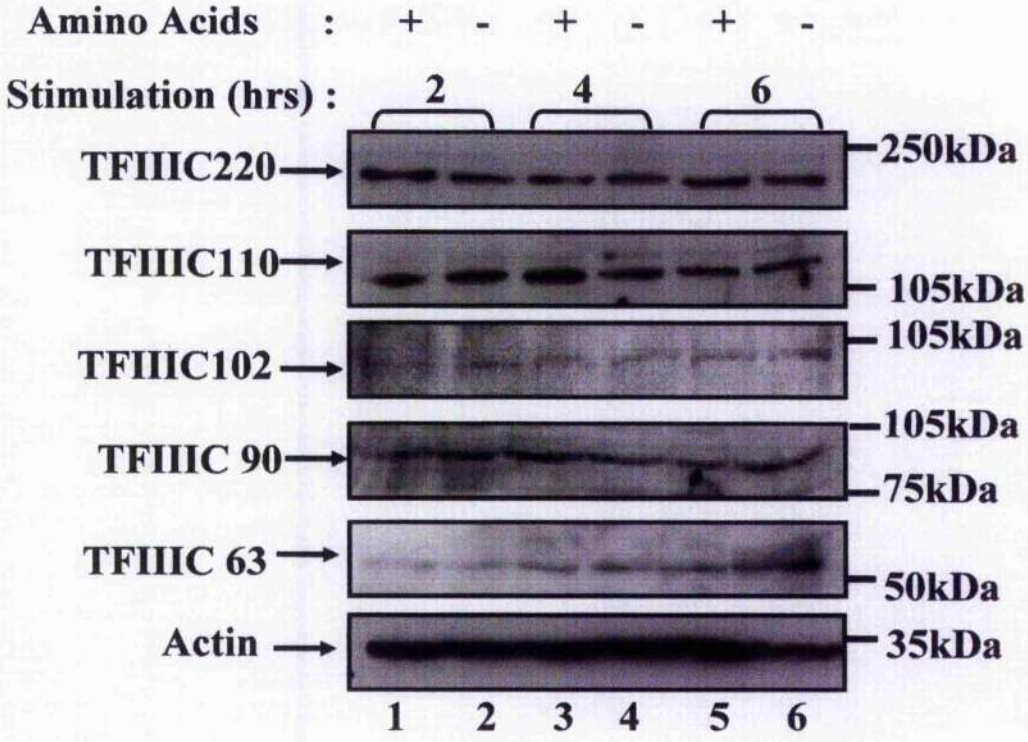
(B) Analysis and representation of transcript levels from three independent experiments, +/-standard deviation. Where lane 1 is designated 1 and other values are calculated as a fold increase of this.

* denotes statistically significant difference from control, at $p < 0.05$

A


Amino Acids : + - + - + -
Stimulation (hrs) : 2 4 6
Bdp1
160kDa
Brf1
105kDa
75kDa
TBP
35kDa
Actin
35kDa
1 2 3 4 5 6

B


Amino Acids : + - + - + -
Stimulation (hrs) : 2 4 6
TFIIIC220
250kDa
TFIIIC110
105kDa
TFIIIC102
105kDa
TFIIIC 90
105kDa
75kDa
TFIIIC 63
50kDa
Actin
35kDa
1 2 3 4 5 6

C

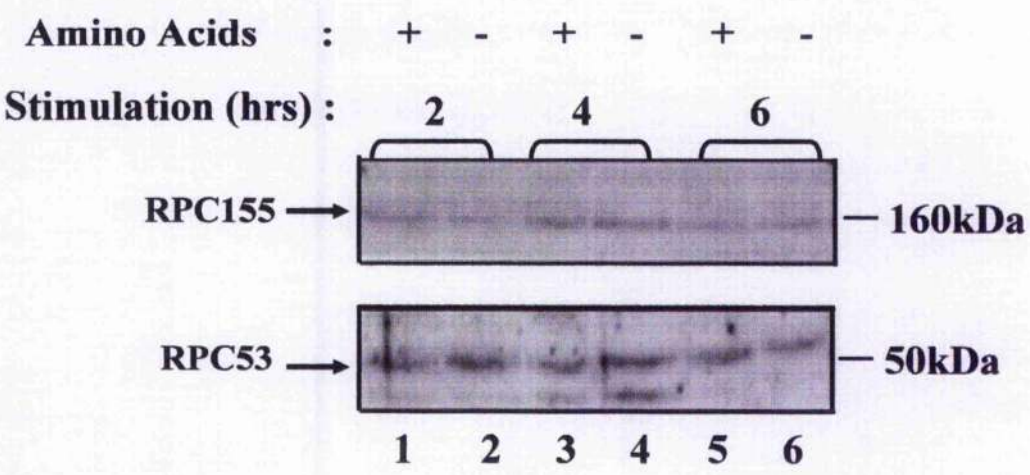


Figure 3.15 : **Decrease in pol III transcription in amino acid deprived cells is not due to reduced TFIIIB, TFIIIC or pol III levels.**

(A) Extracts (50μg) prepared from cells cultured in either DMEM containing 10% dFBS (lanes 1, 3 and 5) or 1 X PBS containing 10% dFBS (lanes 2, 4 and 6) were resolved by SDS-PAGE and analysed by western blotting with antibodies against the TFIIIB subunits; Bdp1 (upper panel), Brf1 (second panel) and TBP (third panel) or in (B) with antibodies against the TFIIIC subunits; TFIIIC220 (upper panel) TFIIIC110 (second panel),TFIIIC102 (third panel) TFIIIC90 (fourth panel) and TFIIIC63 (fifth panel) over the time points indicated. Actin was used as a loading control in each case (A; fourth panel, B; sixth panel). Blots are representative of at least three independent experiments.

(C) Extracts (50μg) were prepared from cells cultured in either DMEM containing 10% dFBS (lanes 1, 3 and 5) or 1 X PBS containing 10% dFBS (lanes 2, 4 and 6) for the times indicated. Samples were subjected to SDS-PAGE analysis and immunoblotted with antibodies to RPC 155 (upper panel) or RPC 53 (lower panel). Blots are representative of two independent experiments.

3.3 Discussion

These data suggest that serum induces an immediate increase in pol III transcription that is regulated, in part, by the mTOR and PI3K signalling cascades. Specific inhibitors of the mTOR and PI3K pathways can reduce pol III transcription within 2 hrs. Inactivation of the mTOR pathway induces a down-regulation in the levels of 5S rRNA and tRNA transcripts as demonstrated by RT- PCR. Primer extension analysis showed that increased signalling through the PI3K and mTOR pathways can increase pol III transcription. Conversely, direct inhibition of the mTOR and PI3K pathways, using a peptide substrate inhibitor shows a concentration-dependent decrease in pol III activity. Signalling through mTOR and PI3K contributes to pol III transcription in asynchronous cells and these effects occur without effect on the abundance of the pol III transcription factors TFIIIB or TFIIIC. Furthermore, we find that pol III transcription is sensitive to amino acid availability and that serum-starved fibroblasts respond to addition of amino acids by quickly up-regulating pol III transcription.

Increased protein synthesis is one of the major anabolic events required for the growth response (Kozma and Thomas, 2002). Since the output of pol I is concerned exclusively with protein synthesis and much of the output of pol III (tRNA and 5S rRNA) is required for the same purpose, it has been found that the two systems are usually co-ordinately regulated (White, 2004b). Interestingly, a functional TOR pathway is required for the co-ordinate regulation of transcription by both pol I and pol III. This was demonstrated by the fact that synthesis of pre-rRNA by pol I and tRNA and 5S rRNA by pol III (Majahan, 1994; Zaragoza *et al.*, 1998; Powers and Walter, 1999) can be inhibited by rapamycin. Recent studies in mammalian cells have identified putative targets of repression by rapamycin in the pol I system (Hannan *et al.*, 2003; Mayer *et al.*, 2004), however, relatively little is published about the regulation of pol III by the mTOR pathway.

Evidence is provided here that pol III transcription is under the control of the mTOR signalling pathway, which is in agreement with previous work in mammals and yeast (Majahan, 1994; Zaragoza *et al.*, 1998; Powers and Walter, 1999). This along with data from pol I regulation (Stefanovsky *et al.*, 2001, Zhao *et al.*, 2003; James and Zomerdijk, 2004) demonstrates further the existence of a direct link between growth factor signalling and ribosome biogenesis in mammals.

Other kinases have been found to regulate pol III transcription. Specifically, a link between a rapid growth factor signalling pathway and the regulation of pol III transcription was recently reported by Felton-Edkins and co-workers. This demonstrated that ERK mitogen–activated protein kinases stimulate the assembly of pol III transcription complexes in mammals (Felton-Edkins *et al.*, 2003a). Specifically, control of pol III activity was found to be through the direct binding and phosphorylation of a subunit of TFIIIB (discussed further in Chapter four) (Felton-Edkins *et al*, 2003a). Previous reports had also shown that the highly conserved protein kinase CK2 regulates pol III transcription via an interaction with TFIIIB. In *S. cerevisiae* CK2 was found to phosphorylate the TBP component of TFIIIB *in vitro* whereas all three human TFIIIB subunits could be phosphorylated directly by CK2 *in vitro* (Ghavidel and Schultz, 1997; Ghavidel *et al.*, 1999; Johnston *et al.*, 2002). Since CK2 is known to form part of the Wnt signalling pathway in *Drosophila* and mammals (Song *et al.*, 2000; Willert *et al.*, 1997) and is required for the proliferation of yeast, worm and mammalian cells (Ghavidel and Shultz, 2001), then these earlier reports provide evidence of the regulation of pol III transcription by a growth signalling pathway.

Northern analysis showed that substantial inhibition of pol III transcription occurred when serum-starved cells were exposed to LY294002 or rapamycin overnight (Fig. 3.3). However, LY294002 treatment reduced B2 transcript levels 10% more than rapamycin treatment. This further reduction of pol III transcription may occur via the inhibition of

another signalling pathway by LY294002, as it is known that PI3K lies upstream of a number of signalling pathways including the MAPK ERK pathway. Interestingly, LY294002 has also been reported to block CK2 activity (Davies *et al.*, 2000) so the negative effects on pol III transcription may occur due to inhibition of CK2, a known positive regulator of pol III (Ghavidel and Schultz, 1997; Ghavidel *et al.*, 1999; Johnston *et al.*, 2002). When cells were exposed to a combination of LY294002 and rapamycin there was a further reduction in the levels of B2 pol III transcript, relative to when either drug was added alone. The effect is not additive, but the combination of drugs show a more complete inhibition of pol III transcription than inhibition of either the PI3K and mTOR pathway alone.

Whilst the Northern and *in vitro* transcription data both indicate that the mTOR pathway controls pol III transcription, the inhibition of pol III appears to have occurred much more quickly in the *in vitro* transcription assay. The Northern analysis ascertains the levels of B2 at the time the cells are harvested. Whilst B2 provides a reliable indication of transcriptional output due to its short half-life, the RNAs inevitably will be present after inhibition of pol III has occurred. In contrast, an *in vitro* transcription assay allows the direct activity of the transcription machinery to be determined in the extracts at a specific time point on a naked template. As the two methods assay different aspects of pol III regulation, this may explain the difference in the time frame of rapamycin inhibition.

RT-PCR analysis showed that substantial decreases in $tRNA^{Leu}$ and $tRNA^{Tyr}$ occurred after exposure to rapamycin or LY294002 for only 6hrs (Fig 3.7). Since these primers hybridise to the introns within unprocessed tRNA precursors, then these short-lived primary transcripts are spliced very rapidly. Consequently, their levels provide a reliable indication of ongoing transcription in a cell (Winter *et al.*, 2000); indicating that type II promoters are sensitive to rapamycin and LY294002 treatment. It was interesting to note that whilst

inhibition of the mTOR and PI3K pathways decreased the RNA levels of both type I (5S rRNA) and type II (tRNA) pol III promoters, levels of the type III promoter (U6), remained unchanged throughout the time course (Fig. 3.7). As type III promoters do not utilise the TFIIIB component Brf1 for promoter recognition or the same form of TFIIIC as the other promoter types, this may implicate Brf1 or TFIIIC as targets of the PI3K and/or mTOR pathways.

PKB has been placed unequivocally upstream of mTOR as a result of several studies in *Drosophila* (Gingras *et al*, 1998; Verdu *et al.*, 1999; Scanga *et al*. 2001; Lizcano *et al*, 2003; Miron *et al*, 2003) and in mice (Peng *et al*, 2003). PKB has emerged as a critical mediator of mTOR activity. This is demonstrated by the fact that growth factors inactivate TSC1/2 through the activation of PKB resulting in TSC2 phosphorylation in both mammalian and *Drosophila* cells (Dan *et al*, 2002; Inoki *et al*, 2002; Manning *et al*, 2002; Potter *et al.*, 2002). This inactivation is considered a critical step in the phosphorylation of S6K1 and 4E-BP1. When a competitive substrate inhibitor of PKB was used in an *in vitro* transcription assay it reduced VA1 transcription in a dose-dependent manner (Fig. 3.8). Without PKB phosphorylation, TSC2 is no longer phosphorylated, so no inhibition of the TSC tumour suppressor complex occurs and mTOR signalling cannot be potentiated. Therefore, this result suggests that active mTOR is required for pol III transcription.

It was possible that the inhibition of pol III transcription which occurred when the mTOR and PI3K pathways were blocked was due to a change in transcription factor abundance. Previous reports have demonstrated that pol III transcription factor levels do change under certain circumstances, for example fibroblasts transformed with polyomavirus or simian virus 40 have abnormally active pol III activity and over-express Bdp1 at both the mRNA and protein level (Felton-Edkins and White, 2002), and elevated TBP levels have been found in colon tumours (Johnson *et al.*, 2003). However, the results presented here show

that inhibition of the mTOR and PI3K pathways does not result in changes in the abundance of the pol III transcription factors TFIIIB or TFIIIC (Fig 3.9 and 3.11). Potentially, changes in the activity of one or several of these components could have occurred through post-translational modifications; therefore, further investigation into this control could involve looking at the phosphorylation status of TFIIIB and TFIIIC, as it has been shown that these factors require phosphorylation to be activated (Hoeffler *et al*, 1998, Felton–Edkins *et al.*, 2003a).

Transcription by pol III inevitably requires the presence and activity of the polymerase, therefore, the levels of two of the pol III subunits and the activity of the enzyme were analysed in cells which had been treated with rapamycin or LY294002. The abundance of the pol III subunits does not vary when the mTOR or PI3K pathways are blocked (Fig. 3.10A and Fig. 3.12A); therefore it is unlikely that lack of pol III is responsible for de-regulating pol III output in response to rapamycin or LY294002 treatment. Although we were unable to determine levels of all pol III subunits, from the available evidence changes in these are also unlikely to explain these results. Just as the abundance of subunits does not alter, neither does the activity of the polymerase in cells where the mTOR or PI3K pathways have been blocked (Fig. 3.10B and Fig. 3.12B). Despite the finding that the activity of the polymerase does not change, its ability to transcribe pol III products does (Fig. 3.5-3.8). Therefore, this result provides further support to the hypothesis that regulation of the pol III transcription by the mTOR and PI3K pathways may occur through changes in the activity of the pol III transcription factors.

It has long been known that nutrients, especially branched chain amino acids, can regulate rates of protein synthesis in animal cells. However, more recently it has become clear that they can do so directly (i.e. the effect is not mediated by hormones such as insulin) and that this involves a signalling pathway implicated in controlling the cell cycle and cell size. The

TOR signalling pathway has been found to couple nutrient availability with cell growth in *S. cerevisiae, D. melanogaster* and mammalian cells (Raught *et al.*, 2001; Rohde *et al.*, 2001; Schmelzle and Hall, 2000). This control was determined through controlling the rate of translation in response to energy and amino acids (Rohde *et al.*, 2001). Furthermore, mammalian cells in culture exhibit an inhibition of overall protein synthesis with depletion of amino acids which is rapidly reversible upon addition of amino acids (Hara *et al.*, 1998). A31 mouse fibroblasts deprived of amino acids for 2, 4, and 6 hrs showed that the phosphorylation status of S6K1 and 4E-BP1 is affected by the absence of amino acids (Fig. 3.13C), confirming these previous studies. Additionally, we find that pol III transcription is sensitive to amino acid availability and a 20% decrease is observed within 2 hrs (Fig 3.13B).

A major mechanism for degrading intracellular proteins in response to amino acid deprivation is autophagy. Autophagy is a major source of endogenous amino acids for gluconeogenesis and other critical pathways early in starvation (Shigemitsu *et al.*, 1999). A large increase in levels of B2 RNA occurred when dialysed serum was added back in the absence of amino acids (Fig. 3.14, lane 5); this effect could only be possible if autophagy had occurred, as the extracellular levels of amino acids have been depleted. By far the biggest increase in pol III transcription occurred when both serum and amino acids were added back together (Fig. 3.14, lane 6 and lane 7), indicating that the full response of pol III transcription to serum depends on both the availability of amino acids and serum. Insulin like growth factor-1 (IGF-1) induction of pol I transcription in HEK293 cells is dependent on PI3K activity and is modulated by mTOR, which coordinates pol I transcription with availability of amino acids (James and Zomerdijk, 2004).

To further investigate whether the response of pol III transcription to serum is dependent on the availability of amino acids, then an inhibitor of autophagy must be included in the

experiment. To verify if amino acid stimulation is dependent on the activities of PI3K and mTOR, these inhibitors could be included in the amino acid add-back experiment. Although PI3K activity is still required for the activation of rapamycin-resistant mutants (Dennis *et al.*, 1996), this role of PI3K in the pol III response to amino acid availability was not investigated due to time constraints. As mTOR is widely regarded as an integrator of both the response to growth factors and the amino acid sensing mechanisms, a pathway involving this kinase may be involved in regulating pol III transcription in response to amino acid availability.

It can be concluded from the experimental data presented in this Chapter that pol III transcription is under the control of the PI3K and mTOR pathways. The following Chapter investigates the possible mechanisms by which the mTOR pathway (in particular), can achieve this control. Regulation of TFIIIB and TFIIIC via phosphorylation has already been identified as an important mechanism (Hoeffler *et al.*, 1998, Felton–Edkins *et al.*, 2003a), so this was further studied. RB, c-MYC and ERK will also be investigated to find if the mTOR pathway exerts its effect through these known regulators of pol III transcription.

Chapter 4

Investigation of the Mechanisms that Regulate Pol III Transcription via the mTOR Pathway

4.1 Introduction

4.1.1 Pol III regulation by RB

The data presented in Chapter 3 show that specific inhibitors of the mTOR and PI3K pathways reduce pol III transcription, and this occurs without influencing the abundance of TFIIIB, TFIIIC or pol III subunits, and without changes in the activity of pol III. There are a number of known regulators of pol III transcription. This Chapter considers whether any of these may be influenced by blocking the mTOR signalling pathway or whether there may be an effect independent of the existing known mechanisms.

RB was the first tumour suppressor gene to be identified and characterised (Friend *et al.*, 1986) and inactivation of RB is associated with a significant proportion of human cancers. These include; familial retinoblastoma, osteosarcomas, small-cell lung, cervical, prostate and breast carcinomas, and some forms of leukaemias (Sellers and Kaelin, 1997). Studies of the retinoblastoma gene have shown that its protein product acts to restrict cell proliferation, inhibit apoptosis and promote cell differentiation (Morris and Dyson, 2001). It does this by binding to and regulating a number of transcription factors (Morris and Dyson, 2001). One example of this control is the regulation of a pol III-specific factor TFIIIB. RB was originally found to repress pol III activity both *in vitro* and *in vivo*, as determined by *in vitro* transcription assays and transient transfection assays (White *et al.*, 1996). It was discovered that the amino acids 379-928 of RB provide this activity and mutations in the pocket domain of RB inhibit the effect (Sutcliffe *et al.*, 1999). It is this portion of RB that was found to bind and inhibit the Brf1 component of TFIIIB (Larminie *et al.*, 1997; Sutcliffe *et al.*, 2000). During the cell cycle maximum pol III activity occurs in late G1 when RB is phosphorylated and inactivated by cyclin-dependent kinases (Larminie *et al.*, 1997; Scott *et al.*, 2001). The importance of RB in regulating pol III transcription is highlighted in RB-knockout mice which are substantially compromised in

their ability to suppress pol III transcription when serum is withdrawn (Scott *et al.*, 2001). The RB-related pocket protein p130 also binds TFIIIB during the G0 and early G1 phases, contributing to its repression in serum-starved cells (Sutcliffe *et al.*, 1999; Scott *et al.*, 2001).

4.1.2 The MAP Kinase ERK pathway regulates pol I and III transcription

Whilst RB and p130 undoubtedly play major roles in growth factor sensitivity of pol III transcription in mammalian cells, some stimulation of pol III transcription has been observed prior to the phosphorylation of RB (Johnson *et al.*, 1974; Mauck and Green, 1974; Scott *et al.*, 2001; Felton-Edkins *et al.*, 2003a). This suggests that other regulatory mechanisms contribute to the mitogenic response. This immediate rise in pol III transcription, as discussed above, occurs within minutes of serum addition to growth-arrested fibroblasts (Felton-Edkins *et al.*, 2003a; Figure 3.2). Similar results have been reported in the regulation of pol I transcription in response to mitogenic stimuli. The pol I transcription factor UBF was found to be phosphorylated in response to growth factors by the MAP kinases ERK1 and ERK2 (Stefanovsky *et al.*, 2001). The immediate early response of pol I transcription (Stefanovsky *et al.*, 2001) was found to coincide with an initial increase in pol III activity (Felton-Edkins *et al.*, 2003a; Scott *et al.*, 2001). A more recent report disputes that UBF is the target of ERK signalling and proposes that TIF1A is targeted by MAPK *in vivo* (Zhao *et al.*, 2003). Regardless of the pol I target, the co-ordinate response of both pol I and pol III to increase production of rRNAs and tRNAs provides a key control point for the regulation of growth and hence proliferation. This is due to their critical role in determining ribosome production, where equimolar amounts of the ribosomal proteins 28S, 18S, 5.8S and 5S are required (Nomura, 1999; Warner, 1999; Brown *et al.*, 2000).

Whilst the role of the ERK signalling pathway was determined in the pol I system, its role in the regulation of pol III transcription was not elucidated until recently. ERK was found to activate pol III transcription by binding and phosphorylating the Brf1 subunit of TFIIIB (Felton-Edkins *et al.*, 2003a). Subsequently, serum induction of pol III transcription can be compromised when substitutions of the ERK docking domain or phospho-acceptor sites in Brf1 are made. ChIP analysis showed that treatment of cells with a specific inhibitor of MEK (the ERK activating kinase), impairs promoter occupancy of Brf1 and pol III at tRNA and 5S rRNA genes. This finding suggests that the MAP Kinase signalling pathway is required for normal pre-initiation complex assembly on pol III templates, thus linking growth factor signalling with pol III activation.

4.1.3 Regulation of pol III transcription by c-Myc

The c-Myc gene was discovered as the cellular homologue of the retroviral v-myc gene 20 years ago (Sheiness *et al.*, 1978; Bishop, 1982). The Myc family members were subsequently considered to be proto-oncogenes, since alterations in their expression play an important, if not essential, role in the development of various animal and human tumours (Dang, 1999). The c-Myc protein contains a basic helix-loop-helix leucine-zipper transcription factor that can act as both a transcriptional activator and repressor (Grandori *et al.*, 2000). The role of Myc in transcription was made more obvious through the identification of a dimerisation partner called Max (Blackwood and Eisenman, 1991; Blackwood *et al.*, 1992). Max is the essential heterodimer partner of Myc proteins and together they are involved in regulating various biological activities including cell proliferation, apoptosis and differentiation (Amati *et al.*, 1992; Oster *et al.*, 2002).

c-Myc has been reported to regulate the expression of several pol III–specific genes (Gomez-Roman *et al.*, 2003). The pol III transcription factor TFIIIB is targeted by c-Myc,

and this appears to be through protein-protein interactions rather than through direct DNA recognition by c-Myc (Gomez-Roman *et al.*, 2003). Chromatin immunoprecipitation assays revealed the presence of c-Myc at tRNA and 5S rRNA genes in untransformed fibroblasts, ovarian epithelial cells (Gomez-Roman *et al.*, 2003) and the transformed cervical cell line HeLa (Felton-Edkins *et al.*, 2003b). Transcription of tRNA and 5S rRNA genes can be induced when exogenous c-Myc is introduced into human diploid fibroblasts (Gomez-Roman *et al.*, 2003). Interestingly, specific depletion of endogenous c-Myc from HeLa cells using RNA interference (RNAi) results in a marked decrease in expression of tRNA and 5S rRNA (Felton-Edkins *et al.*, 2003b). The profound effect of c-Myc on pol III transcription in cells may provide an important component of its growth-promoting activity. This may occur by increasing protein synthesis through the targeting of the protein synthetic apparatus by c-Myc (Felton-Edkins *et al.*, 2003b). This hypothesis is consistent with studies that show c-Myc target genes include those involved in ribosome biogenesis (Oster *et al.*, 2002; O'Connell *et al.*, 2003), and the finding that c-Myc can regulate rRNA processing (Schlosser *et al.*, 2003).

In summary, the objectives of this Chapter were to examine the changes in pol III activity by proteins known to interact with this machinery in response to blocking the mTOR signalling cascade. This should establish whether this pathway regulates pol III via existing known mechanisms.

4.2 Results

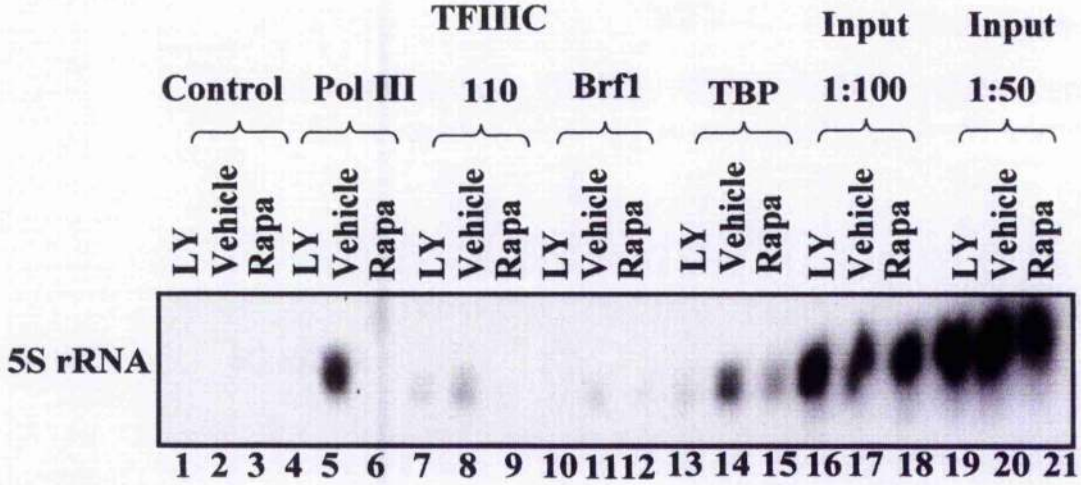
4.2.1. Blocking the mTOR and PI3K pathways affects pol III promoter occupancy

As discussed in Chapter 1, most genes transcribed by pol III require that TFIIIB is brought to the promoter by protein-protein interactions with TFIIIC; in turn, TFIIIB recruits pol III, placing it over the start site so that transcription can commence (Paule and White, 2000; Geiduschek and Kassavetis, 2001). Therefore, TFIIIB is required to bind both TFIIIC and pol III. It was possible that the mTOR and/or PI3K pathways regulate recruitment of pol III via targeted inhibition of one or more of the stages involved in pre-initiation complex formation. Therefore, promoter occupancy of pol III and its related factors was determined in rapamycin- and LY294002-treated cells, to find if blocking the mTOR and/or PI3K pathways affected complex assembly at pol III promoters.

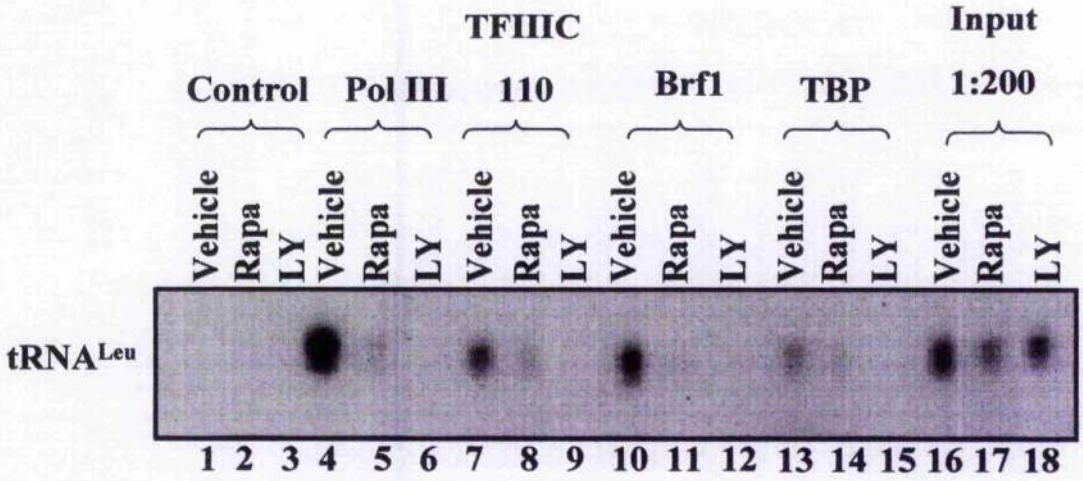
Formaldehyde cross-linked soluble chromatin was prepared from asynchronously growing cells treated for 4 hrs with 50μM LY294002 (Fig. 4.1A, lanes 1, 4, 7, 10, 13, 16 and 19), vehicle (Fig. 4.1A, lanes 2, 5, 8, 11, 14, 17 and 20) or 100nM rapamycin (Fig. 4.1A, lanes 3, 6, 9, 12, 15, 18 and 21). PCR analysis of precipitated DNA showed occupancy of TFIIIC110, Brf1 and TBP is blocked on 5S rRNA promoters when mTOR or PI3K is inactivated. Furthermore, rapamycin and LY294002 decreased promoter-bound pol III. ChIP analysis also shows that binding of TFIIIC110, Brf1 and TBP and pol III to $tRNA^{Leu}$ genes is inhibited by blocking the mTOR and PI3K pathways (Fig. 4.1B, lanes 5, 6, 8, 9, 11, 12, 14 and 15) compared to vehicle-treated control cells (Fig. 4.1B, lanes 4, 7, 10, and 13). The ChIP data for $tRNA^{Leu}$ were quantified and show significant decreases in the occupancy of TFIIIC, TFIIIB and pol III when the mTOR and PI3K pathways are blocked (Fig. 4.1C).

A and B blocks are found in type II promoters (e.g. tRNAs) and are recognised by the multi-subunit complex TFIIIC, where B block binding is the major determinant of binding affinity (White, 2001). To test whether the DNA binding ability of TFIIIC to these promoters was affected by rapamycin or LY294002 treatment, a band shift assay was performed. Microextracts were made from cells which had been grown in the presence of vehicle, rapamycin or LY294002 for 4hrs. Samples were then bound to a radio-labelled B block oligonucleotide in the presence of a competitor (Fig. 4.1D, lanes 2-4) or a non-specific competitor (Fig. 4.1D, lanes 5-7) whilst it migrated through a non-denaturing gel. TFIIIC proteins within the rapamycin and LY294002 extracts displayed increased DNA binding compared to the vehicle–treated extracts (Fig. 4.1D). This increase was quantified as a 2-fold increase for LY294002-treated extracts, and a 3-fold increase for rapamycin-treated extracts (Fig. 4.1E). A bandshift assay determines the DNA binding ability of a protein, whilst a ChIP measures promoter occupancy and is subject to competing influences such as chromatin structure and nucleosome positioning. TFIIIC could be passively released or actively displaced due to changes in the mTOR or PI3K pathway that result in changes in chromatin structure. Confirmation of such changes has been reported during inhibition of the TOR pathway (Rohde and Cardenas, 2003). These data suggest that TOR signalling results in acetylation of histone H4 which alters chromatin conformation allowing active transcription of ribosomal proteins; upon rapamycin treatment, the chromatin returns to a repressed state by recruitment of Rpd-Sin3 histone deacetylase (Rohde and Cardenas, 2003).

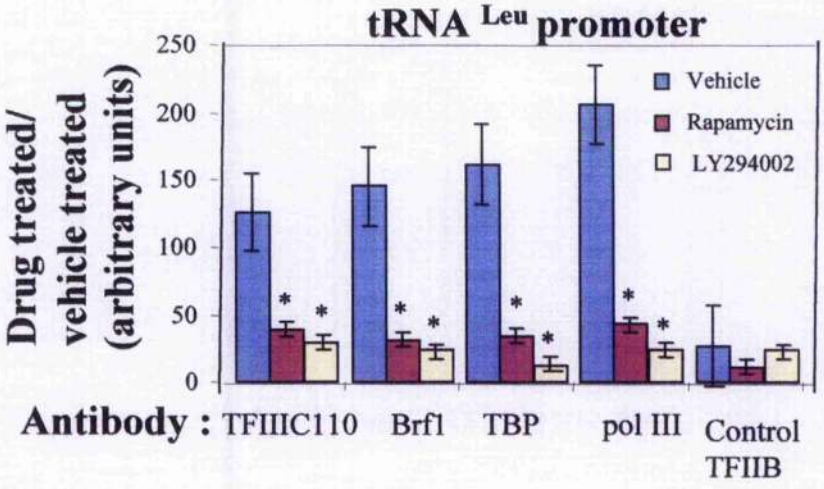
A


TFIIIC
Input
Input
Control
Pol III
110
Brf1
TBP
1:100
1:50
LY
Vehicle
Rapa
5S rRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

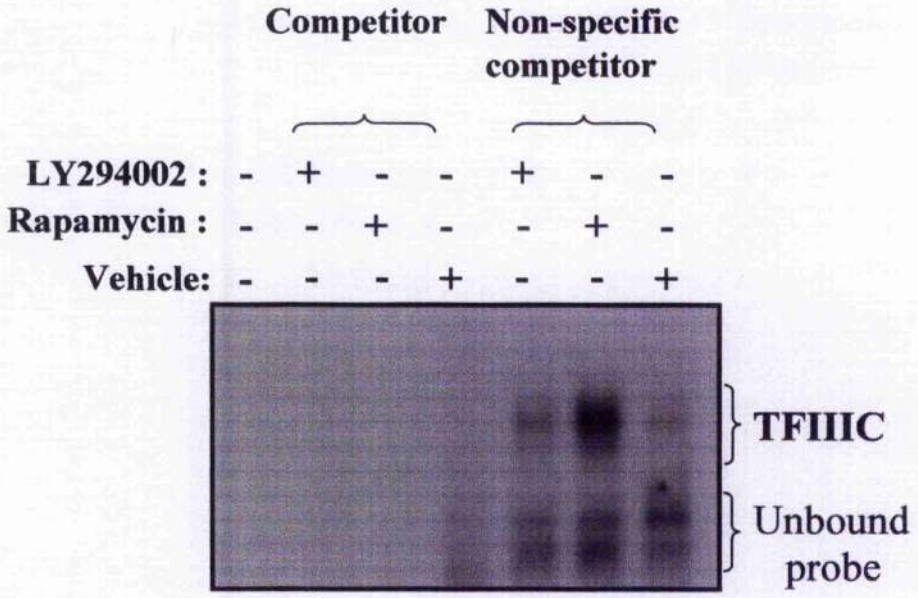
B


TFIIIC
Input
Control
Pol III
110
Brf1
TBP
1:200
Vehicle
Rapa
LY
tRNA Leu
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

C


tRNA Leu promoter
Drug treated/ vehicle treated (arbitrary units)
250
200
150
100
50
0
Vehicle
Rapamycin
LY294002
*
Antibody : TFIIIC110 Brf1 TBP pol III Control TFIIB

D


Competitor
Non-specific competitor
LY294002 : - + - - + - -
Rapamycin : - - + - - + -
Vehicle: - - - + - - +
TFIIIC
Unbound probe

E

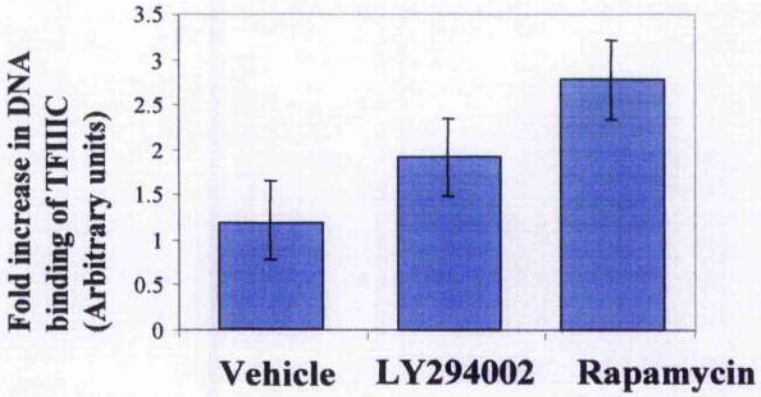

Fold increase in DNA binding of TFIIIC (Arbitrary units)
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
LY294002
Rapamycin

Figure 4.1: **mTOR activity is required for pol III promoter occupancy**

Asynchronous A31 mouse fibroblasts were treated for 4 hrs with vehicle (lanes 2,5, 8, 11, 14, 17 and 20), rapamycin (100nM; lanes 3, 6, 9, 12, 15, 18, 21), or LY294002 (50μM; 1, 4, 7, 10, 13,16 and 19). Association of TFIIIC110, Brf1, TBP and pol III subunit RPC155 with 5S rRNA (A) genes or $tRNA^{Leu}$ genes (B) was then determined by ChIP analysis. This involved semi-quantitative PCR of equivalent DNA input amounts as determined by PCR on diluted input chromatin. Control ChIPs were carried out with TFIIB antibody. PCR reactions incorporated [α-^{32}P] dCTP and products were resolved on denaturing sequencing gels which were subjected to autoradiography. PCR products were quantified by densitometry (C). PCR products from three independent ChIP experiments were quantified for $tRNA^{Leu}$ genes; after normalisation to the input, the mean and standard error of the mean are shown for the drug treated/vehicle treated signals.

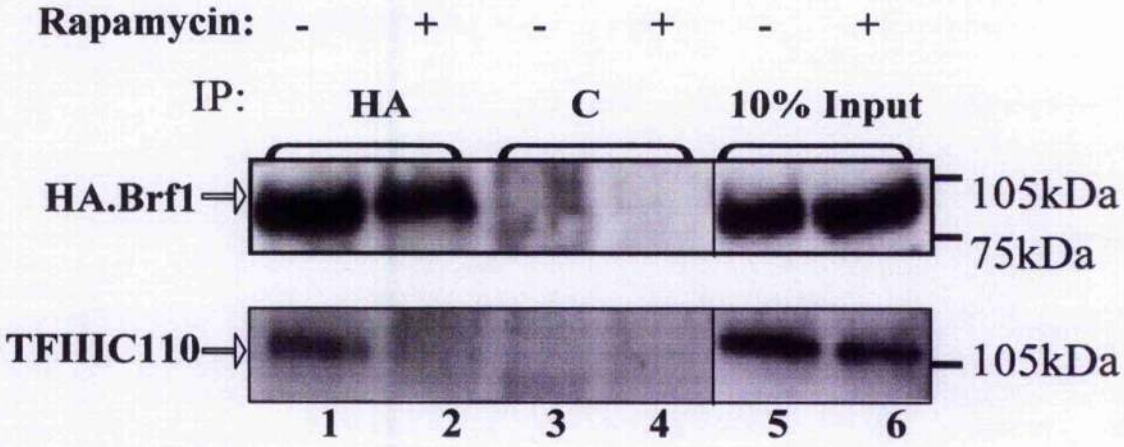
(D) Cell extracts (15μg) which had been vehicle-treated (lanes 4 and 7), rapamycin-treated (100nM; lanes 3 and 6) or LY294002-treated (50μM; lanes 2 and 5) for 4 hrs were used in a band-shift assay. 1ng of radiolabelled B-block probe, and 1 μg of polydI-dC competitor was added to lanes 1-7. Lane 1 is a control and contained no extract. Lanes 2-4 contained 50ng of unlabelled B-block. Values shown in (E) are the average of 2 independent experiments +/- the standard error of the mean.

* denotes statistically significant difference from control, at $p < 0.05$.

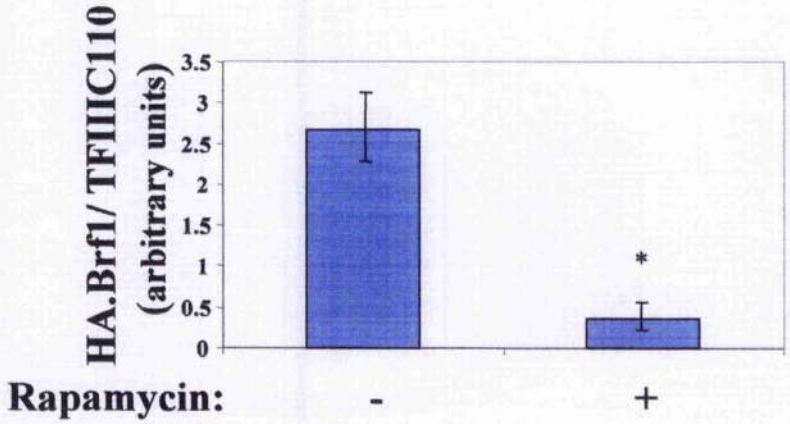
4.2.2. The mTOR pathway controls the interaction of TFIIIB and TFIIIC and the interaction of TFIIIB with pol III

TFIIIB is brought to most pol III promoters by protein-protein interactions with TFIIIC; in turn, TFIIIB recruits pol III placing it over the start site to allow transcription to commence (Paule and White, 2000). Therefore, TFIIIB binds to both TFIIIC and to pol III. To investigate whether the mTOR signalling pathway can influence the interaction between TFIIIB and TFIIIC, co-immunoprecipitations were carried out in Rat1A cells which stably over-express pCDNA3HA.Brf1. In asynchronously growing cells, the Brf1component of TFIIIB stably associates with TFIIIC110 (Fig 4.2A, lane 1). Rapamycin treatment for 4 hrs prevents TFIIIC binding to TFIIIB (Fig 4.2A, lane 2). This was quantified as a 7.6 fold reduction in binding (Fig. 4.2B). This effect is specific since immunoprecipitations with control antibodies showed no interaction between HA.Brf1 and 4E-BP1 (Fig 4.2A, lanes 3 and 4). Since TFIIIB binds to both TFIIIC and to pol III we were interested to know whether the mTOR pathway also regulated the interaction between TFIIIB and pol III. RPC53 (a subunit specific to pol III) associates with the Brf1 subunit of TFIIIB, in asynchronously growing cells (Fig 4.2B, lane 1). Rapamycin blocks the interaction between TFIIIB and pol III (Fig 4.2B, lane 2). The binding of TFIIIC and pol III to TFIIIB was specific since binding wasn't observed when an irrelevant control antibody was used (Fig 4.2B, lanes 3 and 4). These data suggest an important role for mTOR signalling in the regulation of pol III transcription. Having established that rapamycin could inhibit pol III transcription via a block to complex assembly, our attention turned to the possible mechanism by which this was occurring.

A


Rapamycin: - + - + - +
IP: HA C 10% Input
HA.Brf1
105kDa
75kDa
TFIIIC110
105kDa
1 2 3 4 5 6

B


HA.Brf1/ TFIIIC110 (arbitrary units)
3.5
3
2.5
2
1.5
1
0.5
0
*
Rapamycin: - +

C

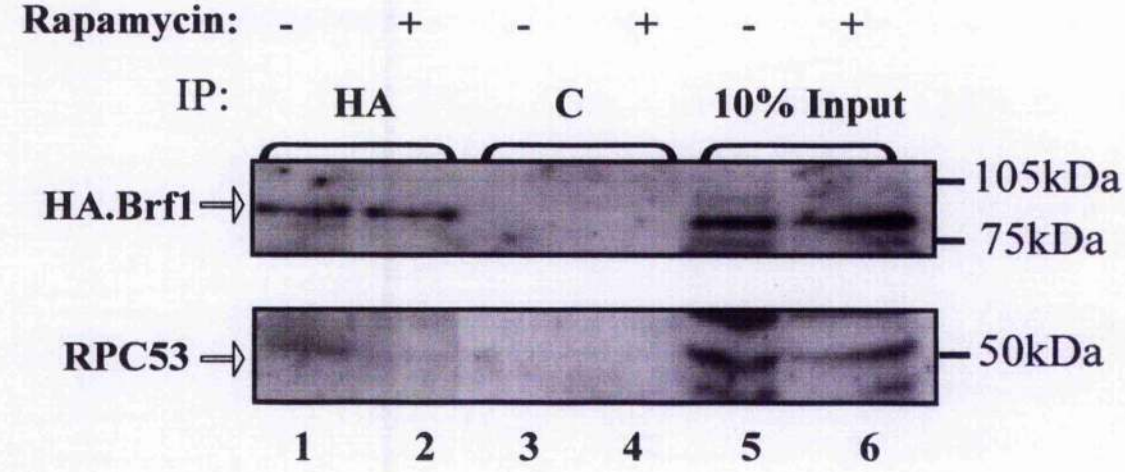

Rapamycin: - + - + - +
IP: HA C 10% Input
HA.Brf1
105kDa
75kDa
RPC53
50kDa
1 2 3 4 5 6

Figure 4.2 : **Inhibition of mTOR compromises binding of TFIIIB to TFIIIC and the binding of TFIIIB to pol III**

(A) Rat1A cells (stably transfected with pCDNA3HA.Brf1) were incubated for 4hrs with vehicle (lanes 1 and 3) or rapamycin (100nM; lanes 2 and 4). Cell extracts (500μg) were immunoprecipitated with anti-HA antibody (lanes 1 and 2) or anti-4EBP1 antibody as a control (C; lanes 3 and 4). Precipitates were resolved by SDS-PAGE and immunoblotted with anti-HA antibody (upper panel) or with anti-TFIIIC110 (lower panel). Lanes 5 and 6 show 10% input of the cell extracts. Results are representative of three independent experiments.

(B) The immunoprecipitations described in (A) was quantified by densitometry and values normalised to the input. The graph shows the mean +/- standard deviations from three independent experiments. Values are arbitrary. * denotes statistically significant difference from control, at $p < 0.05$

(C) Rat1A cells (stably transfected with pCDNA3HA.Brf1) were incubated for 4hrs with vehicle (lanes 1 and 3) or rapamycin (100nM; lanes 2 and 4). Cell extracts (500μg) were immunoprecipitated with anti-HA antibody (lanes 1 and 2) or anti-4EBP1 antibody (c; lanes 3 and 4). Precipitates were resolved by SDS-PAGE and Western blotted with anti-HA antibody (upper panel) or with anti-RPC53 (lower panel). Lanes 5 and 6 show 10% input of the cell extracts. Results are representative of two independent experiments.

4.2.3. Amino acid and rapamycin-mediated effects on pol III transcription are independent of RB

There are several possible mechanisms by which the mTOR pathway could mediate the regulation of pol III transcription. One of these is through regulation of the tumour suppressor protein RB. RB is phosphorylated and de-phosphorylated in a cell-cycle dependent manner and it is clear that these changes regulate its activity (Morris and Dyson, 2001). It is known that pol III transcription is sensitive to the phosphorylation status of RB, and that TFIIIB interacts with the underphosphorylated form of pRB. In this way, RB represses TFIIIB activity and therefore, pol III transcription. Accordingly, TFIIIB is released as pRB becomes hyperphosphorylated by cyclin D- and cyclin E- dependent kinases at G1/S (Scott *et al.*, 2001). This de-repression allows TFIIIB to interact with TFIIIC2 at the promoter of class III genes, and recruit pol III to these templates (Sutcliffe *et al.*, 2000). There is conflicting evidence to support the effect of rapamycin on the phosphorylation of RB and this appears to be cell-type dependent (Chen *et al.*, 1996; Muise-Helmericks *et al.*, 1998; Gaben *et al.*, 2004). Therefore, we determined in the 3T3 cells used in this study, whether the effects of this drug on pol III transcription could be explained through changes in the phosphorylation status of RB. Extracts were prepared from cells incubated with 100nM rapamycin over time. Little or no change was observed in the levels of RB phosphorylated at Ser795 until after 8hrs incubation with the inhibitor, at which time phosphorylation of RB at this site is reduced (Fig. 4.3). This decrease is not due to a reduction in the total levels of RB (Fig. 4.3).

The sensitivity of pol III transcription to amino acid availability (previously described in Chapter 3), is thought to involve a pathway comprising the TOR kinases. Therefore, it was possible that this inhibition of pol III transcription that occurs in the absence of amino acids (Fig. 3.13) was due to changes in the phosphorylation status of RB. Since the effect of amino acid deprivation on the phosphorylation status of RB is unknown, protein extracts

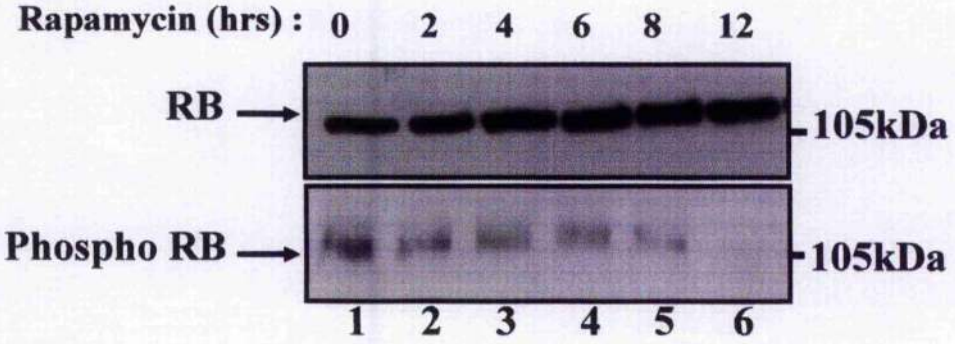


Figure 4.3 : **Effect of rapamycin treatment on pol III transcription is independent of RB at times when pol III transcription is reduced**

Cell extracts (50 μg) were prepared from asynchronous A31 cells treated in the presence of vehicle for 12 hrs (lane 1) or for the various times indicated with rapamcyin (100nM). Samples were resolved by SDS-PAGE and analysed by western blotting with antibodies against total pRB (upper panel), pRB phosphorylated at serine 795 (second panel). Blots are representative of more than three independent experiments.

were made from cells cultured in DMEM containing 10% dialysed serum (Fig. 4.4, lanes 1, 3 and 5) or EBSS containing 10% dialysed serum (which contained no amino acids) (Fig. 4.4, lanes 2, 4 and 6) over a time course. Western analysis shows that RB remained phosphorylated at Ser795 throughout the time course, and total levels of RB remain are also constant (Fig. 4.4, upper two panels). This suggests that early effects of amino acid deprivation on pol III transcription are independent of RB.

The key event that regulates the G1/S transition is the inactivation of RB via sequential phosphorylation by Cdk/cyclin complexes (Sherr, 1996). Regulation of Cdk activity, which is crucial for the orderly initiation and progression of the cell division cycle, involves modulation of the levels of cyclins and Cdk inhibitors (Morgan, 1995; Sherr and Roberts, 1995). Subsequently, the levels of the G1 cyclins, cyclin D and cyclin E were investigated in amino acid-deprived cells. Western analysis shows that the levels of cyclin E proteins are unchanged when the mTOR pathway is blocked for up to 6 hrs; however, levels of cyclin D1 decrease after 4 hrs amino acid deprivation (Fig. 4.4, third and fourth panels). D-type cyclins (D1, D2, D3) are expressed sequentially and are induced in response to growth factor stimulation (Sherr, 1995). p16 is a specific inhibitor of cyclin D1-dependent kinases and, as such, is termed a Cdk inhibitor (CKI). CKIs act stochiometrically, therefore, oscillations in their levels can have a profound effect on cell proliferation (Toyoshima and Hunter, 1994). Interestingly, western blotting for p16 shows that its level does not alter with up to 6hrs of amino acid deprivation, and blotting for actin shows that equal amounts of protein were in each lane (Fig. 4.4, fifth and sixth panel). Therefore, cyclin D1 regulation appears to be independent of p16. The importance of cyclin D1 regulation in the amino acid sensitivity of pol III transcription is undetermined but does not appear to modulate RB phosphorylation at early time points.

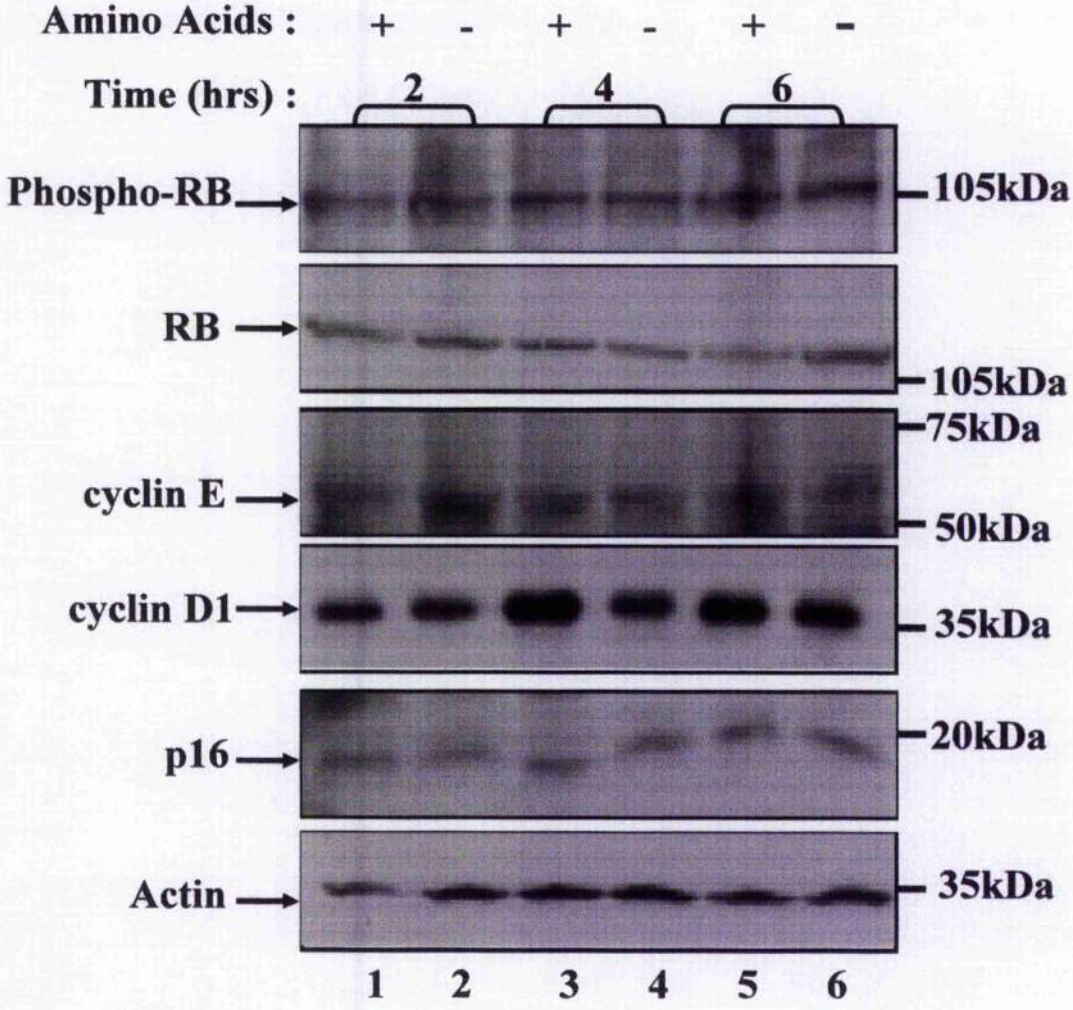


Figure 4.4 : **Effects of amino acid deprivation on pol III transcription are independent of RB**

Protein cell extracts (50 μg) were prepared from cells grown in DMEM containing 10% dialysed FBS (lanes 1, 3 and 5) or EBSS plus 10% dialysed FBS and without amino acids (lanes 2, 4 and 6). Samples were resolved by SDS-PAGE and analysed by Western blotting with antibodies against RB phosphorylated at serine 795 (upper panel), RB (second panel), cyclin E (third panel), cyclin D1 (fourth panel), p16 (fifth panel), or actin (lower panel). Blots are representative of more than three independent experiments.

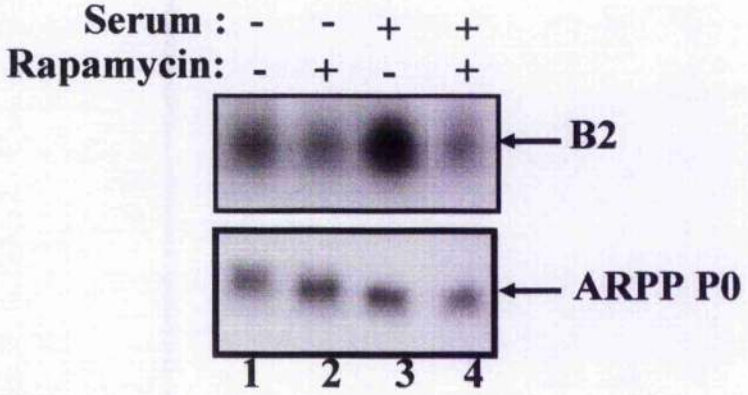
4.2.4 Rapamycin and LY294002 inhibit pol III transcription independent of the effect on proliferation

Although the effects of rapamycin on pol III transcription appear to be independent of an effect on the phosphorylation status of RB, it has previously been shown that this drug causes a G1 arrest (Abraham and Weidcrrecht, 1996; Zhang *et al.*, 2000) and that expression of a rapamycin-resistant mutant of mTOR alleviates the effect of rapamycin on cell cycle progression (Hay and Sonenberg, 2004). We therefore wanted to confirm that the observed rapamycin effect is not due to an indirect consequence of regulation of cell cycle progression. The ability of rapamycin to inhibit pol III transcription was therefore examined in primary cultures of post-mitotic neonatal cardiomyocytes.

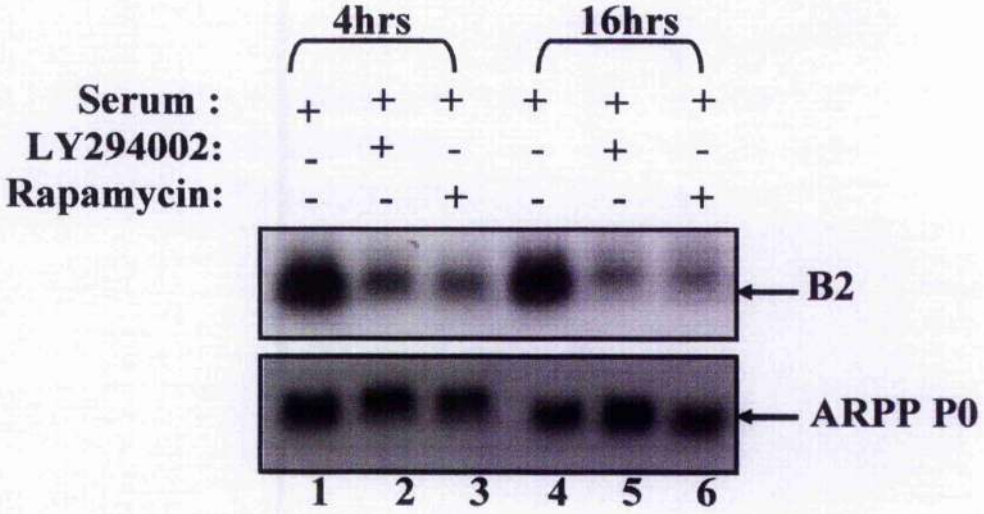
Upon stimulation with Gq/G11 linked agonists such as phenylephrine (PE), endothelin-1 (ET-1) and angiotensin II (Ang II), cardiomyocyte size increases, resulting in increased mass (growth) of the heart (Proud, 2004). Cardiomyocytes do not proliferate or undergo DNA synthesis in response to growth factor stimulation but instead undergo hypertrophic growth (Chien *et al.*, 1991). Increased protein synthesis is a key feature of cardiac hypertrophy and probably underlies the increased cell and organ size observed under this condition (Hannan, 2003). Several signalling pathways have been implicated in cardiac hypertrophy including those involving PI3K (Wang *et al.*, 2001; Wang *et al.*, 2002; Tee *et al.*, 2003a).

Primary cultures of rat neonatal cardiomyocytes were cultured in 10% serum-containing medium (Fig. 4.5A, lanes 3 and 4) or serum-free medium (Fig 4.5A, lanes 1 and 2), for 16hrs. Cells were then incubated in the presence (Fig. 4.5A, lanes 2 and 4) or absence of rapamycin (100nM; Fig. 4.5A, lanes 1 and 3) for 16hrs and RNA was extracted. Northern analysis was performed and shows that levels of the pol III transcript B2 increase upon serum stimulation and this increase can be blocked by rapamycin treatment, whereas

A


Serum : - - + +
Rapamycin: - + - +
B2
ARPP P0
1 2 3 4

B


4hrs
16hrs
Serum : + + + + + +
LY294002: - + - - + -
Rapamycin: - - + - - +
B2
ARPP P0
1 2 3 4 5 6

C

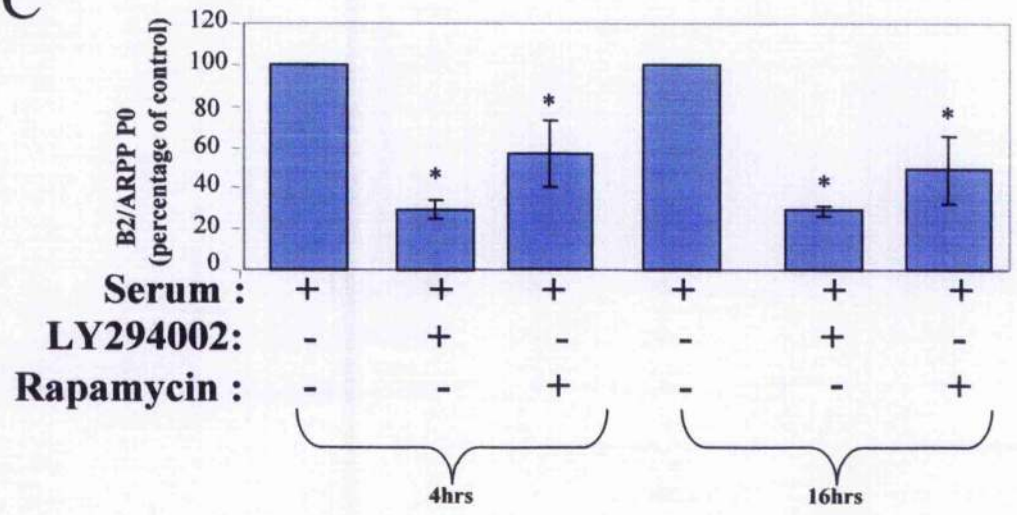

B2/ARPP P0 (percentage of control)
120
100
80
60
40
20
0
Serum : + + + + + +
LY294002: - + - - + -
Rapamycin : - - + - - +
4hrs
16hrs

Figure 4.5: **The effects of the mTOR and PI3K signalling pathways on pol III transcription are independent of cell cycle.**

(A) Primary cultures of rat neonatal cardiomyocytes were grown in 10% FCS (lanes3 and 4) or were serum-starved (lanes 1 and 2) for 16hrs. This was carried out in the presence of either vehicle (lanes 1 and 3), or rapamycin (100nm; lanes 2 and 4). RNA was extracted and Northern analysis was performed using the B2 probe (upper panel), the lower panel shows the same blot that has been stripped and re-probed with the ARPP P0 gene. Experiment (A) was carried out by Fiona Cairns and is representative of more than three independent experiments.

(B) Primary cultures of rat neonatal cardiomyocytes were treated for 16hrs with serum to induce hypertrophic growth. The cells were also incubated with either vehicle (lanes 1and 4), LY294002 (50μM; lanes 2 and 5) or rapamycin (100nM;lanes 3 and 6) for 4 hrs (lanes 1-3) or 16hrs (lanes 4-6). RNA was extracted and Northern analysis was performed using the B2 probe (upper panel), the lower panel shows the same blot that has been stripped and re-probed with ARPP P0.

(C) The B2 signals from vehicle, LY294002 and rapamycin treated cells as shown in (B) were quantified by densitometry and normalised against the ARPP P0 signal. The graph shows means +/- the standard deviations from three independent experiments; values obtained for cells grown in serum for either 4 hrs or 16hrs were set as 100 and other values were calculated as a percentage of this.

* denotes statistically significant difference from control, at $p < 0.05$

rapamycin treatment has only a slight reduction in the levels of B2 when cells are cultured in serum-free medium (Fig. 4.5A). This effect is specific, since levels of the ARPP P0 transcript are unchanged (Fig. 4.5A). To compare the effects of blocking the PI3K and mTOR pathway on pol III transcription during hypertrophy in cardiomyocytes, primary cultures of rat neonatal cardiomyocytes were serum-stimulated for 4hrs or 16hrs, with or without vehicle, rapamycin (100nM) or LY294002 (50μM) and RNA was extracted. Levels of the pol III transcript B2 was reduced by 40% or 50% when exposed to rapamycin for 4hrs and 16hrs, respectively (Fig 4.5B and C). By 4hrs, the drug LY294002 reduces levels of B2 by 70%, compared to untreated myocytes and this 70% reduction is maintained up to 16 hrs of LY294002 exposure (Fig 4.5B and C). Thus, the ability of mTOR to regulate pol III transcription is independent of its effects on cell cycle. This is consistent with the evidence that mTOR-dependent growth of fibroblasts continues despite a blockade of cell cycle progression (Fingar *et al.*, 2002).

4.2.5. Amino acid and rapamycin-mediated effects on pol III transcription are independent of ERK and c-Myc

Another known regulator of pol III transcription in serum-stimulated cells includes the ERK mitogen-activated protein kinase. Some reports have implicated a connection between the MAPK and mTOR signalling pathways (Wang *et al.*, 2001; Wang *et al*, 2002; Tee *et al.*, 2003a). In adult cardiomyocytes the GPCR agonists PE and ET-1 were shown to activate S6K1 and 2 and inactivate 4EBP1. These effects can be blocked by inhibition of the ERK and mTOR pathways, but are independent of PI3K signalling (Wang *et al.*, 2001; Wang *et al.*, 2002). In addition, the phosphorylation and inactivation of TSC2 (an upstream regulator of mTOR) has also been shown to occur via a PI3K-independent mechanism that possibly involves MEK-regulated pathways (Tee *et al.*, 2003a), which provides further support for connections between MEK/ERK and mTOR signalling. Consequently, the

decrease in pol III activity observed during amino acid deprivation or rapamycin treatment is potentially due to inhibition of the ERK pathway. Recent work has shown that ERK in its active forms can interact with and phosphorylate the Brf1 subunit of TFIIIB *in vitro* and *in vivo*, thus activating pol III transcription (Felton-Edkins *et al.*, 2003a). It is possible that treatment of cells with rapamycin or amino acid deprivation could be blocking ERK activation through cross talk of the mTOR and ERK pathways. To this end, the phosphorylation status of the p42 and p44 isoforms of ERK was measured during a rapamycin time course and the effect of amino acid deprivation was also investigated. Protein extracts were made from cells cultured in DMEM containing 10% serum and 100nM rapamycin over a time course. Western analysis shows that there is no effect of rapamycin treatment on ERK phosphorylation for up to 12hrs, and that total levels of ERK remained unchanged (Fig 4.6A, first and second panels). Actin is used as a loading control to show equal protein levels throughout (Fig. 4.6A, lower panel). Similarly, protein extracts made from cells cultured in DMEM containing 10% dialysed serum (Fig. 4.6B, lanes 1, 3 and 5) or EBSS containing 10% dialysed serum (Fig. 4.6, lanes 2, 4 and 6) over a time course. Amino acid deprivation does not affect the abundance or phosphorylation of ERK (Fig. 4.6).

The proto-oncogene c-Myc has also recently been discovered as a regulator of pol III transcription (Felton-Edkins *et al.*, 2003b; Gomez-Roman *et al.*, 2003). c-Myc has the potent capacity to stimulate cell growth (Dang, 1999; Schmidt, 1999). Indeed, in some instances, c-Myc has been found to drive protein synthesis and growth independently of cell cycle progression (Schuhmacher *et al.*, 1999; Kim *et al.*, 2000). It follows that lack of c-Myc results in diminished growth and protein synthesis (Schmidt, 1999; Johnston *et al.*, 1999). In human cells, the translation of c-Myc is cap-dependent and is suppressed by rapamycin (Hosoi *et al.*, 1998; Gera *et al.*, 2003). The mechanism appears to be through a shift of c-Myc RNA from the polysomal fraction to the translationally inactive pool (Gera

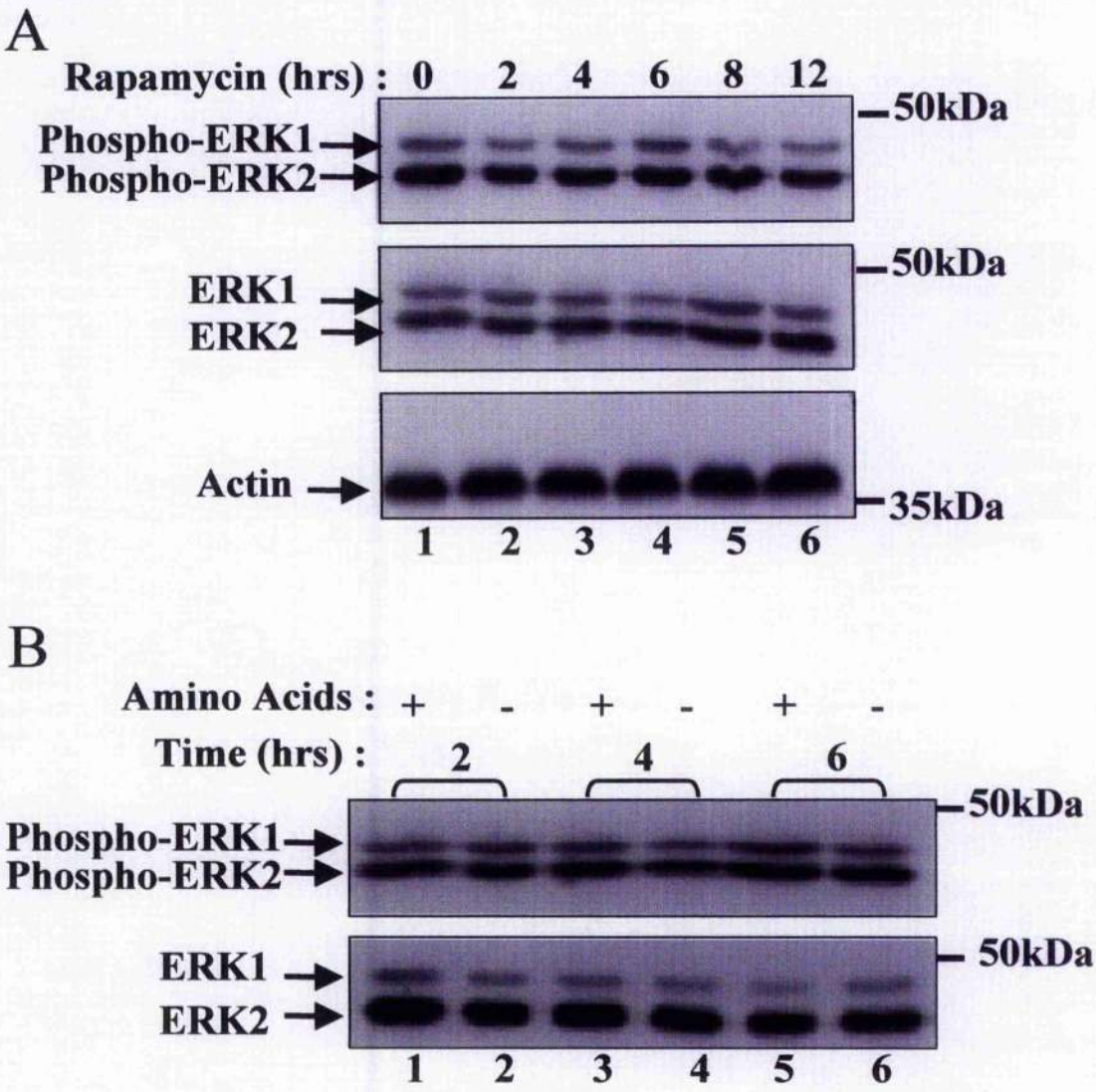


Figure 4.6 : **Effects of rapamycin treatment on pol III activity are independent of ERK signalling**

(A) Protein cell extracts (50μg) were prepared from cells grown in DMEM plus 10% serum for 12hrs (lane 1) or for the various times indicated with rapamycin (100nM). Samples were resolved by SDS-PAGE and analysed by western blotting with antibodies against ERK 42 and 44kDa isoforms (upper panel) or total ERK (middle panel). Actin was used as a loading control (lower panel).Blots are representative of more than three independent experiments.

(B) Protein cell extracts (50 μg) were prepared from cells grown in DMEM and containing 10% dialysed FBS (lanes 1, 3 and 5) or EBSS plus 10% dialysed FBS (lanes 2, 4 and 6). Samples were resolved by SDS-PAGE and analysed by western blotting with antibodies to p42 and p44 isoforms of ERK (upper panel), or total ERK (second panel). Blots are representative of more than three independent experiments.

et al., 2003). Since withdrawal of amino acids mimics rapamycin treatment, both rapamycin exposure and amino acid deprivation could potentially cause a decrease in levels of c-Myc.

Extracts of cells grown in the presence of rapamycin (100nM) were prepared for Western analysis. Protein levels of c-Myc are substantially decreased after 6hrs exposure to rapamycin (Fig. 4.7A, upper panel). The effect is specific, since levels of actin are constant (Fig. 4.7, lower panel). Cells extracts grown in DMEM containing 10% dialysed serum (Fig. 4.7B, lanes 1, 3, 5 and 7) or EBSS containing 10% dialysed serum (Fig. 4.7B, lanes 2, 4, 6 and 8) were harvested for protein. Subsequent immunoblotting shows levels of c-Myc decrease by 8 hrs in the absence of amino acids (Fig 4.7B upper panel). Thus, the effects of rapamycin on pol III transcription cannot be explained through changes in the abundance of c-Myc or phosphorylation of ERK.

4.2.6. Phosphorylation of TFIIIB and TFIIIC is due in part to the mTOR signalling pathway

Previous results have shown that inhibition of the mTOR pathway does not result in changes in the abundance of the pol III transcription factors TFIIIB or TFIIIC (Fig. 3.9). However, changes in the activity to one or several of these components could have occurred through a post-translational modification, such as phosphorylation. In fact, it has already been shown that TFIIIB and TFIIIC require phosphorylation to be activated (Hoeffler *et al.*, 1998, Felton–Edkins *et al.*, 2003a). In addition, mutating an ERK phosphorylation site on Brf1 reduces serum-stimulated pol III transcription, indicating that phosphorylation of this subunit is required for maximal activity (Felton-Edkins *et al.*, 2003a). Therefore, we investigated whether the phosphorylation status of TFIIIB and TFIIIC changes in response to rapamycin treatment.

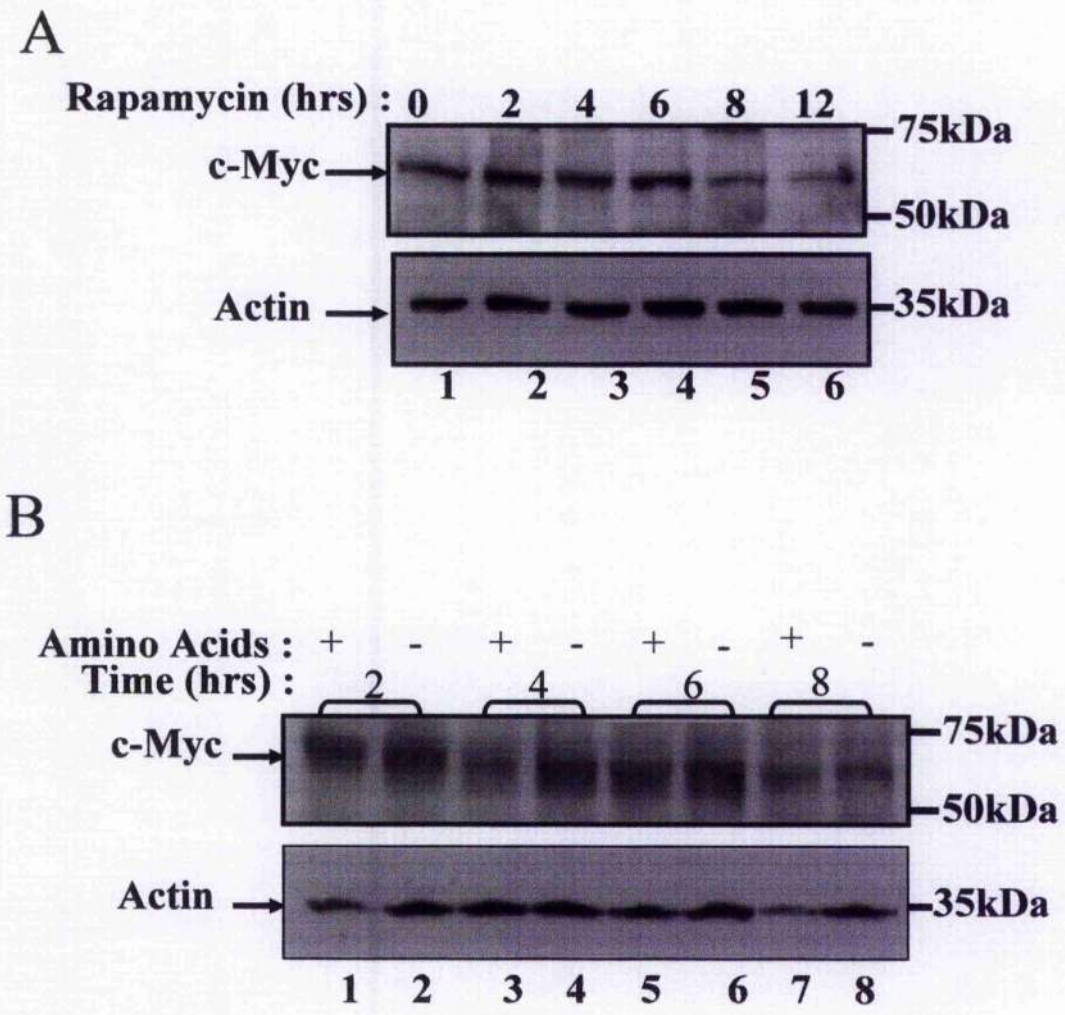


Figure 4.7 : **Effects of amino acid deprivation on pol III transcription are independent of c-Myc**

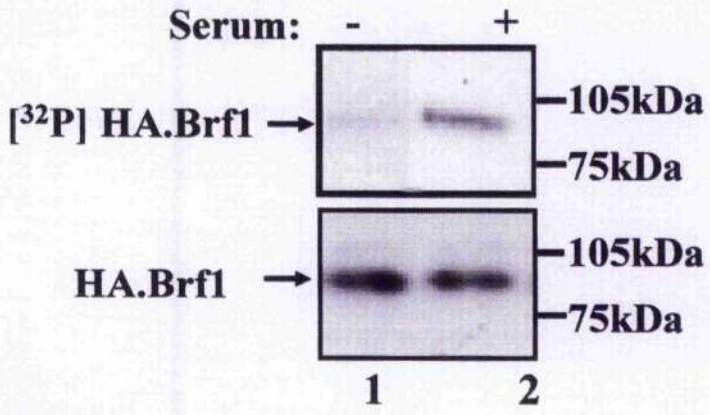
(A) Protein cell extracts (50μg) were prepared from cells grown in DMEM containing 10% FBS, for 12hrs (lane 1) or for the various times indicated with rapamycin (100nM; lanes 2-6). Samples were resolved by SDS-PAGE and analysed by Western blotting with antibodies against c-Myc (upper panel) or actin (lower panel) as a loading control. Blots are representative of at least three independent experiments.

(B) Protein cell extracts (50 μg) were prepared from cells grown in DMEM and 10% dialysed FBS (lanes 1, 3 and 5) or EBSS plus dialysed FBS (lanes 2, 4 and 6) were resolved by SDS-PAGE and analysed by Western blotting with antibodies for c-Myc (upper panel) or actin (lower panel). Blots are representative of at least three independent experiments.

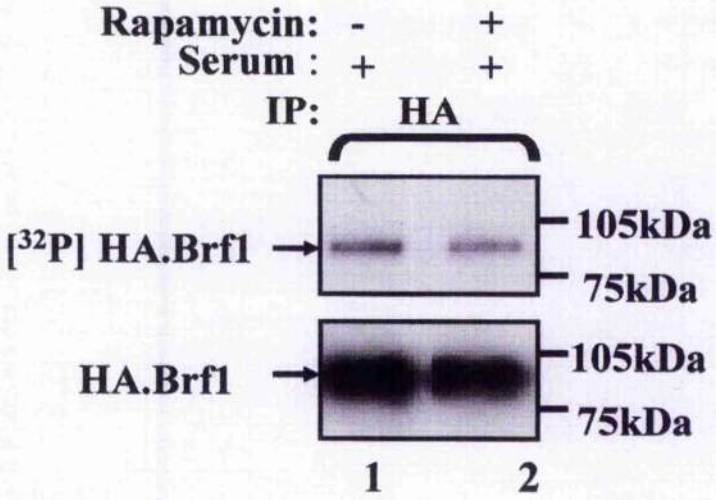
CCL39 fibroblasts were transfected for 48hrs with pCDNA3HA.Brf1 and transferred to phosphate-free DMEM with 250μCi/ml [^{32}P] orthophosphate for 3hrs. This was carried out in asynchronously growing cells or those that were starved of serum for the final 16hrs of transfection. Extracts were immunoprecipitated using an HA antibody, run on SDS-PAGE gels and either subjected to autoradiogaphy or immunoblotted for HA. Phosphorylation of Brf1 increased significantly in the presence of serum (Fig. 4.8A, upper panel); this effect was not due to decreased levels of Brf1 in the serum-free media (Fig. 4.8A, lower panel). To establish whether the mTOR pathway blocks the phosphorylation of Brf1 growing cells were labelled with [^{32}P] orthophosphate, similar to the previous experiment but this time in the presence or absence of rapamycin. Rapamycin was found to decrease the phosphorylation of Brf1 by 30% (Fig. 4.8B and C). This effect is not due to changes in the abundance of the protein since overall levels of Brf1 were not affected by rapamycin treatment (Fig. 4.8B, lower panel).

To investigate whether the TFIIIC subunit TFIIIC110 was phosphorylated in response to serum, asynchronously growing cells were transferred to phosphate-free media, and incubated for 3hrs with [^{32}P] orthophosphate in the presence or absence of serum. Extracts were immunoprecipitated with an antibody for TFIIIC110, and after SDS-PAGE analysis either exposed to film or immunoblotted for TFIIIC110. Phosphorylation of TFIIIC110 increased significantly in the presence of serum (Fig. 4.9A, upper panel) whilst immunoprecipitated levels of TFIIIC110 were only slightly decreased in the serum-free media (Fig. 4.9A, lower panel). To test whether TFIIIC110 phosphorylation was sensitive to rapamycin treatment, cells growing in a medium containing 10% serum were transfected with the construct pCDNA3HA.TFIIIC110. Cells were transferred to phosphate-free media 48hrs later, and incubated for 3hrs with [^{32}P] orthophosphate in the presence or absence of rapamycin (100nM). Extracts were immunoprecipitated with HA antibody, run on SDS-PAGE gels and either subject to autoradiogaphy or immunoblotted for HA.

A


Serum: - +
[32P] HA.Brf1
105kDa
75kDa
HA.Brf1
105kDa
75kDa
1 2

B


Rapamycin: - +
Serum : + +
IP: HA
[32P] HA.Brf1
105kDa
75kDa
HA.Brf1
105kDa
75kDa
1 2

C

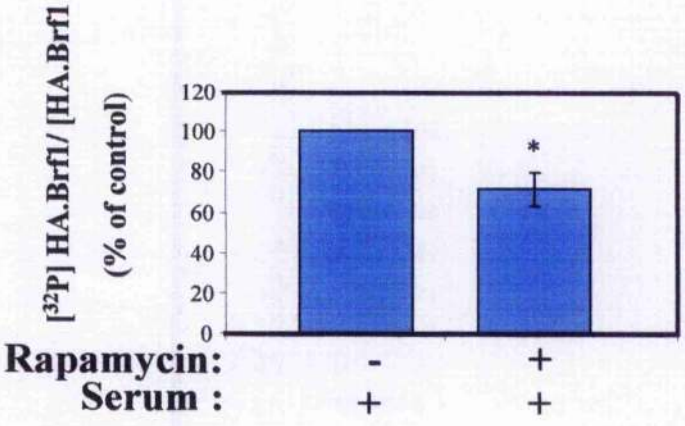

[32P] HA.Brf1/ [HA.Brf1
(% of control)
120
100
80
60
40
20
0
*
Rapamycin: - +
Serum : + +

Figure 4.8: **Phosphorylation of Brf1 is inhibited by blocking the mTOR pathway**

(A) CCL39 cells transiently transfected with pCDNA3HA.Brf1for 48hrs, were labelled with [^{32}P] orthophosphate for 3 hrs. Cells in lane 1 were transferred to serum-free medium for 24 hrs prior to labelling. Cell extracts were prepared and Brf1 was immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE, and either visualised by autoradiography (upper panel), or transferred to PVDF membrane followed by Western blotting with HA antibody (lower panel). Results are representative of more than three independent experiments.

(B) CCL39 cells growing in 10% serum were transiently transfected with pCDNA3-HA.Brf1 (10μg) and labelled 48 hrs later with [^{32}P] orthophosphate for 3 hrs in the absence (lane 1) or presence (lane 2) of rapamycin (100nM). Cell extracts were prepared and Brf1 was immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE, and either visualised by autoradiography (upper panel) or transferred to PVDF membrane followed by western blotting with anti-HA (lower panel).

(C) The phospholabelling result described in (B) was quantified by densitometry and values normalised to the immunoprecipitation. The graph shows the mean +/- standard deviations from three independent experiments, where the untreated sample is 100% and the rapamycin treatment is calculated as a percentage of the control.

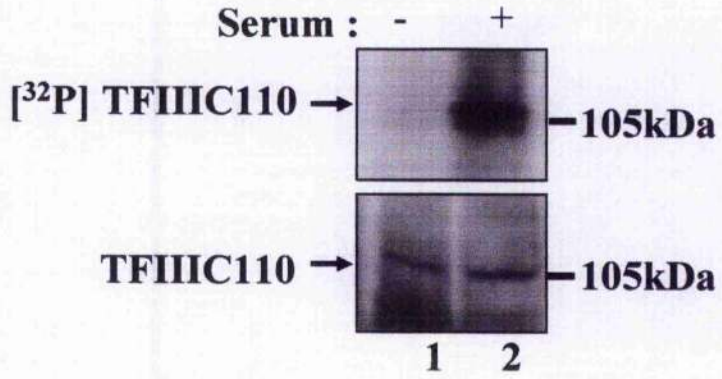
* denotes statistically significant difference from control, at $p < 0.05$

Phosphorylation of TFIIIC110 significantly decreased during 3hrs rapamycin treatment (Fig. 4.9B, upper panel), whilst levels of immunoprecipitated TFIIIC110 did not alter (Fig. 4.9B, lower panel). This decrease was quantified as a 75% reduction in phosphorylation of TFIIIC110 (Fig. 4.9C). The findings that both Brf1 and the TFIIIC110 subunit of TFIIIC were phosphorylated in response to serum, supports previous work in our laboratory (P. Scott, unpublished).

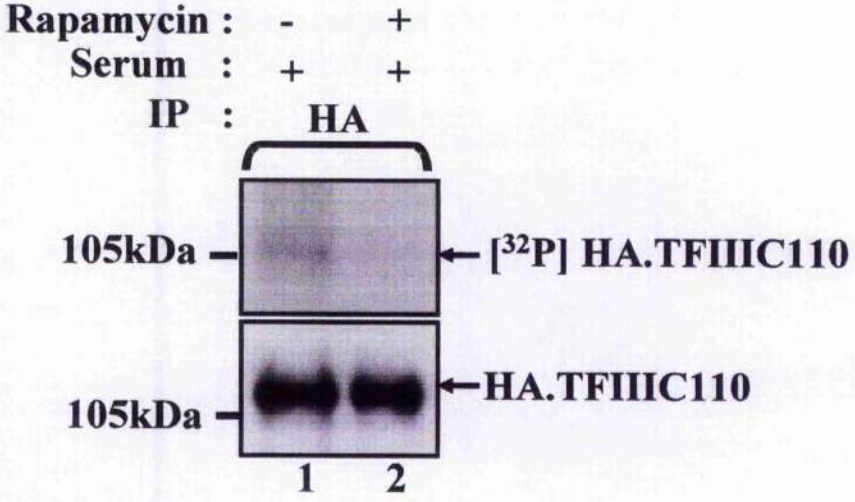
4.2.7. Regulation of pol III transcription by mTOR is due in part to signalling through S6K1

The implication that the mTOR pathway was involved in controlling the phosphorylation status of TFIIIB and/or TFIIIC led to the search for an upstream kinase that could mediate this. Recent evidence has reported that the pol I transcription factor UBF is regulated by an mTOR pathway (Hannan *et al.*, 2003). They showed that mTOR, via S6K1, can activate rDNA transcription through phosphorylation of UBF. With this in mind, the regulation of pol III transcription by mTOR, via S6K1, was examined via primer extension. $pCDNA_3$ or a constitutively active, rapamycin-insensitive mutant of S6K1 (S6KED3E) were transfected into cells for 48hrs along with a VA1 pol III reporter construct and GFP as a control. Cells were either left growing in serum or were transferred to serum-free medium for the final 16hrs of transfection. Vehicle or rapamycin were then added for a further 4hrs in the presence and absence of serum. In the $pCDNA_3$ transfected cells, VA1 transcription is stimulated upon addition of serum (Fig. 4.10, compare lanes 1 and 3), and this effect can be blocked by incubating the cells with rapamycin (Fig. 4.10, lane 4). Levels of the pol II reporter GFP remained relatively stable in each condition (Fig. 4.10, lower panel). In serum-starved S6KED3E-transfected cells, higher levels of VA1 transcription by pol III were shown (Fig. 4.10, compare lanes 1 and 5). However, there was a further increase in pol III activity when S6KED3E-transfected cells were growing in serum (Fig. 4.10, lanes 5

A


Serum : - +
[32P] TFIIIC110
105kDa
TFIIIC110
105kDa
1 2

B


Rapamycin : - +
Serum : + +
IP : HA
105kDa
[32P] HA.TFIIIC110
105kDa
HA.TFIIIC110
1 2

C

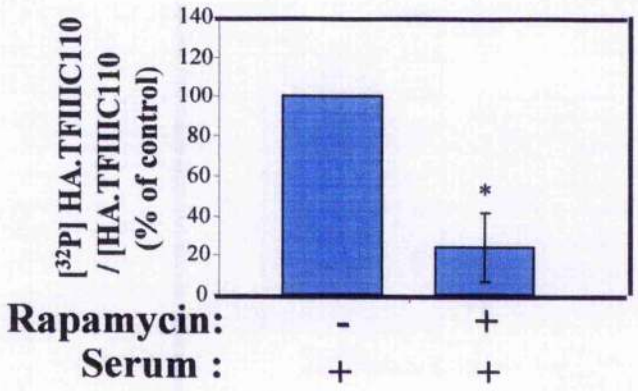

[32P] HA.TFIIIC110 / [HA.TFIIIC110 (% of control)
140
120
100
80
60
40
20
0
*
Rapamycin: - +
Serum : + +

Figure 4.9: **Phosphorylation of TFIIIC110 is inhibited by blocking the mTOR pathway**

(A) CCL39 cells were labelled with [^{32}P] orthophosphate for 3 hrs. Cells in lane 1 were transferred to serum-free medium for 24 hrs prior to labelling. Cell extracts were prepared and TFIIIC110 was immunoprecipitated with an anti-TFIIIC110 antibody, resolved by SDS-PAGE, and either visualised by autoradiography (upper panel), or transferred to PVDF membrane followed by Western blotting with anti-TFIIIC110 (lower panel). Results are representative of more than three independent experiments.

(B) CCL39 cells growing in 10% serum were transiently transfected with pCDNA3-HA.TFIIIC110 (10μg) and labelled 48 hrs later with [^{32}P] orthophosphate for 3 hrs in the absence (lane 1) or presence (lane 2) of rapamycin (100nM). Cell extracts were prepared and TFIIIC110 was immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE, and either visualised by autoradiography (upper panel) or transferred to PVDF membrane followed by western blotting with anti-HA (lower panel).

(C) The phospholabelling result described in (B) was quantified by densitometry and values normalised to the immunoprecipitation. The graph shows the mean +/- standard deviations from three independent experiments, where the untreated sample is 100% and the rapamycin treatment is calculated as a percentage of the control.

* denotes statistically significant difference from control, at $p < 0.05$

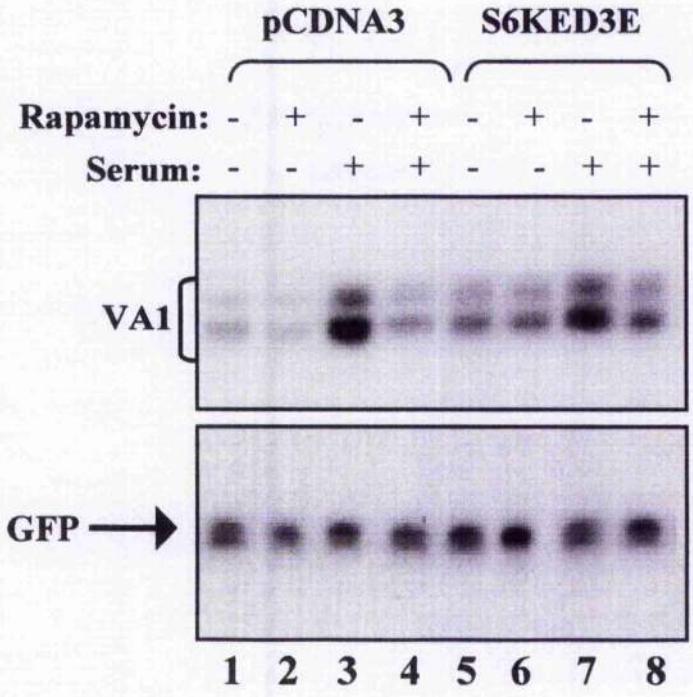


Figure 4.10 : **Rapamycin inhibition of pol III activity may be partially mediated by S6K1 activity**

Asynchronously growing A31 fibroblasts were transfected for 48hrs with pVA1 (0.25μg; all lanes), pGFP (0.25μg; all lanes), pCDNA3 (1.5μg; lanes 1- 4), pCDNA3-S6KED3E (1.5μg; lanes 5-8). During the final 16hrs of transfection, the cells were either maintained in the same medium (lanes 3, 4, 7 and 8), or in a medium containing 0.5% serum (lanes 1, 2, 5 and 6). For the final 4hrs either vehicle (lanes 1, 3, 5 and 7) or rapamycin (100nm; lanes 2, 4, 6 and 8) was added. VA1 (upper panel) and GFP levels (lower panel) were assayed by primer extension are shown. Results are representative of at least two independent experiments.

and 7). This effect is only reduced to basal levels in the presence of rapamycin (Fig. 4.10, compare lanes 6, 7 and 8). These results suggest that regulation of pol III transcription by mTOR is due in part to signalling through S6K1.

4.3 Discussion

Pol III transcription is reduced when the mTOR signalling pathway is blocked. We have shown that transcription initiation complex assembly on pol III promoters is also reduced. This appears to be regulated at the level of both TFIIIB and TFIIIC recruitment, since both transcription factors are prevented from binding in the presence of the mTOR inhibitor rapamycin. Blocking mTOR activity also prevents TFIIIB binding to both pol III and to TFIIIC using co-immunoprecipitation studies. Since more is known about how TFIIIB is regulated in cells, the activities of known TFIIIB regulators were investigated in the current Chapter. This demonstrated that the effect of rapamycin on pol III transcription occurs prior to observing changes, if any, on the activation of ERK and c-Myc, and the inactivation of the TFIIIB repressor RB. Although the precise mechanism of how mTOR links to these transcription factors was not identified, phosphorylation of both TFIIIB and TFIIIC was reduced when this pathway is blocked, providing the basis for future work considerations.

In order to investigate the role of the mTOR and PI3K pathways in controlling transcription by pol III, we explored the possibility that mTOR and/or PI3K downstream effectors were involved in the regulation of binding of pol III and its transcription factors to the promoters of pol III-transcribed genes. ChIP analysis showed that binding of TFIIIC, TFIIIB and pol III to $tRNA^{Leu}$ genes (type II promoter) is inhibited by rapamycin and LY294002 treatment (Fig. 4.1B). These results suggest that both transcription factors could be targets of the mTOR and PI3K pathways. ChIP analysis also determined that binding of

TFIIIC, TFIIIB and pol III to 5S rRNA genes is inhibited by blocking the mTOR or PI3K pathway (Fig. 4.1A). The productive recruitment of TFIIIC to 5S rRNA promoters requires the presence of the gene-specific factor TFIIIA. TFIIIA serves as an adaptor, providing a platform that allows recruitment of TFIIIC onto 5S rRNA genes for which it has little affinity (White, 1996). Therefore, TFIIIA could be also be a target for the mTOR and PI3K pathways in this promoter type.

In other systems, a strong interaction between TFIIIC and tRNA genes was identified during nutrient deprivation (Roberts *et al.*, 2003). Although mTOR was not implicated in this paper, the fact that mTOR activity is also down-regulated upon nutrient starvation (Hara *et al.*, 1998; Kim *et al.*, 2002) suggests a possible involvement of mTOR under such conditions in yeast when pol III is inactivated. In this Chapter, binding of TFIIIC to tRNA genes is blocked when mTOR activity is inhibited, suggesting that the regulation of TFIIIC binding to promoters is different in the two systems.

As described previously, transcription by pol III requires multiple general initiation factors that assemble on the promoter in an ordered fashion; recruitment of pol III to its promoters has been found to be primarily by interactions with TFIIIB (Schramm & Hernandez, 2002), however, TFIIIC attachment to the promoter region must precede this. Therefore, one explanation of the ChIP data is that by blocking the mTOR or PI3K pathways, modification of TFIIIC and/or TFIIIB can no longer occur. Two outcomes are then possible; the interaction of TFIIIC with the promoter is prevented, and/or the interaction between TFIIIC and TFIIIB is blocked. The bandshift data supports the idea that the DNA binding activity of TFIIIC does not decrease after LY294002 or rapamycin-treatment; in fact, 2-fold and 3-fold increases, respectively, are found (Fig. 4.1E). In contrast, the ChIP data shows TFIIIC is released from tRNA promoters. This can be explained by findings reported in Lasser *et al.* (1983), which show that although TFIIIC binds tightly on a tRNA

gene, the stability of the DNA binding is dependent on the presence of TFIIIB, which has the ability to transform an otherwise unstable complex into a stable one (Lasser *et al.*, 1983). Thus, it is postulated that the inability of TFIIIB to occupy tRNA promoters after rapamycin and LY294002-treatment prevents the stabilisation of TFIIIC and leads to the release of TFIIIC, despite the fact that TFIIIC can bind the DNA.

The interactions of the pol III transcription factors were also investigated in response to rapamycin treatment. The association of TFIIIB with TFIIIC was ablated in the presence of rapamycin (Fig. 4.2), which suggests that the mTOR pathway controls this association. Other signalling pathways have been found to regulate the interaction between TFIIIB and TFIIIC, including the Wnt (Johnston *et al.*, 2002) and MAPK signalling pathways (Felton-Edkins *et al.*, 2003a). Phosphorylation and activation of TFIIIB by CK2 appears to be necessary for the interaction between TFIIIB and TFIIIC both *in vitro* and *in vivo* (Johnston *et al.*, 2002). More recent work demonstrated that the interaction of TFIIIB with TFIIIC is sensitive to ERK activity. Moreover, promoter occupancy by Brf1 and pol III at endogenous tRNA and 5S rRNA genes decreases when cells are treated with the MEK inhibitor PD98059 (Felton-Edkins *et al.*, 2003a). In fact, both CK2 and ERK appear to play a role in the regulation of TFIIIB phosphorylation in serum-stimulated cells. Previous work has shown that TFIIIB and TFIIIC are phosphorylated in response to serum stimulated fibroblasts (Felton-Edkins *et al.*, 2003a; Scott *et al.*, unpublished); this finding was verified in the phospholabelling experiments seen in Fig. 4.8A and 4.9A. Interestingly, phosphorylation of both the Brf1 component of TFIIIB and the TFIIIC110 subunit of TFIIIC are reduced by 30% and 75%, respectively, after exposure of cells to rapamycin for 4 hrs (Fig. 4.8B and 4.9B). Hence, the mTOR signalling pathway may also contribute to the phosphorylation status of these transcription factors.

The mTOR pathway has also been found to link to other transcription factors. Recent studies in the pol I system have demonstrated that when the mTOR pathway is blocked, phosphorylation on certain sites of TIF1A can be enhanced, whilst others are reduced (Mayer *et al.*, 2004). This reduction in TIF1A phosphorylation may be due to blocking the downstream kinase activity of the mTOR pathway via rapamycin treatment. However, enhancement of phosphorylation can be explained by the fact that mTOR controls the activity of PP2A, a phosphatase which is known to be inactivated by mTOR signalling (Peterson *et al.*, 1999). This finding was substantiated in the pol I system, where PP2A was found to regulate TIF1A in an mTOR-dependent manner (Mayer *et al.*, 2004). Upon rapamycin treatment, PP2A dephosphorylates and inactivates TIF1A *in vivo*, whereas inhibition of PP2A with calyculin A protects TIF1A from inactivation by rapamycin. Intriguingly, the same study showed that localisation of TIF1A altered in response to rapamycin treatment; blocking mTOR signalling caused a translocation of TIF1A from the nucleus to the cytoplasm (Mayer *et al.*, 2004). It remains possible that TFIIIB and TFIIIC, in addition to phosphorylation and activation by the mTOR pathway, undergo nucleo-cytoplasmic transport which is regulated reversibly by phosphorylation. Indeed, regulation of subcellular localisation of transcription factors has already been reported in response to TOR (Beck and Hall, 1999).

As well as contributing to the phosphorylation of TFIIIB and TFIIIC, the mTOR signalling pathway may also affect known regulators of pol III transcription such as ERK, c-Myc and RB. Hence, the observed effect of blocking mTOR could be explained by a change in activity of one of these proteins. In particular, analysis of the 5'UTR of the human cyclin D1 gene has revealed a tract of oligopyrimidines (TOP) proximal to the cap structure (Herber *et al.*, 1994). The mTOR pathway increases the translation of mRNAs containing such a tract and therefore cyclin D1 is considered rapamycin-sensitive (Jefferies *et al.*, 1994; Terada *et al.*, 1994). The result of rapamycin treatment is therefore decreased

translation of cyclin D1, resulting in decreased phosphorylation of RB (Hashemolhossein *et al.*, 1998). In the present study, rapamycin treatment of mammalian cells blocked the hyper-phosphorylation of RB; however, this inhibition only occurred after 12 hrs of rapamycin treatment (Fig. 4.3). The block to RB hyper-phosphorylation by rapamycin may be due to decreased levels of cyclin D1, as has been previously reported (Jefferies *et al.*, 1994; Terada *et al.*, 1994), and could account for the reduction of pol III activity observed at the 12hr time point. There is however, a decrease in pol III activity that occurs prior to this when this signalling cascade is blocked (Fig. 3.5 and 3.6). These data suggest that effects on pol III activity via mTOR signalling are independent of RB.

Since mTOR is regarded as a sensor of amino acids, we also looked at the effect that amino acid deprivation has on the phosphorylation status of RB. RB phosphorylation is not blocked in cells which are cultured in amino acid-free medium for up to 6 hrs (Fig. 4.4). However, levels of cyclin D1 were inhibited after 4hrs amino acid deprivation (Fig. 4.4). The effect of mTOR on cell cycle progression is mediated at least in part, by increased translation of positive regulators of the cell cycle, such as cyclin D1 (Gera *et al.*, 2004). Moreover, pulse-chase experiments have determined that rapamycin treatment decreases the half-life of cyclin D1 (Hashemolhossein *et al.*, 1998), which may explain the decrease in cyclin D1 levels. Since the abundance of p16, a specific inhibitor of cyclin D1, is not altered by rapamycin treatment, the amino acid sensing pathway via mTOR may control the stability of cyclin D1. These data suggest that the reduction in pol III transcription in the absence of amino acids is independent of RB at early time points.

Since activation of the ERK signalling pathway is known to regulate pol III transcription (Felton-Edkins *et al.*, 2003a), it remained possible that mTOR and ERK pathways were involved in cross-talk, as has been previously reported (Wang *et al.*, 2001; Wang *et al.*, 2002; Tee *et al.*, 2003a), and that rapamycin was blocking ERK activation. However,

phospho-ERK was not affected by rapamycin treatment in these cells (Fig. 4.6A), indicating that the inhibitory effects of rapamycin on pol III transcription were mediated by another mechanism. Similarly, amino acid deprivation over a time course of 6hrs did not affect phosphorylation of ERK (Fig 4.6B), indicating that the amino acid sensitivity of pol III transcription is independent of ERK signalling.

c-Myc has recently been reported as a regulator of pol III transcription (Gomez *et al.*, 2003). TFIIIB was reported as the target of c-Myc, and this appears to be through protein-protein interactions, rather than through direct DNA recognition by c-Myc. Since rapamycin has previously been reported to suppress the translation of c-Myc (Hosoi *et al.*, 1998; West *et al.*, 1998; Gera *et al.*, 2004), this inhibition of pol III activity by rapamycin or by amino acid starvation could be via the degradation or down regulation of c-Myc. mTOR signalling via 4E-BP1 was found to relieve the translational repression imposed on *c-myc* mRNA by its structured 5'untranslated region (UTR). Therefore, rapamycin treatment is expected to block the phosphorylation of 4E-BP1 and abolish the translational component of the c-Myc response. This could account for the decrease in levels of c-Myc observed between 6- 8hrs of rapamycin exposure (Fig. 4.7A). Similarly, amino acid starvation is sensed by mTOR and results in the dephosphorylation of 4E-BP1 and thus a translational repression of c-Myc mRNA (Proud, 2002). The results indicate that levels of c-Myc are down regulated between 6 and 8hrs (Fig. 4.7 B). Whilst the decrease of c-Myc observed in both the rapamycin and amino acid starvation time courses could account for the reduction of pol III activity observed at this time, there is a decrease in pol III activity that occurs within 4 hrs of rapamycin treatment (Fig. 3.5 and 3.6). These data suggest that inhibition of pol III transcription by rapamycin and amino acid starvation are also independent of c-Myc at early time points.

It is shown that expressing a constitutively active form of S6K (S6KED3E) can increase pol III transcription (Fig. 4.10), therefore, the effector of the mTOR pathway that regulates pol III may indeed be S6K. This is particularly interesting since the pol I factor UBF was proposed to be regulated, at least in part, by S6K1 (Hannan *et al.*, 2003). Therefore, S6K1 regulation of pol III transcription was investigated by studying the effect of S6KED3E mutant on pol III activity in serum-starved cells. In this way, this constitutively active construct could be considered in the absence of other cell signalling pathways. It was found that expression of active S6K1 is sufficient to induce VA1 transcription by pol III. Thus, the ability of mTOR to regulate pol III transcription is mediated, at least in part, through S6K1.

To examine whether the effects of mTOR on pol III transcription might be the indirect consequence of regulation of cell cycle progression, the ability of rapamycin to block pol III activity was tested in primary cultures of neonatal cardiomyoctes. Since these cells are non-dividing but can undergo hypertrophic growth then the effect of rapamycin on pol III transcription can be tested independently of effects on the cell cycle. Rapamycin treatment for either 4 hrs or 16hrs reduces pol III transcription by around 40% (Fig. 4.5B). A role for signalling through mTOR in cardiac hypertrophy has already been identified, as rapamycin blocks the increases in cardiomyocyte size induced by agonists such as PE and Ang II (Sadoshima and Izumo, 1995). The findings reported here imply that the aforementioned effect of rapamycin could be occurring through the inhibition of pol III transcription. Moreover, this evidence corroborates the findings that rapamycin inhibition of pol III transcription occurs independently of cell cycle effects (Fingar *et al.*, 2002). Strong inhibition of pol III that occurs upon exposure of cardiomyocytes to the PI3K inhibitor LY294002 may be explained by the fact that this kinase lies upstream of a number of signalling pathways including the Raf/ERK/MAP kinase pathway. Hence, the effect of this drug on pol III transcription may not be due solely to blocking mTOR. It is known that the

hypertrophic response caused by agonists such as PE, ET-1 and Ang II can increase the proportion of Ras in its active GTP-liganded form (Sugden and Clerk, 2000), which in turn can activate signalling through both the Raf/ERK/MAP kinase and PI3K pathways. Therefore, both mTOR and ERK pathways may play an important role in the regulation of pol III transcription during cardiomyocyte hypertrophy.

In summary, the first two results Chapters have shown that pol III transcription is under the control of the PI3K and mTOR pathways; levels of pol III transcripts were reduced *in vivo* and pol III activity was reduced *in vitro* in response to inhibition of the pathways. Conversely, increased signalling through the PI3K and mTOR pathways was found to increase pol III transcription. Investigation into the possible mechanisms by which the mTOR pathway can achieve this control showed that the mTOR pathway regulates phosphorylation of TFIIIB and TFIIIC. Additionally, when the mTOR pathway is blocked by rapamycin, the interactions between TFIIIB and TFIIIIC, and TFIIIB and pol III are ablated. This is supported by the finding that mTOR activity is required for normal promoter occupancy at pol III promoters. It is shown that inhibition of pol III transcription by rapamycin is independent of effects on the abundance of the pol III transcription factors TFIIIB and TFIIIC or indeed of pol III itself. Additionally, rapamycin-mediated inhibition is independent of known regulators of pol III, that is, c-Myc, RB or ERK signalling. However, the inhibitory effect of rapamycin on pol III transcription is due in part to signalling through the translational effector S6K1.

It can be concluded from the experimental data presented in these Chapters that like the pol I system, the control of pol III transcription is under the regulation of the mTOR signalling pathway in mammalian cells. The importance of this is highlighted, when we consider that the majority of transformed and tumour cell types display abnormally elevated levels of the

pol III products (Brown *et al.*, 2000) and that components of the mTOR signalling pathway have been identified as targets for oncogenic activation (Shamji *et al.*, 2003).

Chapter 5

Involvement of Maf1 in the Regulation of Mammalian Pol III Transcription

5.1 Introduction

5.1.1 Discovery of Maf1; a regulator of pol III transcription

Transcription by pol III is tightly coupled with cell growth; increasing in response to serum and decreasing when nutrients or serum factors are limiting (Brown *et al.*, 2000). It is known that key aspects of this regulation involve RB, c-Myc and signalling through the ERK pathway. In addition, pol III transcription is regulated by a wide variety of conditions that perturb cellular processes or the environment of the cell (Zaragoza *et al.*, 1998; Powers and Walter, 1999; Ghavidel and Schultz, 2001; Li *et al.*, 2000), such as drug treatment, DNA damage and secretory pathway defects. These are sensed by different signalling pathways that converge on the transcription machinery to affect regulation (Upadhya *et al.*, 2002). The understanding of these signalling pathways, their mechanisms of transcriptional regulation and the ultimate targets of the transcription machinery were largely unknown until recently. Studies in yeast identified a structurally novel, and phylogenetically conserved protein, Maf1 (Pluta *et al.*, 2001), as an essential and specific mediator of pol III regulation under a variety of conditions (Upadya *et al.*, 2002). Maf1 was isolated and characterised in yeast based on the ability of maf1-1 mutants to decrease the efficiency of SUP11 ($tRNA^{Tyr}$) suppression and cause temperature-sensitive respiratory growth (Boguta *et al.*, 1997). Genetic methods were employed to identify trans-acting elements involved in tRNA biosynthesis which could convert a tRNA gene into a nonsense repressor. Phenotypic loss of suppression was then used to identify genes coding for the elements essential for tRNA biosynthetic activities. RPC160 genes with 3' deletions in their open reading frames were found to suppress the maf1-1 phenotypes when overexpressed, which identified Maf1 as playing an important role in the regulation of tRNA biosynthetic capacity (Boguta *et al.*, 1997).

5.1.2 Maf1 is an essential mediator of diverse signals that repress transcription by pol III

Maf1 is a novel hydrophilic protein of around 395 amino acids (Boguta *et al.*, 1997), which is conserved from yeast to humans, sharing around 30% identity at the amino acid level (Willis *et al.*, 2004). It is a non-essential, nuclear protein in yeast, where it was found to interact with pol III and act as a negative effector of pol III transcription (Pluta *et al.*, 2001). *In vivo* studies have shown that maf1-1 mutant phenotypes result in elevated levels of tRNAs, and that the rate of pol III transcription *in vitro* is significantly increased in maf1-1 mutant cells (Pluta *et al.*, 2001). Subsequent studies in yeast found that secretion-defective mutants cause transcriptional repression of the ribosome and result in repression of tRNA synthesis (Upadyha *et al.*, 2002). Importantly, deletion of Maf1 was found to block the repression of pol III transcription in secretion-defective cells (Upadyha *et al.*, 2002), indicating a requirement for Maf1 to mediate the pol III response.

In agreement with these studies, Maf1 was found to be an essential mediator of repression of transcription by pol III in response to a diverse range of cellular conditions. This repression was demonstrated in cells following DNA damage, after growth to stationary phase and by treatments with rapamycin or the anti-fungal compound chlorpromazine (CPZ) (Upadhya *et al.*, 2002; Willis *et al.*, 2004; Desai *et al.*, 2004). This suggests that distinct pathways involving secretory signalling, TOR kinase and DNA damage response pathways are involved in regulating Maf1. However, other conditions have since been reported to require Maf1 for the repression of pol III and include oxidative stress, endoplasmic reticulum (ER) stress and carbon source starvation (Desai *et al.*, 2004). Taken together, these data suggest a convergence of signals that mediate transcriptional repression of ribosome synthesis and tRNA synthesis. Since many of these treatments are not pol III-specific, it is thought that the repression is achieved via activation of distinct signalling pathways that converge at or above Maf1. The idea of a common mediator of

stress lying upstream of Maf1 is intriguing; however, the identity of any such protein is unknown.

5.1.3 Targets of Maf1 in yeast

After the discovery that diverse conditions require Maf1 in order to down-regulate pol III transcription, attempts began to elucidate the mechanism by which this occurred. Upadhya *et al.* (2002) established an *in vitro* system to study Maf1-dependent repression of pol III transcription, and chose to focus on repression by CPZ. Whole cell extracts prepared after CPZ treatment of logarithmically-growing cells were found to have 15-fold less pol III activity compared to untreated extract (Upadhya *et al.*, 2002). To examine the possibility that CPZ-induced signalling could target a component of the pol III transcription machinery, transcription reactions were supplemented with partially purified yeast TFIIIB, TFIIIC or pol III. No effect of adding TFIIIC or pol III was seen in either control or CPZ-treated extract, whereas adding TFIIIB to CPZ-treated extract restored transcription to similar levels found in the control extract (Upadhya *et al.*, 2002). These findings were the first to implicate TFIIIB as the target of transcriptional repression by CPZ. A mechanism for this repression was later discovered, when CPZ was found to inhibit pol III transcription *in vitro* by affecting TFIIIB complex assembly (Desai *et al.*, 2004). The Brf1 subunit of TFIIIB was proposed as a likely target of repression in yeast, as the specific activity of Brf1 decreases 3-fold in the TFIIIB fraction from CPZ-treated extracts and supplementation of CPZ-treated extracts with Brf1 minimizes the difference in transcriptional activity between CPZ-treated and control extracts (Desai *et al.*, 2004).

Based on the direct interaction of Maf1 with pol III in yeast (Pluta *et al* 2001), and the extensive range of conditions that have been found to be dependent on the presence of Maf1 to repress pol III transcription, Maf1 has been placed at or near the end of several

signalling pathways (Upadhya *et al.*, 2002; Willis *et al.*, 2004; Desai *et al.*, 2004). This suggests that a common mechanism functioning downstream of Maf1 acts to repress the pol III transcription machinery in yeast. Indeed, several studies have shown that this regulation is via TFIIIB (Upadya *et al.*, 2003; Willis *et al.*, 2004; Desai *et al.*, 2004). Consistent with this conclusion, TFIIIB had previously been identified as a likely target of repression by rapamycin treatment and DNA damage in yeast (Zaragoza *et al.*, 1998; Ghavidel *et al.*, 2001).

Since the role of Maf1 in the regulation of pol III had not been investigated in mammalian cells, this chapter focuses on this putative regulation of pol III by Maf1 in fibroblasts and HeLa cells and identifies a possible mechanism by which this is achieved.

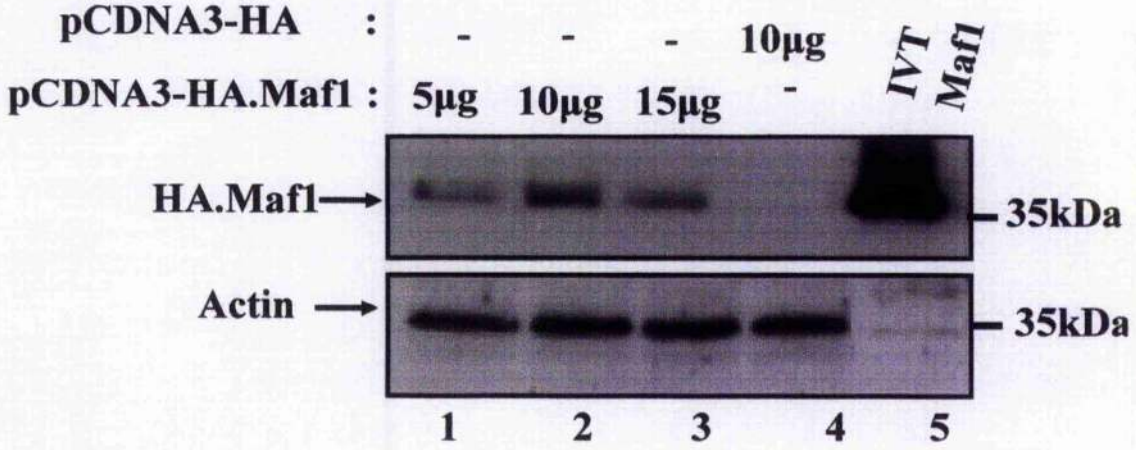
5.2 Results

5.2.1 Maf1 inhibits pol III transcription

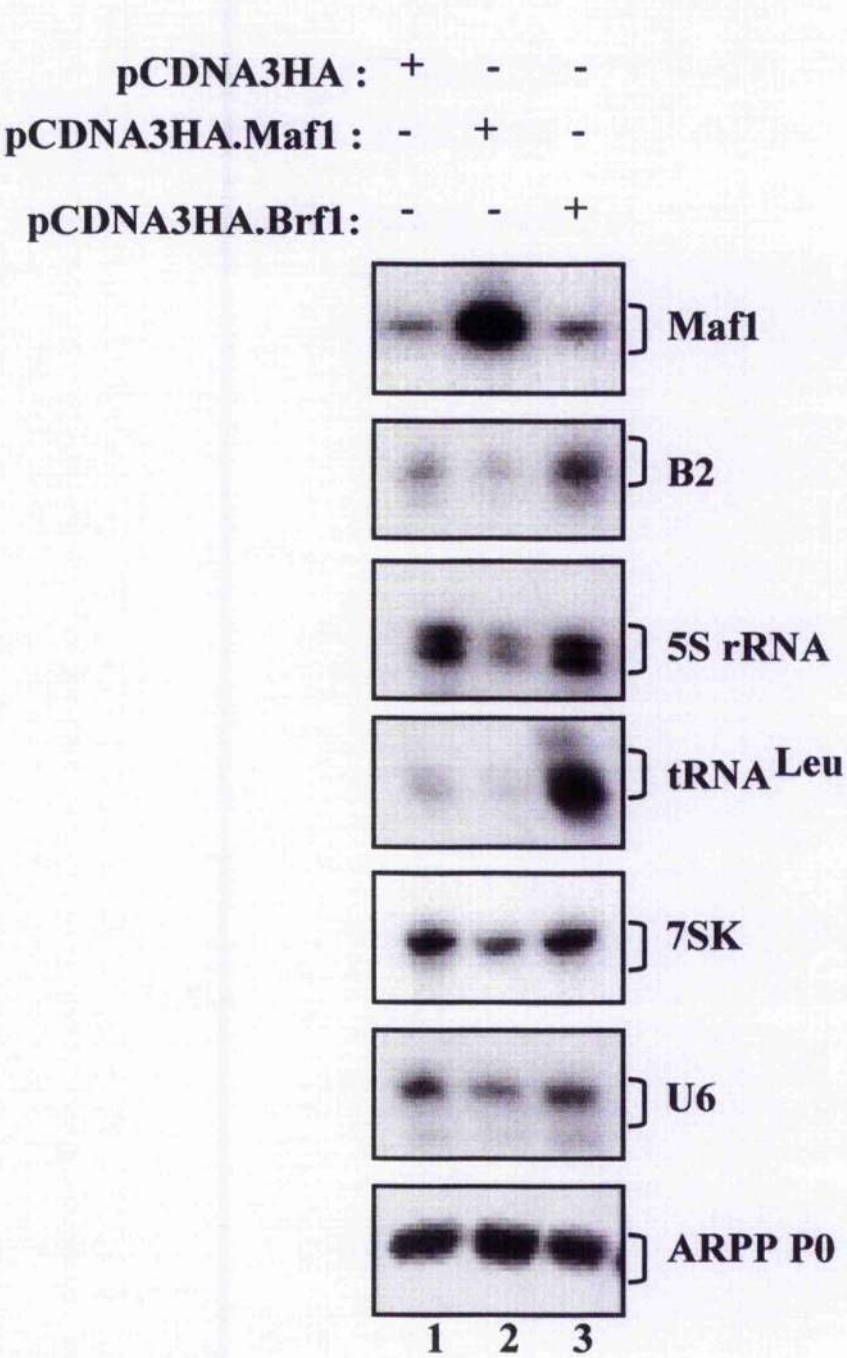
To study the effect of Maf1 on pol III transcription in cells, I overexpressed *Mus Musculus* (Mm) Maf1 (Maf1 cDNA from Olivier Lefebvre, Seclay, France) in fibroblast cells, following sub-cloning of the construct into pCDNA3HA. Asynchronous fibroblasts were transiently transfected with pCDNA3-HA (Fig. 5.1A, lane 4) or different concentrations of pCDNA3HA.Maf1 (Fig. 5.1A, lanes 1-3) for 48hrs and protein extracts were made. Samples were run on SDS-PAGE gels and immunoblotted with an anti-HA antibody. The optimum transfection efficiency was achieved by transfecting 10μg of pCDNA3HA.Maf1 into cells (Fig. 5.1A lane 2). *In vitro* radiolabelled Maf1 was synthesised *in vitro* by translation with a reticulate lysate in the presence of [^{35}S] Cysteine (Fig. 5.1A lane 5). This runs at the same size as Maf1 expressed in cells and acts as a positive marker for this protein.

To test if Maf1 affected the product levels of different pol III promoter types, cDNAs were prepared from RNA extracted from Maf1-transfected cells and compared to untransfected cells or those transfected with pCDNA3HA.Brf1. Subsequent PCR analysis of the cDNAs showed a substantial increase in the RNA levels of Maf1 following transient transfection of pCDNA3HA.Maf1. The increase in levels of Maf1 accompanied a decrease in B2, 5S rRNA, tRNA^{Leu}, 7SK and U6 (Fig. 5.1B, lane 2). These effects were specific, since there was no change in the level of the class II gene product ARPP P0 mRNA (Fig. 5.1B, lower panel). Brf1 has previously been found to be limiting for pol III transcription in fibroblast cells (Felton-Edkins *et al.*, 2003a). Therefore, Brf1 was used as a positive control to induce pol III activity. Increases in B2, 5S rRNA and tRNA^{Leu} RNAs were observed in Brf1-transfected cells compared to un-transfected cells (Fig. 5.1B, lanes 1 and 3). In contrast to

A


pCDNA3-HA :
pCDNA3-HA.Maf1 :
5µg
10µg
15µg
10µg
IVT Maf1
HA.Maf1
35kDa
Actin
35kDa
1
2
3
4
5

B


pCDNA3HA :
pCDNA3HA.Maf1 :
pCDNA3HA.Brf1:
Maf1
B2
5S rRNA
tRNA Leu
7SK
U6
ARPP P0
1
2
3

C

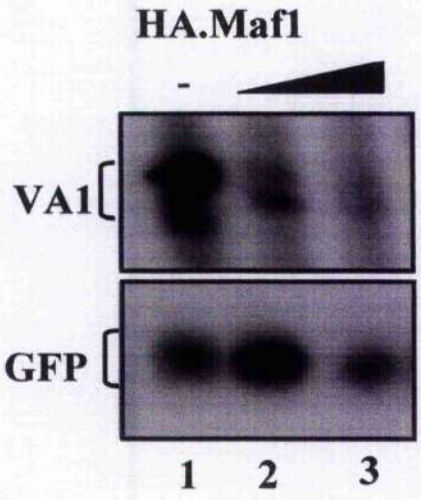


Fig 5.1: **Mafl represses all tested class III genes *in vivo***

(A) CCL39 cells growing in 10% serum were transiently transfected with pCDNA3-HA (10μg; lane 4), pCDNA3HA-MmMaf (5μg; lane 1), pCDNA3HA-MmMaf (10μg; lane 2) or pCDNA3HA-MmMaf (15μg; lane 3) for 48 hrs and protein extracts were made. [^{35}S]-labelled Mafl (5μl; lane 5) was synthesised *in vitro* using a reticulocyte lysate. An aliquot of this was used as a positive control. Samples were subjected to SDS-PAGE analysis and Western blotting and were probed with an anti-HA antibody (upper panel) or actin (lower panel). Blots are representative of more than three independent experiments.

(B) RNA was extracted from asynchronously growing CCL39 cells which had been transiently transfected with pCDNA3HA (10μg; lane 1), pCDNA3HA-MmMaf (10μg; lane 2) or pCDNA3HA-Brfl (10μg; lane 3) for 48hrs. cDNAs were generated by reverse transcription of the extracted RNA. These cDNAs were PCR amplified by using primers for Mafl (upper panel), B2 (second panel), 5S rRNA (third panel), $tRNA^{Leu}$ (fourth panel), 7SK (fifth panel), U6 (sixth panel) and ARPP P0 (lower panel). Results are representative of at least three independent experiments.

(C) Asynchronously growing CCL39 fibroblasts were transfected for 48hrs with pVA1 (0.25μg; all lanes), pGFP (0.25μg; all lanes), pCDNA3HA (2μg, lane 1), pCDNA3HA.Mafl (1ug, lane 2; 2ug, lane 3) and RNA extracted. VA1 (upper panel) and GFP levels (lower panel) were assayed by primer extension as shown. Results are representative of two independent experiments.

the type I (5S rRNA) and type II (tRNA and B2) promoter products, the level of the type III promoter products U6 and 7SK were not altered when cells were transfected with pCDNA3HA.Brf1 (Fig. 5.1B, compare lanes 1 and 3). This is in agreement with findings that type III promoters do not use Brf1 in transcription complex assembly (Schramm and Hernandez, 2002). To further investigate the reduction in pol III transcription by over expressing Maf1, this construct was transiently transfected into fibroblast cells along with the adenoviral VA1 gene and a pol II-transcribed GFP control gene. VA1 and GFP RNA levels were assayed by primer extension. Transfection of Maf1 into cells causes a significant decrease of VA1 transcription by pol III compared to un-transfected cells (Fig 5.1C). The pol II reporter GFP was used to check transfection efficiency and shows that decreased VA1 transcription was not accompanied by an effect on GFP transcription (Fig. 5.1C). These data suggest that pol III transcription in mammalian cells is repressed by Maf1.

5.2.2 Knockdown of Maf1 using siRNA increases pol III transcript levels

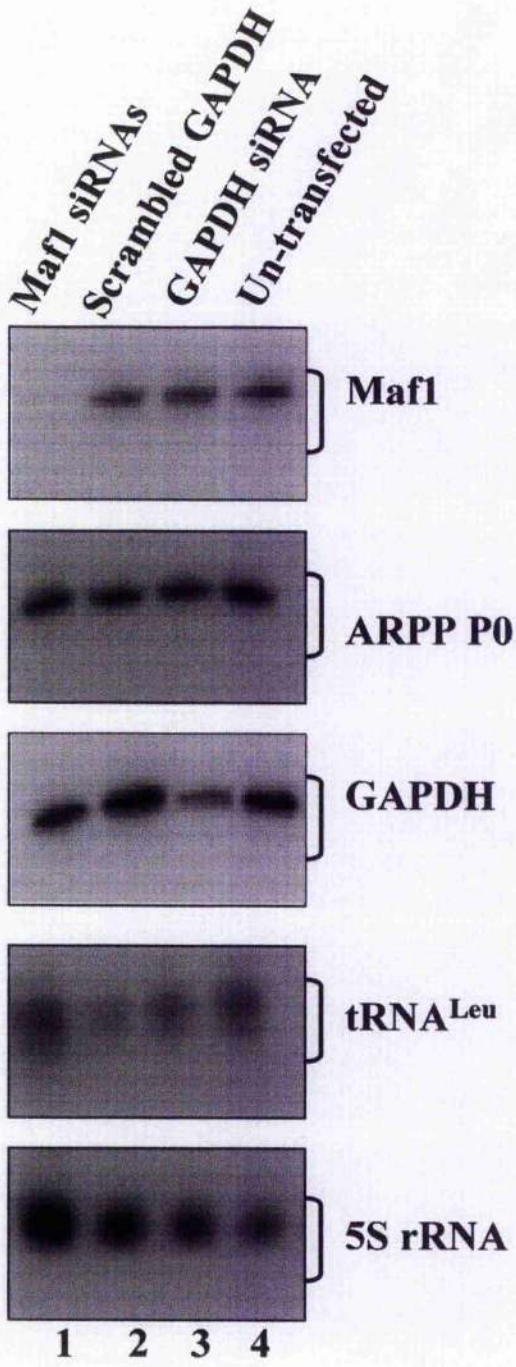
Overexpression of Maf1 decreases pol III transcription; however, the function of endogenous Maf1 can be investigated using an RNA-mediated interference (RNAi) approach. The term RNAi describes the use of double stranded RNA to target specific mRNAs for degradation, thereby silencing their expression (Zamore, 2000). The siRNA design involved the selection of nucleotide sequences within the Maf1 target mRNA which began with an AA dinucleotide, were 21 nucleotides in length and had less than a 50% GC content. These sequences were then entered into the BLAST search engine to check that they did not have significant homology to other genes in the mouse genome database. Four siRNAs targeted to different regions of the Maf1 gene were designed and prepared by *in vitro* transcription using Ambions *Silencer*^{TM} siRNA Construction Kit. The Maf1 siRNA cocktail was introduced directly into mammalian cells by transient transfection, as were

Silencer™ GAPDH siRNA and *Silencer*™ scrambled GAPDH siRNA (all at 50nM final concentration). RNA was harvested 48hrs later and levels of Maf1, ARPP P0, GAPDH, B2, 5S rRNA and $tRNA^{Leu}$ were assayed by RT-PCR. The cocktail of Maf1 siRNAs resulted in a substantial reduction in Maf1 mRNA compared to controls (Fig 5.2A, upper panel) and this accompanied an increase in the levels of both 5S rRNA and $tRNA^{Leu}$ pol III transcripts (Fig 5.2A, panels 4 and 5). This effect was specific, since levels of the pol II transcript ARPP P0 remained virtually unchanged (Fig. 5.2A, panel 2). Transfection of *Silencer*™ GAPDH siRNA reduced the levels of the GAPDH mRNA, verifying that siRNAs had been transfected efficiently (Fig. 5.2A, panel 3). Scrambled GAPDH siRNA did not reduce the level of Maf1 mRNA as compared to that of untransfected cells. This verifies that the siRNA delivery method does not result in changes to the gene expression profile of Maf1, as scrambled GAPDH siRNA does not code for any target and should not decrease mRNA level. These siRNA data suggest that targeted inhibition of Maf1 by siRNA can increase pol III transcript levels.

5.2.3 Knockdown of Maf1 using siRNA does not alter Maf1 protein levels

Since Maf1 siRNAs led to a specific depletion of the targeted mRNA and consequently increased levels of pol III transcripts, it was important to determine that Maf1 protein levels had also been reduced. Protein extracts prepared (in parallel to the RNA in Fig. 5.1A) post-transfection of siRNAs were subjected to SDS-PAGE analysis and were immunoblotted with an antibody against Maf1 (SK-2966) (Fig. 5.2B, lanes 1-4). Radiolabelled Maf1 was prepared *in vitro* by translation with a reticulocyte lysate as before and was used to check the size of the protein (Fig 5.2B, lane 5). Actin was used as a loading control (Fig 5.2B, lower panel). There was a slight decrease in the level of Maf1 protein in the siRNA Maf1-treated cells compared to the un-transfected control cells; however, the level of Maf1 protein was comparable in all the siRNA-treated cells (Fig.

A


Maf1 siRNAs
Scrambled GAPDH
GAPDH siRNA
Un-transfected
Maf1
ARPP P0
GAPDH
tRNA^Leu
5S rRNA
1 2 3 4

B

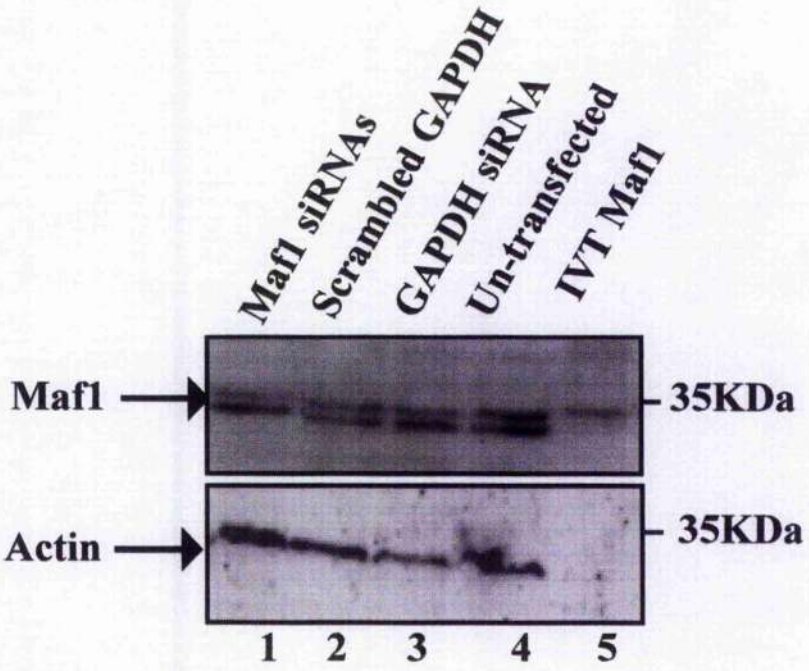


Fig 5.2: **Depletion of endogenous Maf1 by RNAi increases $tRNA^{Leu}$ and 5S rRNA gene expression**

(A) Growing A31 cells were transfected using siPORT lipid (Ambion), with siRNAs against Maf1 (lane 1), scrambled GAPDH siRNA (lane 2) or GAPDH (lane 3) that were purchased from Ambion. All siRNAs were used at 50nM final concentration.Un-transfected cells were used as a negative control (lane 4). RNA was extracted 48hrs post-transfection and RT-PCR was performed. PCR was carried out using the templates Maf1 (upper panel), ARPP P0 (second panel),GAPDH (third panel), $tRNA^{Leu}$ (fourth panel) or 5S rRNA (lower panel). Results are representative of at least three independent experiments.

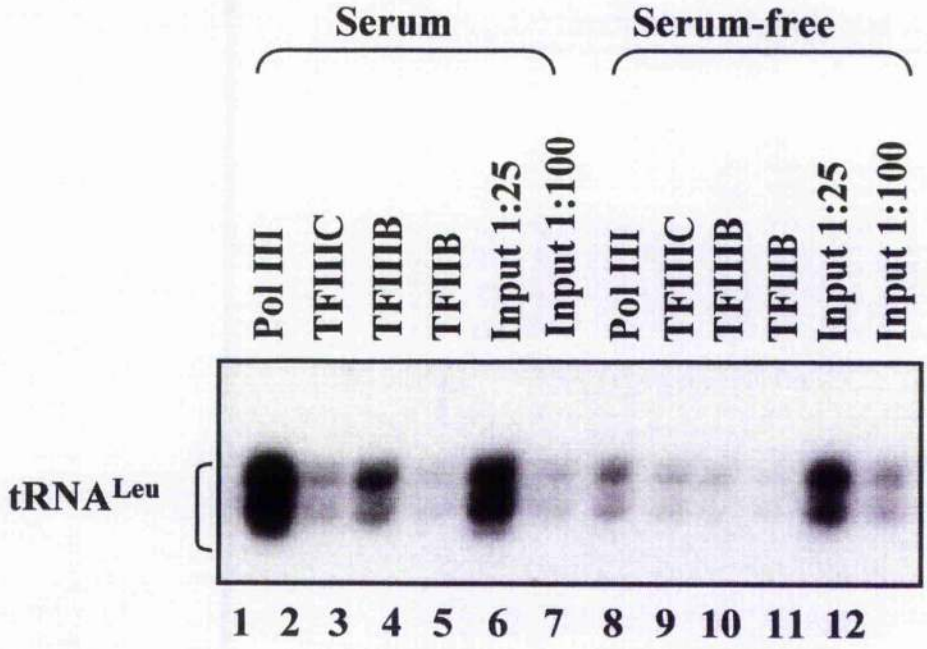
(B) Protein extracts prepared, following transfection of Maf1 or control siRNAs (as described in [A]) were subjected to SDS-PAGE analysis and were immunoblotted with an antibody against Maf1 (SK-2966, upper panel) or actin (lower panel). Methionine-labelled Maf1 prepared by *in vitro* transcription and translation using reticulocyte lysate, was used as a size marker (lane 5). Blots are representative of two independent experiments.

5.2B). Since the RNA levels of the $tRNA^{Leu}$ and 5S rRNA pol III transcripts increased when Maf1 siRNA was knocked down, this implies that some decrease in the level of Maf1 protein must have occurred. This result may also imply that pol III transcripts are extremely sensitive to small changes in Maf1 concentration. As the Maf1 protein was not knocked down this may suggest it is stable and has a long half-life. Long-term siRNA treatment could overcome this problem and can be achieved with either repeated transfections of the siRNA or the stable incorporation of the siRNA using a siRNA expression vector.

5.2.4 Maf1 occupancy on $tRNA^{Leu}$ promoters is reduced in serum-starved cells

Recently, ChIP analysis has shown that CPZ-treatment in yeast caused a marked Maf1-dependent reduction in the occupancy of tRNA genes by pol III (Desai *et al.*, 2004), suggesting that the control of pol III occupancy on DNA is a Maf1-dependent regulatory mechanism. Pol III transcription is known to be sensitive to serum and both TFIIIB and TFIIIC have already been shown to be phosphorylated (and activated) in response to serum addition (Felton Edkins, unpublished; Scott *et al.*, unpublished; Fig 4.8A and 4.9A). In light of this, the occupancy of the pol III factors at tRNA promoters was investigated in cells which were subject to serum starvation to repress pol III transcription. CCL39 cells growing in either the presence (Fig 5.3A lanes 1-6), or absence of serum (Fig 5.3A, lanes 7-12), were formaldehyde cross-linked to preserve the *in vivo* protein/DNA interactions. DNA complexes were then immunoprecipitated using antibodies to a pol III subunit, TFIIIB subunit, TFIIIC subunit and control TFIIB. Cross-links were reversed, protein digested and the DNA was screened by PCR to determine whether class III genes were bound by the protein. Pol III occupancy at $tRNA^{Leu}$ promoters is reduced in serum-starved cells (Fig 5.3A, compare lanes 1 and 7) and there is little change to the occupancy of

A


Serum
Serum-free
Pol III
TFIIIC
TFIIIB
TFIIB
Input 1:25
Input 1:100
Pol III
TFIIIC
TFIIIB
TFIIB
Input 1:25
Input 1:100
tRNA^Leu
1 2 3 4 5 6 7 8 9 10 11 12

B

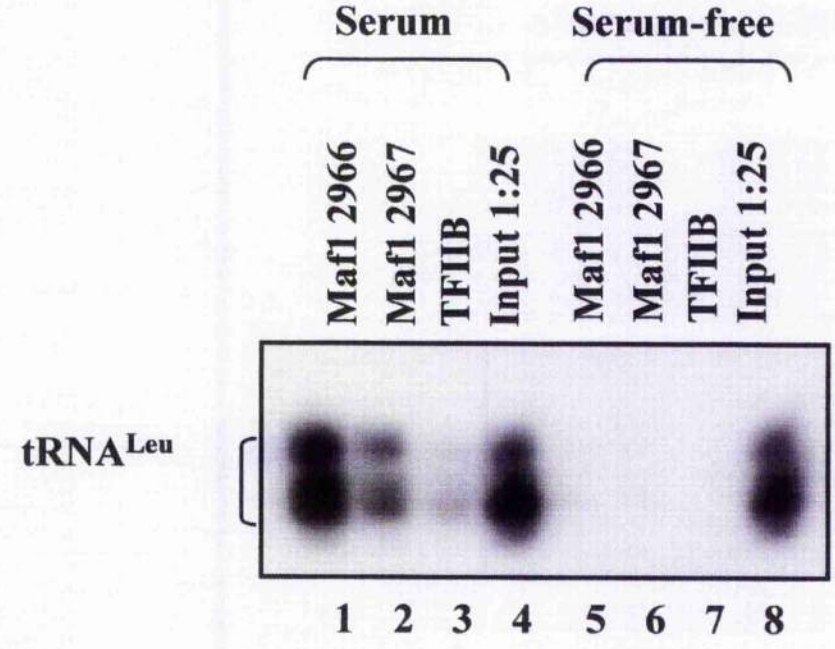

Serum
Serum-free
Maf1 2966
Maf1 2967
TFIIB
Input 1:25
Maf1 2966
Maf1 2967
TFIIB
Input 1:25
tRNA^Leu
1 2 3 4 5 6 7 8

Figure 5.3: **Pol III, Brf1 and Maf1 occupancy at tRNA promoters is reduced in response to serum-starvation**

(A) Asynchronous CCL39 hamster fibroblasts were cultured in the presence (lanes 1-6) or absence of 10% serum (lanes 7-12) for 4 hrs. Association of TFIIIC subunit TFIIIC110, TFIIIB subunit Brf1 and pol III subunit RPC155 with $tRNA^{Leu}$ genes was then determined by ChIP analysis. This involved PCR with equivalent DNA input amounts as determined by PCR on diluted input chromatin. Control ChIPs were carried out with the TFIIB antibody. PCR reactions incorporated [α-^{32}P] dCTP and products were resolved on denaturing sequencing gels which were subjected to autoradiography. Results are representative of two independent experiments.

(B) Asynchronous CCL39 hamster fibroblasts were cultured in the presence (lanes 1-4) or absence of 10% serum (lanes 5-8) for 4 hrs. Association of Maf1 (SK-2966 and SK- 2967) with $tRNA^{Leu}$ genes was then determined by ChIP analysis as described in (A). Control ChIPs were carried out with theTFIIB antibody. PCR reactions incorporated [α-^{32}P] dCTP and products were resolved on denaturing sequencing gels which were subjected to autoradiography. Results are representative of two independent experiments.

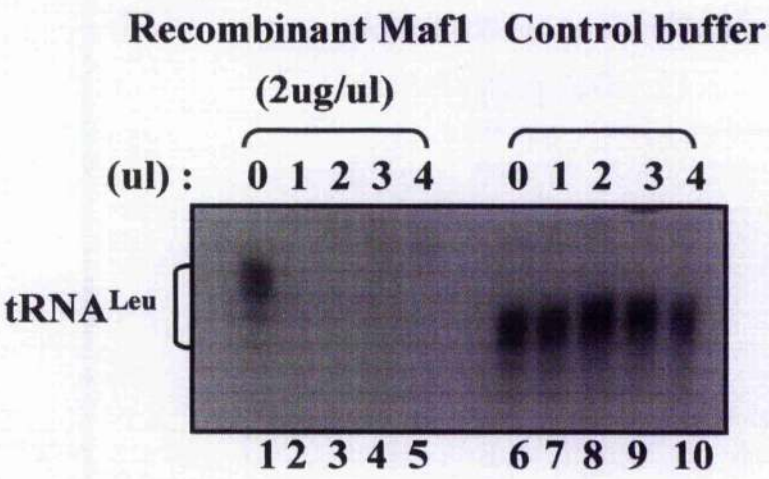
TFIIIC (Fig 5.3A, compare lanes 2 and 8), however, TFIIIB occupancy is reduced in response to serum-starvation (Fig 5.3A, compare lanes 3 and 9). Negligible levels of control TFIIB in both conditions indicated the specificity of the association, and input samples were equal for both conditions (Fig. 5.3A, lanes 4-6 and 10-12). Since Maf1 has been reported to associate with pol III and TFIIIB in yeast (Pluta *et al*, 2001, Desai *et al*., 2004), Maf1 should assume the same pattern of promoter occupancy as these factors under the same environmental conditions. To investigate whether Maf1 is at tRNA promoters in mammalian cells, ChIP assays were used to compare promoter occupancy between serum-rich and serum-starved cells *in vivo*. Formaldehyde cross-linked soluble chromatin was prepared from asynchronously growing cells cultured in 10% serum (Fig. 5.3B, lanes 1-4) or 0% serum (Fig. 5.3B, lanes 5-8) for 16 hours. PCR analysis of precipitated DNA showed that occupancy of Maf1 is blocked on $tRNA^{Leu}$ promoters in response to serum starvation. The SK-2966 was better at immunoprecipitating Maf1 at tRNA promoters than the SK-2967 Maf1 antibody (Fig. 5.3B, compare lanes 1 and 2).

This ChIP data shows that Maf1 is associated with tRNA promoters under normal growth conditions. Upon serum-starvation, Maf1 is displaced from these promoters and this is accompanied by reduced occupancy at tRNA promoters of pol III and the transcription factor TFIIIB. This may suggest that Maf1 interacts with pol III and/or TFIIIB in mammalian cells, under normal growth conditions.

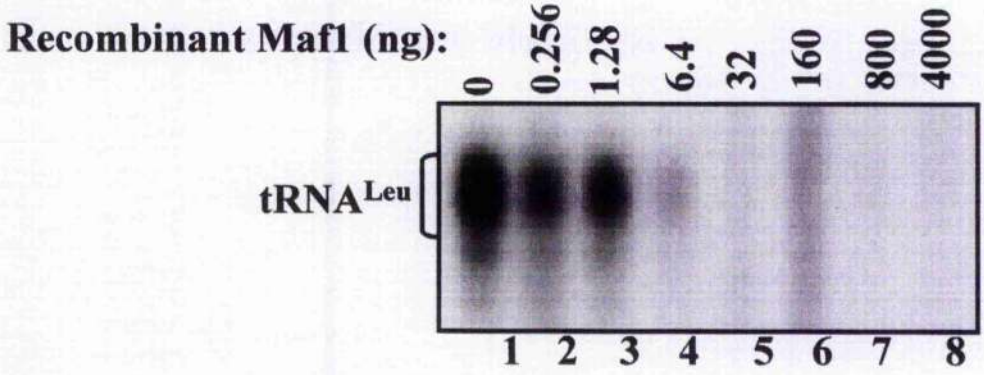
5.2.5 Recombinant Maf1 inhibits pol III transcription *in vitro*

Purified recombinant Human (Hs) Maf1 (HsMaf1.His) was kindly given to us by Olivier Lefebvre and this was used to test whether Maf1 could repress pol III transcription *in vitro* in HeLa cell extract. Varying concentrations (0-8μg) of HsMaf1.His was used in an *in vitro* transcription assay (Fig. 5.4A). HeLa cell extracts treated with just 2μg of HsMaf1.His

A


Recombinant Maf1 (2ug/ul)
Control buffer
(ul) : 0 1 2 3 4 0 1 2 3 4
tRNA^Leu
1 2 3 4 5 6 7 8 9 10

B


Recombinant Maf1 (ng): 0 0.256 1.28 6.4 32 160 800 4000
tRNA^Leu
1 2 3 4 5 6 7 8

C

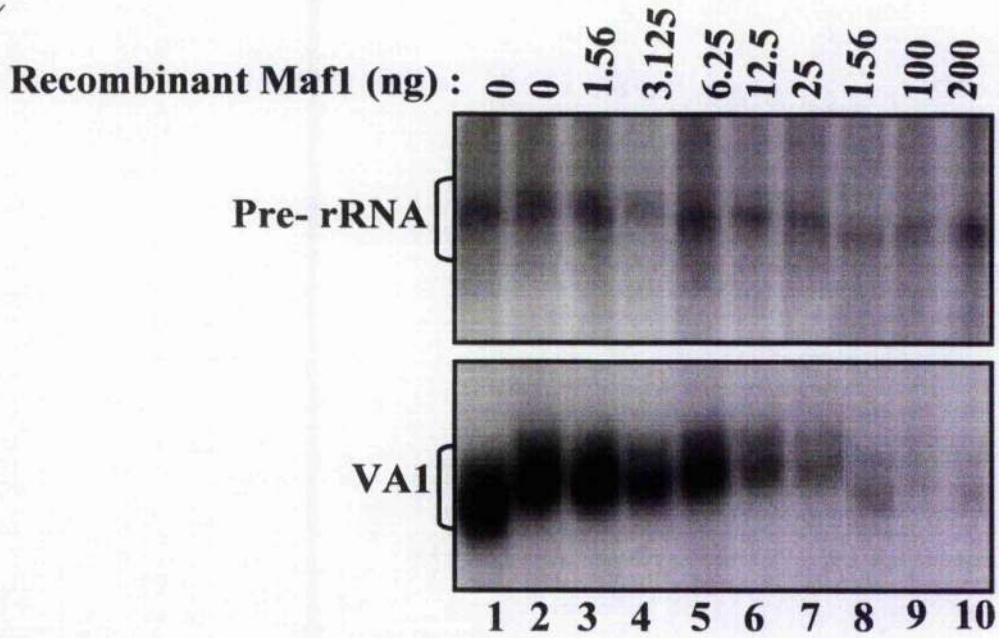

Recombinant Maf1 (ng) : 0 0 1.56 3.125 6.25 12.5 25 1.56 100 200
Pre- rRNA
VA1
1 2 3 4 5 6 7 8 9 10

Fig 5.4 : **Maf1 specifically represses pol III transcription**

(A) $tRNA^{Leu}$ (250ng) was transcribed using 20μg of HeLa nuclear extract together without (lane 1), or with 2, 4, 6, 8μg (lanes 2-5) of recombinant purified HsMaf.His1 or without (lane 6), or with 2, 4, 6, 8μg of control buffer (lanes 7-10). Incorporation of [^{32}P]dUTP was detected by autoradiography after samples were run on a denaturing sequencing gel. Results are representative of more than three independent experiments.

(B) *In vitro* transcription was carried out using 20μg of HeLa nuclear extract plus control buffer (lane 1), 0.256ng (lane 2), 1.28ng (lane 3), 6.4ng (lane 4), 32ng (lane 5), 160ng (lane 6), 800ng (lane 7), and 4000ng (lane 8) of recombinant purified HsMaf1.His on a $tRNA^{Leu}$ template (250ng). Incorporation of [^{32}P] dUTP was detected by autoradiography after samples were run on a denaturing sequencing gel. Results are representative of more than three independent experiments.

(C) HeLa nuclear extract (20μg) was used in an *in vitro* transcription assay using both $tRNA^{Leu}$ (250ng) and pre-rRNA (250ng) templates together with control buffer (lane 1 and 2), 1.56 ng (lane 3), 3.125ng (lane 4), 6.25ng (lane5), 12.5ng (lane 6), 25ng (lane 7), 50ng (lane 8), 100ng (lane 9) or 200ng (lane 10) of recombinant purified Hs.Maf1.His. Incorporation of [^{32}P] dUTP was detected by autoradiography after samples were run on a denaturing sequencing gel. Results are representative of more than three independent experiments.

were unable to support transcription of $tRNA^{Leu}$ genes, whilst extracts treated with a control (from a fraction taken during the purification of HsMaf1 with no His Tag) were unaffected (Fig. 5.4A). Further titrations with the HsMaf1.His protein were carried out using lower concentrations of HsMaf1.His. *In vitro* transcription of $tRNA^{Leu}$ in HeLa cell extracts was abolished with as little as 6.4ng of HsMaf1.His (Fig. 5.4B).

To ensure that the Maf1-mediated repression of transcription was specific to pol III transcription, a pol I template was included in an *in vitro* transcription reaction along with a pol III template. HeLa cell extracts were used to transcribe pol I and VA1 genes during incubation with varying concentrations (0-200ng) of HsMaf1.His. Transcription of VA1 genes was significantly inhibited in HeLa cell extracts treated with 12.5ng of HsMaf1.His whilst transcription by pol I of pre-ribosomal DNA remained unchanged in the presence of up to 200ng of HsMaf1.His (Fig. 5.4C). These data show that Maf1 specifically represses pol III transcription and this is in agreement with RT-PCR analysis of pol III transcript levels (Fig. 5.1). Since RT-PCR analysis had determined that all pol III promoter types were subject to repression by Maf1, it was investigated whether repression of all class III genes also occurred *in vitro*. HeLa cell extracts treated with 200ng of HsMaf1.His (Fig. 5.5, lane 1) or control buffer (lane 2), were assayed for their ability to transcribe a variety of pol III templates. *In vitro* transcription of 5S rRNA (Fig. 5.5, upper panel), $tRNA^{Leu}$ (Fig. 5.5, second panel), B2 (Fig. 5.5, third panel), VA1 (Fig. 5.5, fourth panel), 7SL (Fig. 5.5, fifth panel) and 7SK (Fig. 5.5, sixth panel) were significantly reduced in the presence of 200ng of HsMaf1.His, whilst transcription of pol 1 was unaffected (Fig. 5.5, lower panel). This result confirms that Maf1 can repress all types of class III genes.

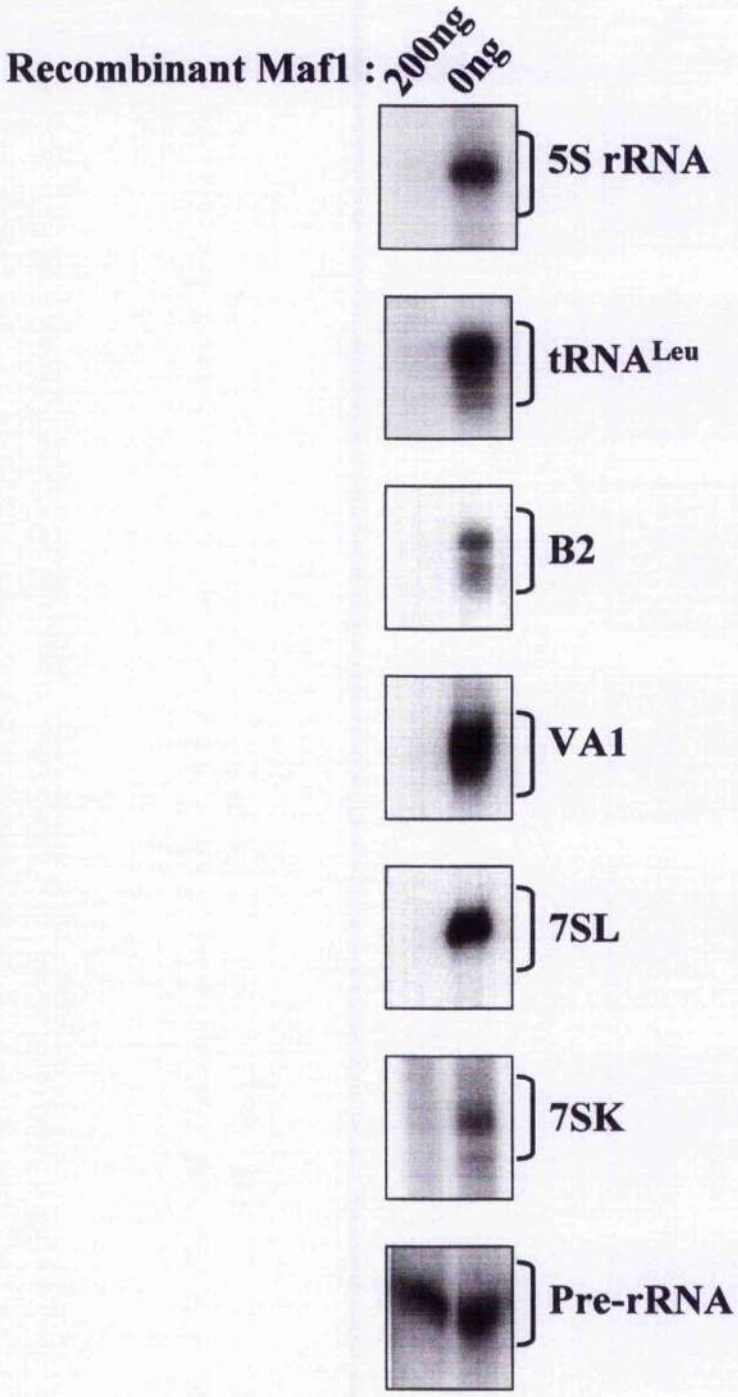


Fig 5.5: ***In vitro* repression of all class III genes by recombinant purified Maf1**
20μg of HeLa nuclear extract was used in an *in vitro* transcription assay using the templates (250ng) 5S rRNA (upper panel), $tRNA^{Leu}$ (second panel), B2 (third panel), VA1 (fourth panel),7SL (fifth, panel), 7SK (sixth panel) or pre-rRNA (lower panel), in the presence (lane 1) or absence (lane 2) of HsMaf1.His (200ng). Incorporation of [^{32}P] dUTP was detected by autoradiography after samples were run on a denaturing sequencing gel. Results are representative at least two independent experiments.

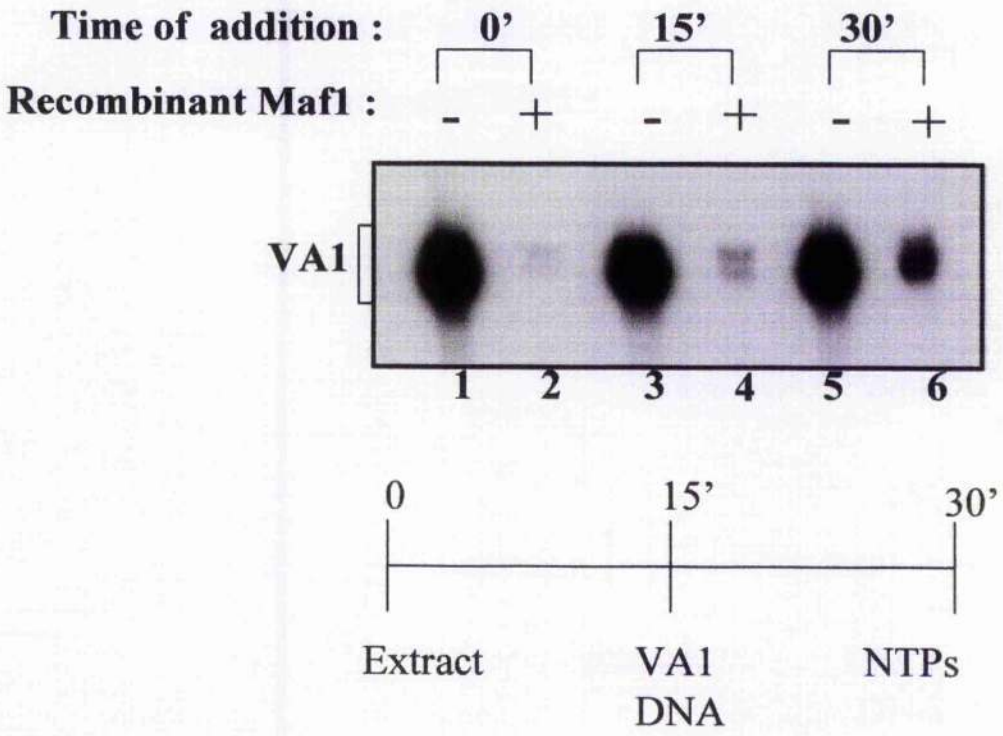
5.2.6 Maf1 can repress pol III transcription whether added before, during or after initiation complex assembly.

Having provided both *in vitro* and *in vivo* evidence that Maf1 can specifically repress pol III transcription we were interested to know the mechanism by which this occurred. We investigated whether assembly of a class III gene into a stable pre-initiation complex can influence the repressive effects of Maf1. HsMaf1.His (200ng) was added to a HeLa nuclear extract either 15mins prior to the addition of a VA1 gene (Fig 5.6A, lanes 1 and 2), simultaneously with the addition of VA1 DNA (Fig. 5.6A, lanes 3 and 4), or 15mins after the addition of VA1 template (Fig. 5.6A, lanes 5 and 6). Nucleotides were then added to allow transcription. Since pol III factors assemble rapidly onto the VA1 gene promoter, this assays tests the ability of Maf1 to repress a pre-formed initiation complex. Maf1 can inhibit transcription of VA1 whether added before, during or after complex assembly (Fig. 5.6A). Relative to HeLa extract alone, Maf1 was found to inhibit VA1 transcription by 90% when added prior to, or during complex assembly (0 and 15mins, respectively). 75% inhibition was observed when Maf1 was added 15mins after the template (Fig. 5.6B). These observations suggest that the class III transcription factors are slightly more susceptible to Maf1 when free in solution but do not receive substantial protection even when assembled into a pre-initiation complex.

5.2.7 TFIIIB is a specific target for repression by Maf1

Since Maf1 can regulate each of the three pol III promoter types (Fig. 5.1 and 5.5), it seemed likely that it acts upon a general component of the pol III transcription apparatus. It has been suggested that in yeast, Maf1 may function as a stochiometric inhibitor of TFIIIB-DNA complex assembly and of polymerase recruitment through its interactions with Brf1 and pol III, respectively (Pluta *et al.*, 2001; Upadhya *et al.*, 2002; Desai *et al.*,

A


Time of addition :
0'
15'
30'
Recombinant Maf1 :
- + - + - +
VA1
1 2 3 4 5 6
0
15'
30'
Extract
VA1 DNA
NTPs

B

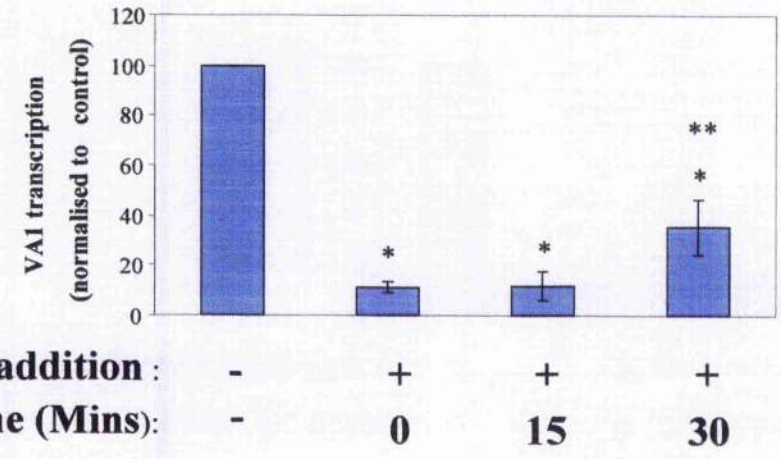

VA1 transcription (normalised to control)
120
100
80
60
40
20
0
*
*
**
*
Maf1 addition : - + + +
Time (Mins): - 0 15 30

Fig. 5.6: **Maf1 represses pol III transcription whether added before, during or after initiation complex assembly.**

(A) HeLa nuclear extract was pre-incubated for 15mins at 30°C (lanes 1 and 2), before the addition of the VA1 DNA (lanes 3 and 4), after a further 15 mins at 30°C, nucleotides were added and transcription was allowed to proceed (lanes 5 and 6). Reactions were carried out in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3 and 5) of HsMaf1.His (200ng). Samples were run on a denaturing gel followed by autoradiography.

(B) The VA1 signals from (A) were quantified by densitometry. The graph shows the mean and standard deviations from three independent experiments; values obtained for cells grown in absence of HsMaf1.His recombinant protein were set as 100 and other values were calculated as a percentage of this.

* denotes statistically significant difference from control, at $p < 0.05$

** denotes statistically significant difference from 15 mins, at $p < 0.05$.

2004). It followed that if Maf1 could repress class III genes by inactivating individual components of the pol III transcription apparatus, then it should be possible to overcome this repression by adding more of that factor to the system. Addback experiments were therefore carried out to determine which fraction would rescue Maf1-induced repression of pol III transcription. A fraction containing partially purified TFIIIB was found to stimulate pol III transcription when titrated into HeLa Extracts treated with recombinant Maf1 (Fig. 5.7, lanes 2-5). Little or no stimulation was observed in response to a fraction containing TFIIIC (Fig. 5.7, lanes 9-11) or pol III (Fig. 5.7, lanes 6-8). The activity of all fractions had previously been confirmed using complementation assays (R.J. White, unpublished data). This implicates TFIIIB as a target for Maf1–mediated repression of pol III transcription in mammalian cells.

5.2.8 Maf1 is a phosphoprotein

Several studies in yeast have proposed that Maf1 is a common mediator of repression for many cellular stresses and have suggested that Maf1 lies near to or is at the end of stress-sensing pathways (Upadhya *et al.*, 2002; Willis *et al.*, 2004; Desai *et al.*, 2004). In fact, a link between the mTOR signalling pathway and Maf1 has been reported in yeast (Upadhya *et al.*, 2002; Willis *et al.*, 2004; Desai *et al.*, 2004). Since many signalling pathways rely on phosphorylation for either activation or repression of their targets, it was interesting to find if Maf1 was regulated in this way. To this end, an *in vitro* kinase assay was carried out. Recombinant Maf1 or a buffer control was bound to Ni^{2+}-NTA-resin , washed and incubated for 2 hrs with extracts from growing fibroblasts that had been vehicle-treated (Fig. 5.8A, lanes 1 and 3) or incubated with 100nM rapamycin (Fig. 5.8A, lanes 2 and 4) for 4hrs. Samples were γ-[^{32}P] ATP-labelled for 30mins. Following incubation, samples were washed extensively, run on SDS-PAGE, and dried gels were exposed to autoradiography. No difference in the level of phosphorylation of Maf1 was shown when

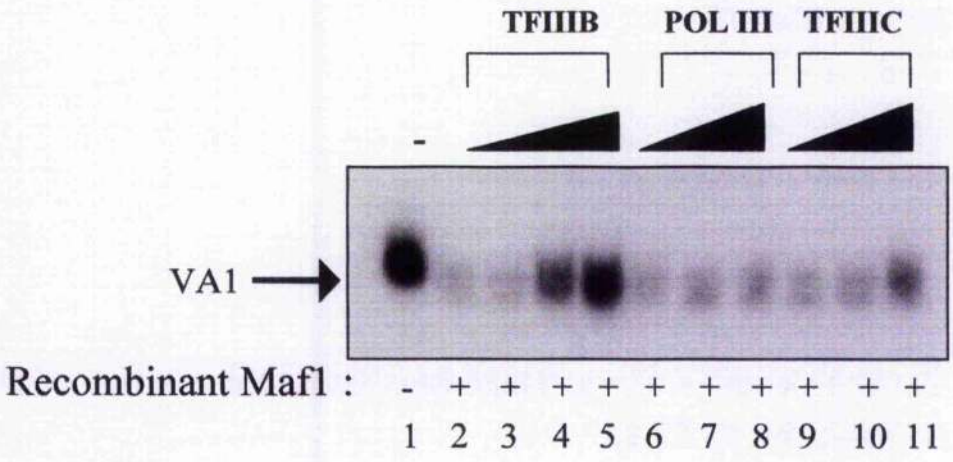


Fig 5.7 : **A component of TFIIIB is a target for repression of pol III transcription by Maf1**

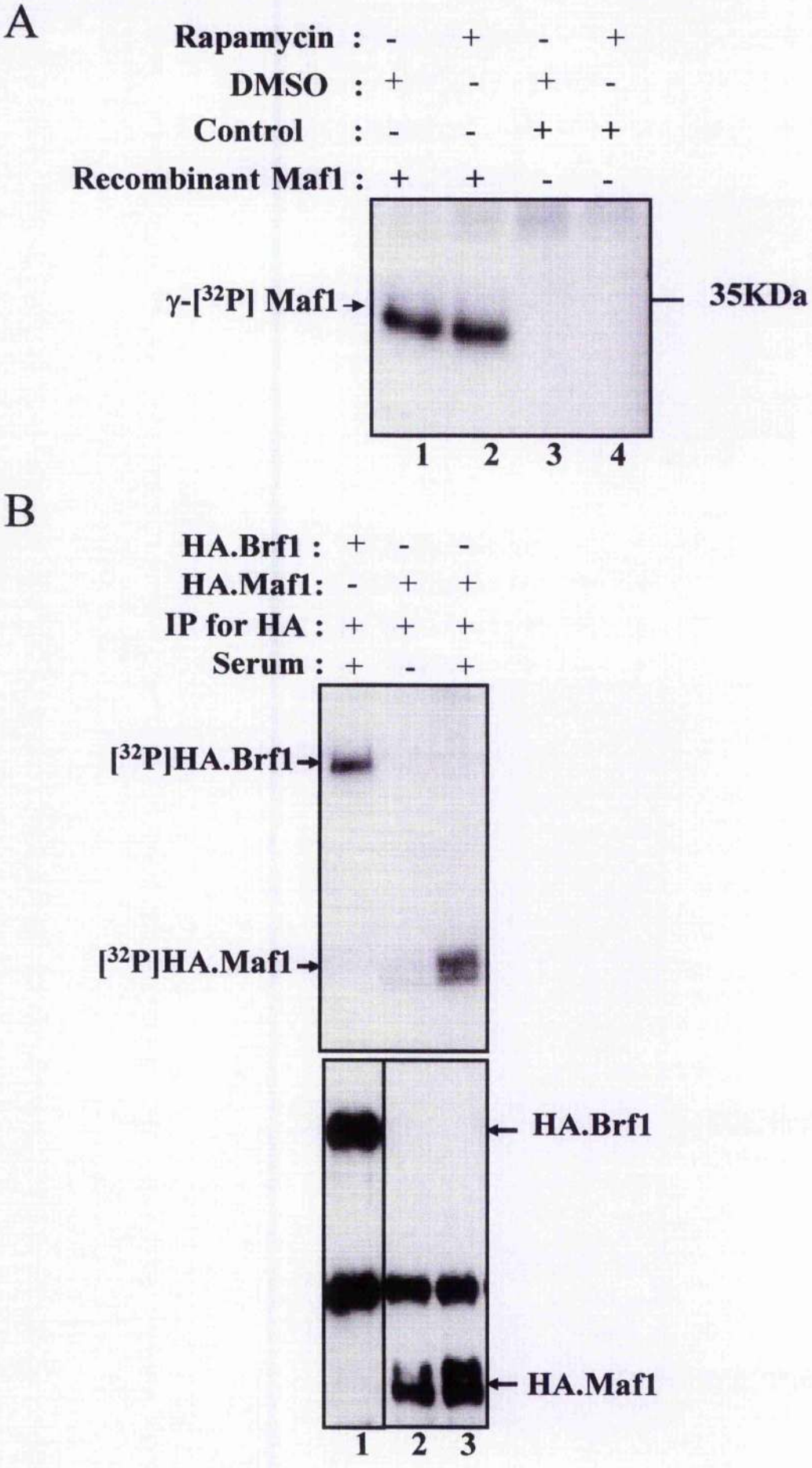
(A) 20μg of HeLa nuclear extract, was used in an *in vitro* transcription assay using the VA1 template (250ng) in the presence of HsMaf1.His (50ng ; all lanes). In addition the reactions were supplemented with 0, 1, 2, 3 or 4μl of fractions TFIIIB (A25 (15)16) (lanes 2-5), pol III (A25 (1.0)13), (lanes 6-8), or TFIIIC (CHep 1-15) (lanes 9-11). Samples were then run on a denaturing gel and exposed to film. Results are representative of more than three independent experiments.

the recombinant protein was incubated with either the vehicle-treated or rapamycin-treated extracts (Fig. 5.8A, lanes 1 and 2). This effect is specific, since samples which contained only vehicle-treated and rapamycin-treated extracts could not be phosphorylated (Fig. 5.8A, lanes 3 and 4).

5.2.9 The *in vivo* phosphorylation of Maf1 is reduced in serum-starved cells

The finding that Maf1 could be phosphorylated *in vitro* prompted an investigation as to whether it could also be phosphorylated *in vivo*. Previous ChIP data had suggested that Maf1 occupancy is sensitive to serum (Fig. 5.3). Therefore, we determined the phosphorylation status of Maf1 when the cells were grown in the presence or absence of serum. CCL39 cells growing in a medium containing 10% serum were transfected with the construct pCDNA3HA.Brf1 as a positive control, or pCDNA3HA.Maf1. Following 24hrs of transfection, cells were transferred to fresh media containing 10% serum (Fig. 5.8B, lanes 1 and 3) or to DMEM without serum (Fig. 5.8B, lane 2). Cells were transferred to phosphate-free DMEM 24hrs later, and incubated for 3hrs with [^{32}P] orthophosphate in the presence or absence of serum. Extracts were immunoprecipitated with HA antibody, run on SDS-PAGE gels and either subjected to autoradiography or immunoblotted for HA.

As shown previously, Brf1 was found to be phosphorylated when cells were grown in the presence of serum (Felton-Edkins *et al.*, 2003a; Fig. 4.8A). Phosphorylation of Maf1 decreased significantly in the absence of serum (Fig. 5.8B upper panel, compare lanes 2 and 3). Some of this effect could be due to the finding that immunoprecipitated Maf1 levels decreased slightly in the serum-free media, (Fig. 5.8B lower panel, lanes 2 and 3). However, quantification of [^{32}P]-labelled HA.Maf1 and immunoprecipitated HA.Maf1


A
Rapamycin : - + - +
DMSO : + - + -
Control : - - + +
Recombinant Maf1 : + + - -
γ-[32P] Maf1
35KDa
1 2 3 4
B
HA.Brf1 : + - -
HA.Maf1: - + +
IP for HA : + + +
Serum : + - +
[32P]HA.Brf1
[32P]HA.Maf1
HA.Brf1
HA.Maf1
1 2 3

C

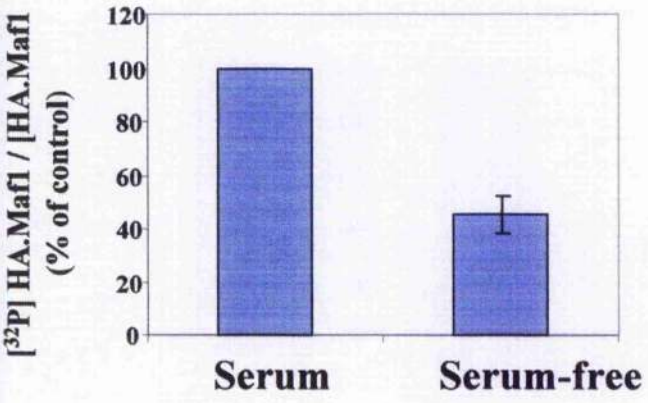


Figure 5.8: **Maf1 can be phosphorylated *in vitro* and *in vivo***

(A) CCL39 extracts (50μg) which had either been vehicle treated (lanes 1 and 3), or rapamycin (100nM) treated for 4 hours (lane 2 and 4), were incubated in the presence of recombinant HsMaf1.His (2μg) (lanes 1 and 2) or control buffer (2μg) (lanes 3 and 4) an *in vitro* kinase assay was carried out using [γ-^{32}P] ATP to label. Results are representative of more than three independent experiments.

(B) CCL39 cells transiently transfected with pCDNA3HA.Brf1 (lane 1) or pCDNA3HA.Maf1 (lanes 2 and 3) for 48hrs, were labelled with [^{32}P] orthophosphate for 3 hrs. Cells in lane 2 were transferred to serum-free medium for 24 hrs prior to labelling. Cell extracts were prepared and Brf1 or Maf1 was immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE, and either visualised by autoradiography (upper panel, lanes 1-3) or transferred to PVDF membrane, followed by Western blotting with HA antibody (lower panel, lanes 1-3).

(C) The phospholabelling result described in (B) was quantified by densitometry and values normalised to the immunoprecipitation. The graph shows the average +/- the standard error of the mean from two independent experiments, where the cells grown in media containing serum is designated 100% and the serum-starved treatment is calculated as a percentage of this.

show that serum-starved cells had 55% less Maf1 phosphorylation than those cells grown in the presence of serum (Fig. 5.8C). This result suggests that during growth (serum-rich conditions), Maf1 is phosphorylated and does not repress pol III transcription. However, under repressive growth conditions (in the absence of serum); Maf1 is de-phosphorylated and acts as a negative effector of pol III transcription.

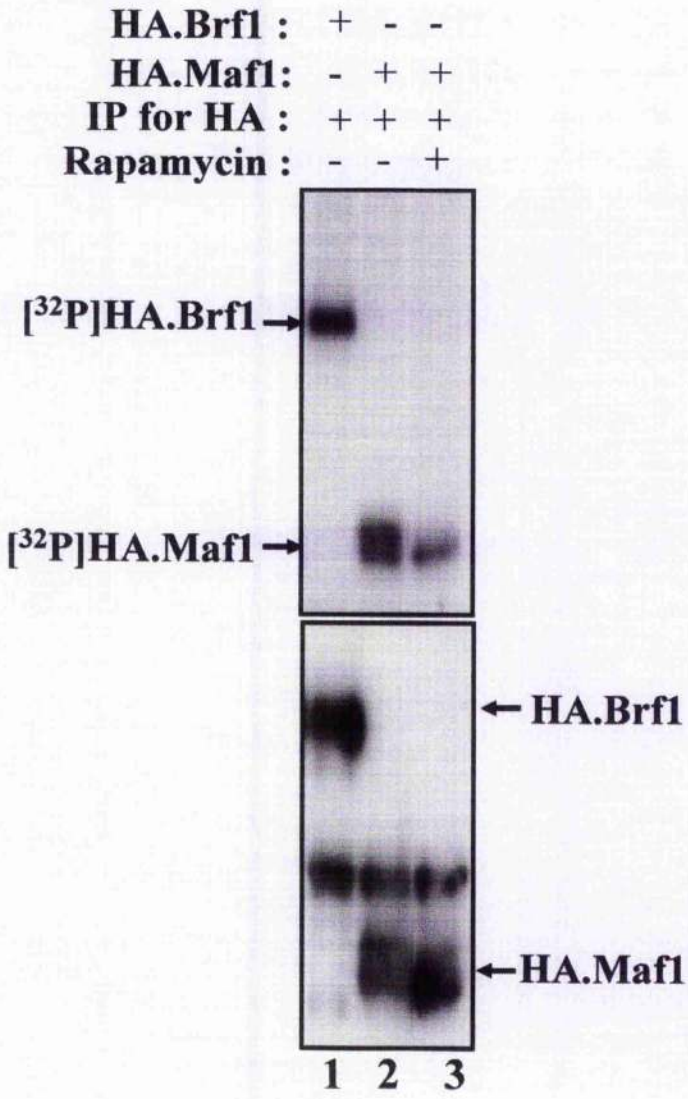
5.2.10 Maf1 phosphorylation *in vivo* is due in part to the mTOR pathway

A link between the mTOR signalling pathway and Maf1 has been reported in yeast (Upadhya *et al.*, 2002; Willis *et al.*, 2004; Desai *et al.*, 2004), whereby rapamycin treatment was found to repress pol III transcription, as had been previously reported (Mahajan, 1994; Powers and Walter, 1999; Zaragoza *et al.*, 1998; Chapter 3 and 4). Remarkably, this inhibition can be blocked by deleting Maf1, which places Maf1 downstream of the TOR kinases in yeast (Upadhya *et al.*, 2002). We were interested to find out whether the de-phosphorylation of Maf1 was a common response to other conditions known to repress pol III transcription in mammalian cells. Since inhibition of pol III has been reported by blocking the mTOR pathway (Mahajan, 1994; Chapter 3 and 4) and the effects of rapamycin on reducing pol III transcription was found to be a Maf1-dependent response in yeast (Upadyha *et al.*, 2002; Desai *et al.*, 2004), rapamycin was used to treat cells to determine if Maf1 phosphorylation was under the control of the mTOR pathway. Cells growing in a medium containing 10% serum were transfected with the construct pCDNA3HA.Maf1. They were then transferred to phosphate-free DMEM 48hrs later, and incubated for 3hrs with [^{32}P] orthophosphate in the presence or absence of rapamycin. Extracts were immunoprecipitated with HA antibody, run on SDS-PAGE gels and either subject to autoradiography or immunoblotted for HA. Phosphorylation of Maf1 decreased greatly in the presence of rapamycin (Fig. 5.9A upper panel, compare lanes 2 and 3). In fact, quantification of [^{32}P]-labelled HA.Maf1 and immunoprecipitated HA.Maf1 shows

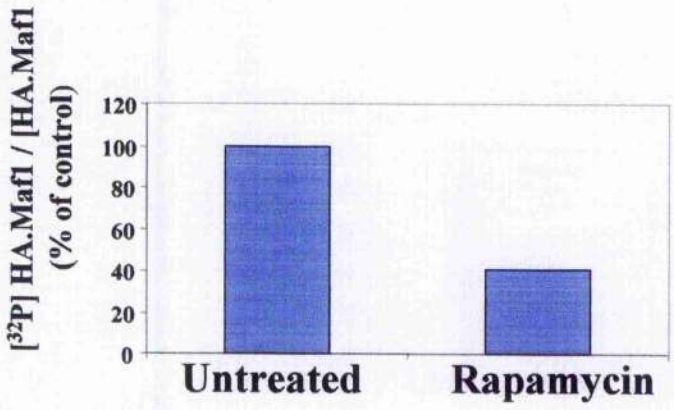
that rapamycin-treated cells had 55% less Maf1 phosphorylation than those cells grown in the presence of serum (Fig. 5.9B). This result suggests that during growth, mTOR signalling results in the phosphorylation and inhibition of Maf1. However, when mTOR signalling is blocked, Maf1 is de-phosphorylated and acts as a negative effector of pol III transcription. This finding is inconsistent with the *in vitro* kinase result, whereby recombinant Maf1 could still be phosphorylated when incubated with rapamycin-treated extracts (Fig. 5.8A). This difference could be due to the protein extraction method and will be discussed further.

To investigate whether the mTOR signalling pathway can influence the interaction between Maf1 and pol III, co-immunoprecipitations were carried out in CCL39 cells which were transiently transfected with pCDNA3-HA.Maf1. In asynchronously growing cells, Maf1 stably associates with the BN51 subunit of pol III (Fig. 5.9C, lane 1). Rapamycin treatment for 4 hrs does not prevent Maf1 binding to pol III (Fig. 5.9C, lane 3). This effect is specific, since immunoprecipitations with control antibodies showed no interaction between HA.Maf1 and pol III (Fig. 5.9C, lanes 2 and 4). The results presented in Figure 5.9 suggest that the mTOR pathway does not regulate the interaction between Maf1 and pol III, and that pol III can associate with Maf1 in its active (dephosphorylated), or inactive (phosphorylated) state.

A


HA.Brf1 : + - -
HA.Maf1: - + +
IP for HA : + + +
Rapamycin : - - +
[32P]HA.Brf1
[32P]HA.Maf1
HA.Brf1
HA.Maf1
1 2 3

B


[32P] HA.Maf1 / [HA.Maf1 (% of control)
120
100
80
60
40
20
0
Untreated
Rapamycin

C

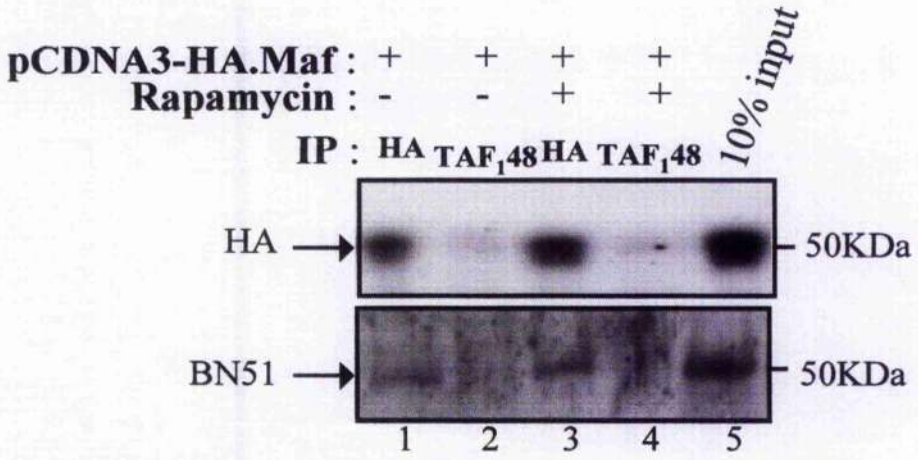


Figure 5.9: **Maf1 phosphorylation is controlled by the mTOR pathway**

(A) CCL39 cells growing in 10% serum were transiently transfected with pCDNA3HA.Brf1 (lane 1) or pCDNA3-HA.Maf1 (lanes 2 and 3) and labelled 48 hrs later with [^{32}P] orthophosphate for 3 hrs in the absence (lanes 1 and 2), or presence (lane 3) of rapamycin (100nM). Cell extracts were prepared and Brf1 or Maf1 was immunoprecipitated with an anti-HA antibody, resolved by SDS-PAGE, and either visualised by autoradiography (upper panel, lanes 1-3) or transferred to PVDF membrane followed by western blotting with anti-HA (lower panel, lanes 1-3). Results are representative of one experiment.

(B) The phospholabelling result described in (A) was quantified by densitometry and values normalised to the immunoprecipitation. The graph shows quantification from one experiment, where the cells grown in media containing serum are designated 100% and the cells that are rapamycin-treated are calculated as a percentage of this.

(C) CCL39 cells transiently transfected with pCDNA3HA.Maf1 were incubated for 4hrs with vehicle (lanes 1 and 2) or rapamycin (100nM; lanes 3 and 4). Cell extracts (500μg) were immunoprecipitated with anti-HA antibody (lanes 1and 3) or anti-TAF$_1$48 antibody as a control (lanes 2 and 4). Precipitates were resolved by SDS-PAGE and immunoblotted with anti-HA antibody (upper panel) or with anti-BN51 (lower panel). Lane 5 shows a 10% input of the vehicle –treated cell extract as a size marker. Blots are representative of two independent experiments.

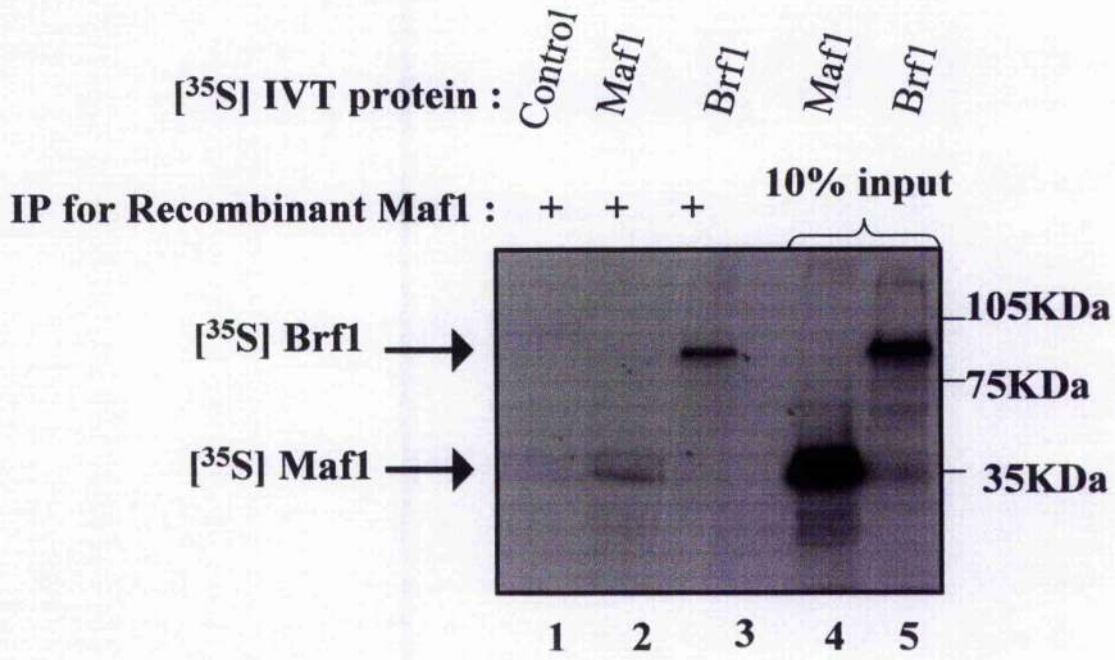
5.2.11 Maf1 interacts with Brf1 *in vitro*

The add-back experiment suggests that Maf1 may target a component of TFIIIB in order to act as a repressor of pol III transcription (Fig. 5.7). This possibility was tested in pull-down assays using recombinant Maf1 bound to Ni^{2+}-NTA-resin. Radiolabelled Maf1, Brf1 or luciferase control were synthesised *in vitro* by translation with a reticulocyte lysate in the presence of [^{35}S]-Methionine and [^{35}S]-Cysteine. These were incubated with His.Maf1.His (pre-bound to Ni^{2+}-NTA-resin), under stringent ionic conditions to prevent non-specific binding. After extensive washing, the bound proteins were analysed by SDS-PAGE and autoradiography (Fig. 5.10A). [^{35}S]-Maf1 was found to bind to Brf1 but not to control luciferase, indicating a specific interaction between Maf1 and Brf1 under these conditions (Fig. 5.10A, compare lanes 1 and 3).

A variation of the pull-down experiment was carried out to further test the interaction of Maf1 and Brf1 *in vitro*. Unlabelled HA.Brf1 was bound to anti-HA insoluble agarose beads. [^{35}S]-Maf1 or [^{35}S]-STP3 control were incubated with Brf1-HA resin under stringent ionic conditions to prevent non-specific binding. After extensive washing, the bound proteins were analysed by SDS-PAGE and autoradiography. [^{35}S]-Maf1 was found to bind Brf1 (Fig. 5.10B, lane 1) but did not bind to [^{35}S]-labelled luciferase control (Fig. 5.10B, lane 2), indicating the specificity of the interaction. [^{35}S]-labelled Maf1 and Brf1 were loaded as size markers (Fig. 5.10B, lanes 4 and 5).

In an attempt to determine which region of Brf1 that Maf1 bound to, a pull-down assay was carried out with recombinant fragments of the Brf1 protein. The amino terminal half of Brf1 shares a high degree of sequence similarity with TFIIB, hence these domains are referred to as TFIIB repeat domains (I and II) (see Fig. 5.10C). The C-terminal half comprises of three Brf1 homology domains which are responsible for most of its binding affinity with TBP (Wang and Roeder, 1995). Zinc finger-, H1- and H2-GST fusion

A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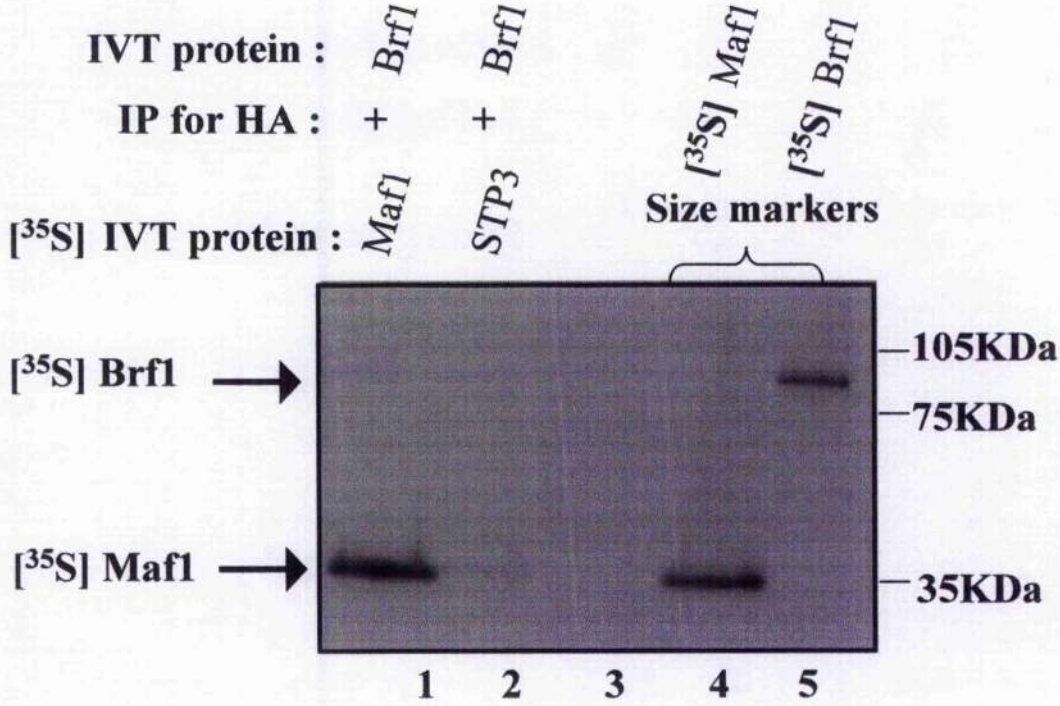
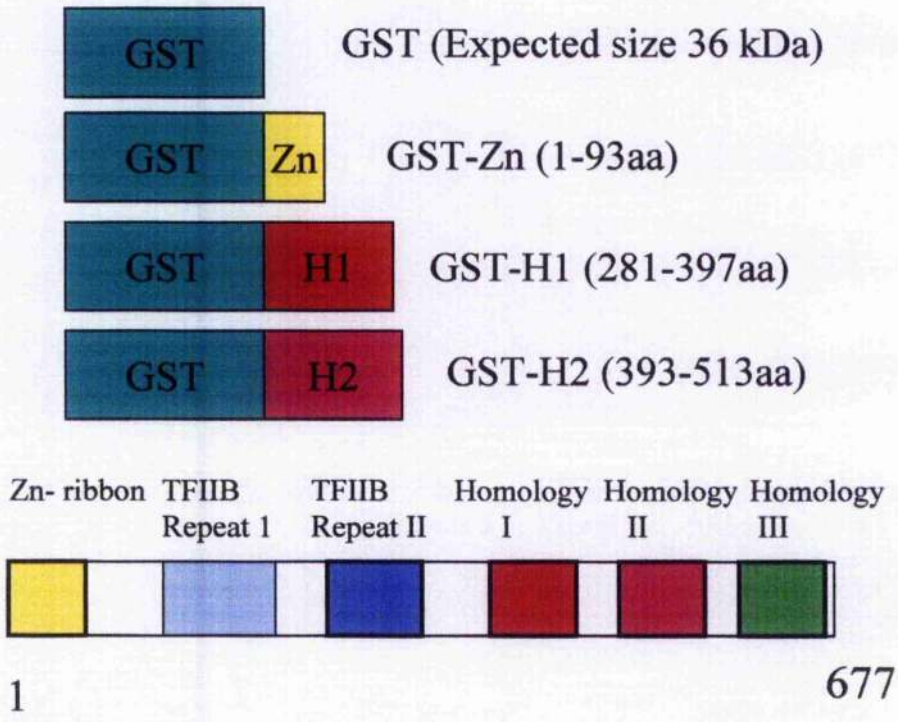
Control
Mafl
Brfl
Mafl
Brfl
IP for Recombinant Maf1 : + + +
10% input
[35S] Brf1
[35S] Maf1
105KDa
75KDa
35KDa
1 2 3 4 5

B


IVT protein : Brfl Brfl
IP for HA : + +
[35S] IVT protein : Mafl STP3
[35S] Mafl
[35S] Brfl
Size markers
[35S] Brf1
[35S] Maf1
105KDa
75KDa
35KDa
1 2 3 4 5

C


GST
GST (Expected size 36 kDa)
GST
Zn
GST-Zn (1-93aa)
GST
H1
GST-H1 (281-397aa)
GST
H2
GST-H2 (393-513aa)
Zn- ribbon
TFIIB Repeat 1
TFIIB Repeat II
Homology I
Homology II
Homology III
1
677

D

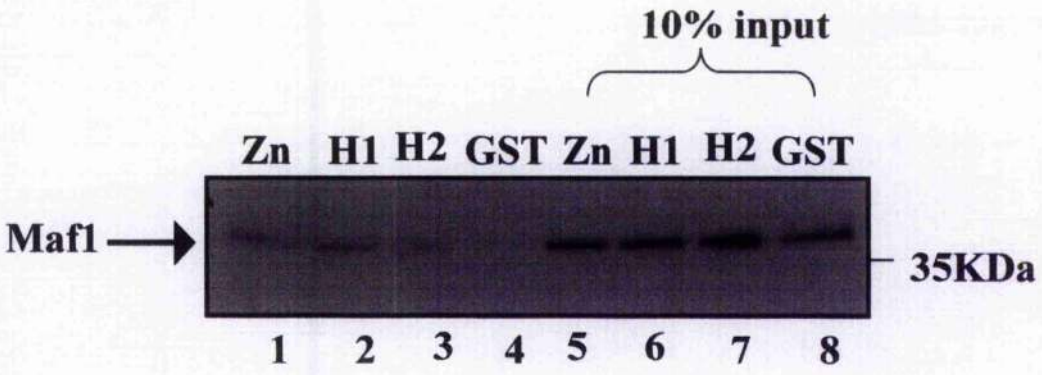

10% input
Zn H1 H2 GST Zn H1 H2 GST
Maf1
35KDa
1 2 3 4 5 6 7 8

Fig 5.10: **Recombinant Maf1 associates with Brf1 *in vitro***

(A) [^{35}S]- Methionine-labelled Maf1 (lane 2), luciferase (lane 1) or Brf1 (lane 3) prepared using *in vitro* transcription and translation with reticulocyte lysate were incubated with Ni^{2+}- bound MmMaf1.His at 4°C for 3 hrs under stringent ionic conditions. After extensive washing the bound proteins were analysed by SDS-PAGE and subjected to autoradiography. Lanes 4 and 5 are 10 % inputs of [^{35}S]-labelled Maf1and Brf1used in the pull down assay. Results are representative of more than three independent experiments.

(B) [^{35}S]- Methionine-labelled Maf1 (lane 1) or luciferase (lane 2) prepared using *in vitro* transcription and translation with reticulocyte lysate was incubated at 4°C for 3 hrs with Brf1-HA resin under stringent ionic conditions. After extensive washing the bound proteins were analysed by SDS-PAGE and subjected to autoradiography. [^{35}S]-labelled Maf1 and Brf1 were loaded as size markers (lanes 4 and 5). Results are representative of more than three independent experiments.

(C) The structural motifs of Brf1. Sequence similarity of its N-terminal half with TFIIB and three conserved regions in the C-terminal half are indicated.

(D) Zinc finger-, H1- and H2-GST fusion proteins of Brf1 produced from bacterial cultures were immobilised onto glutathione-agarose beads. Pre-cleared Recombinant Maf1 protein was incubated at 4°C with each of the Brf1-GST fusion proteins (lanes 1-3) and with GST alone (lane 4) for 30mins in a pull down assay. After stringent washes, samples were subjected to SDS PAGE and coomassie-stained. [^{35}S] signals were amplified and gels dried and exposed to film. Inputs of the Brf1-GST-fusion proteins are shown in lanes 5-8. Results are representative of three independent experiments.

proteins produced from bacterial cultures were immobilised onto glutathione-agarose beads. Radiolabelled Maf1 was synthesised *in vitro* by translation with a reticulocyte lysate in the presence of [^{35}S]-Methionine and [^{35}S]-Cysteine. [^{35}S]-Maf1 protein which had been pre-cleared with glutathione-agarose beads was incubated with each of the Brf1-GST fusion proteins and with GST alone. After stringent washes to prevent non-specific binding, samples were subjected to SDS-PAGE and coomassie-stained. [^{35}S] signals were amplified and gels dried and exposed to film. Maf1 bound to H1 most strongly, although some interaction with the Zn-ribbon region was observed (Fig 5.10D, lanes 1 and 2). This effect was specific, since Maf1 did not bind to GST alone and inputs show that equal amounts of the Brf1-GST-fusion protein were used (Fig. 5.10D, lanes 5-8).

5.3 Discussion

Maf1 repression of pol III was investigated in mammalian cells where it was found to repress transcription of all class III genes, both *in vitro* and *in vivo*. Phospho-labelling and *in vitro* kinase assays determined that Maf1 is a phosphoprotein. Moreover, serum-starvation led to reduced Maf1 phosphorylation and decreased occupancy of Maf1, pol III and Brf1 on tRNA^{Leu} promoters. Maf1 was found to repress pol III transcription whether it was added before, during or after initiation complex assembly, and *in vitro* experiments determined Maf1 interactions with a component of TFIIIB as being central to this regulation. Further *in vitro* studies identified that the Brf1 subunit of TFIIIB is a binding partner of Maf1. Consistent with these findings, TFIIIB has previously been identified as a likely target of repression by rapamycin-treatment, CPZ-treatment and DNA damage in yeast (Zaragoza *et al.*, 1998; Ghavidel *et al.*, 2001; Desai *et al.*, 2004). Limited studies with rapamycin show that phosphorylation of Maf1 is reduced in mammalian cells when the mTOR signalling pathway is blocked (Fig. 5.9A). Since Maf1 was found to interact

with Brf1 and with pol III, this places Maf1 near or at the end of at least two signalling pathways, and suggests that Maf1 may be a common mediator of stress signals.

An early study carried out in yeast, demonstrated that maf1-1 deletion mutants had increased levels of tRNAs and this correlated with increased tRNA synthesis rate *in vitro* (Pluta *et al.*, 2001). In light of this, a Maf1 construct was transfected into cells to find if it could repress levels of pol III transcripts in a mammalian system. All class III genes were found to be repressed by Maf1 (Fig. 5.1), a finding which was backed up by *in vitro* findings (Fig. 5.5). In fact, it was determined that Maf1 could repress transcription from all three promoter types at low ng quantities (Fig 5.5), without effects on pol I transcription (Fig. 5.4 and 5.5), which strengthens the notion of Maf1 being a highly specific and potent inhibitor of pol III transcription. All three promoter types are repressed by Maf1, which suggests that the polymerase may be targeted by this protein, since this is the common factor to all three promoters. However, the addback experiments show that adding more pol III made no difference to Maf1-mediated repression. Therefore, Maf1 may have more than one target.

ChIP data established that serum-starvation reduced the occupancy of Maf1 at $tRNA^{Leu}$ promoters, which coincided with reduced occupancy of pol III and TFIIIB (Fig. 5.3A and B). These findings suggest that Maf1 may be directly bound to one or both of these factors in mammalian cells; indeed support for associations of Maf1 with both Brf1 and pol III have been reported in yeast (Pluta *et al.*, 2001; Upadhya *et al.*, 2002; Desai *et al.*, 2004). Interestingly, reports in yeast find that Maf1-dependent transcriptional repression of pol III by CPZ-treatment does not detectably affect the occupancy of TFIIIC but does reduce the occupancy of Brf1 and Bdp1 (Desai *et al.*, 2004), which is in agreement with the ChIP data found from serum-starving mammalian cells (Fig. 5.3A and B). A striking reduction in polymerase occupancy was noted on tRNA genes after CPZ-treatment (Desai *et al.*, 2004),

which was also consistent with the data obtained in serum-starved cells (Fig. 5.3). Polymerase occupancy was not reduced after CPZ-treatment of a Maf1-deletion strain, and therefore it was concluded that Maf1 is required for reduced pol III occupancy on tRNA genes (Desai *et al.*, 2004). The pattern of pol III, TFIIIB and Maf1 occupancy observed in serum-rich and serum-starved fibroblasts is similar, which first suggested an association of Maf1 with one or both of these components in a mammalian system.

Using siRNA to target Maf1 does increase pol III transcript levels (Fig. 5.2A). However, despite successful knockdown of Maf1 at the mRNA level, a corresponding change in protein levels was not observed (Fig. 5.2B), possibly due to a long half-life of the protein. Changes to the siRNA Maf1 method may allow future success in the knockdown of Maf1 and allow Maf1-dependent processes to be identified.

Fully-formed pol III transcription complexes are extremely stable and support multiple rounds of transcription without dissociating from the template (Lassar *et al.*, 1983). However, Maf1 is able to repress transcription when added before, or during initiation complex assembly and to a slightly lesser extent when added to pre-initiation complexes that had already assembled onto the adenoviral VA1 gene (Fig. 5.6), suggesting that fully formed complexes are somewhat protected from the inhibitory effects Maf1. It has been suggested that in yeast, Maf1 may function as a stochiometric inhibitor of pol III complex assembly through its interactions with Brf1 (Desai *et al.*, 2004). Consistent with this, TFIIIB fractions were found to restore transcription of Maf1-treated extract (Fig. 5.7) which provided the possibility that the activity of one or more components of the TFIIIB subunits was compromised in serum-free extracts. Indeed this is the case in yeast, where changes in the activity of Brf1 underscored transcriptional differences between untreated and CPZ-treated extracts (Desai *et al.*, 2004).

A specific interaction between Maf1 and Brf1 was demonstrated by *in vitro* pull-downs and this is consistent with pull-down assays in yeast, where recombinant Brf1 associated with [^{35}S]-labelled Maf1 *in vitro* (Desai *et al.*, 2004). The Zn-ribbon and H1 regions of Brf1 were found to interact with Maf1 by *in vitro* pull-down. Unfortunately, all the regions of full length Brf1 were not available at the time of carrying out this experiment; therefore, it is possible that Maf1 also interacts with additional regions of Brf1. It is known from work in mammalian systems that Brf1 can be both activated and inhibited by phosphorylation (Johnston *et al.*, 2002; Fairley *et al.*, 2003; Felton-Edkins *et al.*, 2003a). Therefore, it remains possible that through its interaction with Maf1, the activity of Brf1 could be targeted for post-translational modifications. Interestingly, the phosphorylation of Maf1 was found to be serum-dependent (Fig. 5.10A), and since serum-starvation is known to decrease pol III transcription (Mauck and Green, 1974), this links Maf1 activity with the regulation of pol III transcription.

Studies in yeast have placed Maf1 downstream of the TOR pathway (Upadhya *et al.*, 2002), however, the rapamycin-treated fibroblast cell extracts used in the *in vitro* kinase assay remained able to phosphorylate recombinant Maf1 (Fig. 5.8). In contrast, the *in vivo* phosphorylation of Maf1 was reduced when cells were treated with rapamycin (Fig. 5.9A). This apparent contradiction in findings may be explained by the recent deduction that the mTOR/raptor complex is sensitive to disruption by detergents (Fingar and Blenis, 2004). This review of recent experimental findings suggests that the *in vitro* kinase activity of mTOR may not correlate with the activation of downstream targets *in vivo*; therefore, protein should be extracted under different conditions to preserve the mTOR/raptor association. Also, the detergent-soluble extracts that were used in the *in vitro* kinase assay would contain rapamycin-sensitive as well as insensitive enzymes and so there may well be other kinases present in these crude extracts that could have phosphorylated the recombinant Maf1. In support of this, the *in vivo* labelling assay showed that rapamycin

does not completely block Maf1 phosphorylation and so other rapamycin-insensitive kinases must also act to phosphorylate Maf1. In conclusion, these data indicate that the control of pol III transcription by the mTOR pathway may be via Maf1.

In summary, Maf1 represses transcription of all class III genes in mammalian cells and this repression can be relieved by the addition of a purified fraction of TFIIIB. Maf1, pol III and Brf1 follow the same pattern of promoter occupancy on $tRNA^{Leu}$ genes in response to stress; moreover, Maf1 was found to interact directly with both pol III and Brf1. These data, taken together with the finding that phosphorylation of Maf1 is inhibited by both serum-starvation and rapamycin-treatment of cells, suggests that Maf1 lies near to or at the end of a stress signalling pathway. Therefore it is possible that Maf1 receives signals from this signalling pathway to co-ordinate pol III activity, and hence the growth capacity of the cell, with nutrient availability.

Chapter 6

Final Discussion

6.1 Introduction

In yeast and higher eukaryotes, cell growth is co-ordinately regulated with transcription of the protein synthetic machinery by pols I and III (Reviews by Grummt, 2003; White, 2004b). The effectors of this co-ordinate regulation include p53, RB and its relatives p107 and p130. These repressors function by binding directly to components of the pol I and pol III transcription machinery and thereby preventing protein–protein interactions necessary for transcription. In addition, specific components of the pol I and pol III machinery were found to be substrates for phosphorylation by the MAPK ERK signalling pathway (Stefanovsky *et al.*, 2001; Zhao *et al.*, 2003; Felton-Edkins *et al.*, 2003a), providing a connection between growth factor signalling and the biosynthetic pathways that underlie growth.

Further studies have identified that, in addition to growth factor signalling by the ERK pathway, the mTOR pathway can also couple production of the biosynthetic machinery with the needs of the cell. mTOR has been found to co-ordinate pol I transcription in mammals with the availability of nutrients and/or growth factors (James and Zomerdijk, 2004). Since the mTOR signalling pathway regulates components of pol I (Hannan *et al.*, 2003; Mayer *et al.*, 2004), and there appears to be a common pol I target for growth factors and nutrient-dependent activation of ribosome biogenesis (Wang *et al.*, 2001; Mayer *et al.*, 2004), it has been suggested that cross talk of the ERK and mTOR signalling pathways may be facilitated by components of the pol I machinery.

Since pol I transcription is regulated by mTOR (Hannan *et al.*, 2003; Mayer *et al.*, 2004), the initial objectives of this project were to establish if pol III transcription is also under the control of the mTOR signalling pathway, and to investigate possible mechanisms by which this regulation occurs. A further objective, which developed during the course of this work, was to identify whether Maf1 is a negative regulator of pol III transcription in mammalian

cells and to investigate how this regulation is achieved. This thesis describes how each of these aims has been met.

6.2 mTOR and PI3K inhibit pol III transcription

By using the inhibitors LY294002 and rapamycin, pol III transcription was shown to be under the control of both the PI3K and mTOR signalling pathways. This conclusion was drawn from evidence based on Northern analysis, *in vitro* transcription data, and RT-PCR and primer extension analysis (Chapter 3). Whilst all the available evidence showed that this down-regulation in pol III transcription occurs within a few hours of blocking the mTOR and PI3K pathways, the RT-PCR analysis, in particular, allowed some clue as to how this regulation was occurring. This was due to the finding that inhibition of the mTOR and PI3K pathways results in promoter-specific decreases in RNA levels. Those transcripts driven by type I and type II pol III promoters had decreased RNA levels, whereas levels of RNA driven by a type III promoter remain unaffected after incubation with LY294002 or rapamycin (Fig. 3.7). These findings were the first to suggest that the TFIIIB component Brf1, and/or TFIIIC are targets of the PI3K and/or mTOR pathways, since type III promoters do not utilise Brf1 or TFIIIC.

As PI3K lies upstream of mTOR, LY294002 would prevent signalling through mTOR; however, there is also an mTOR-independent effect, since Northern analysis shows that the combination of rapamycin and LY294002 causes a further inhibition of pol III transcription than either drug added alone (Fig. 3.3). The PI3K inhibitor LY294002 could be exerting these effects through modulation of CK2 activity (Davies *et al.*, 2000) and /or ERK signalling, as these are published regulators of pol III transcription (Johnston *et al.*, 2002; Felton-Edkins *et al.*, 2003a). Alternatively, partial inhibition of the PI3K and mTOR pathways may occur when LY294002 or rapamycin are added individually. If this is the

case, a more complete inhibition of the mTOR pathway may occur when both inhibitors are added together.

6.3 Rapamycin-mediated inhibition of pol III transcription is not due to decreased abundance of TFIIIB or TFIIIC, or decreased activity or abundance of pol III

Previous reports have demonstrated that pol III transcription factors can become over-expressed during transformation or infection by viruses, which serves to increase pol III transcriptional output (Felton-Edkins and White, 2002; Johnson *et al.*, 2003). In contrast, specific transcription factors are decreased when cells differentiate, resulting in a down-regulation of pol III transcription (Alzuherri and White, 1999). In light of this, Western analysis was used in an attempt to clarify whether the inhibition of the PI3K or mTOR pathways results in a decrease in the abundance of TFIIIB or TFIIIC. This was not found to be the case (Fig 3.9 and 3.11). Moreover, the abundance and activity of pol III was also unaffected by inhibition of the mTOR and PI3K pathways (Fig. 3.10 and 3.12). Several alternative possibilities by which this inhibition occurred were considered. For example, the activity of one or more of the transcription factors might be compromised, mTOR signalling might be modulating protein-protein interactions between either the basal transcription machinery or co-regulators of pol III transcription, or signalling through mTOR could be modulating the accessibility of DNA.

6.4 Rapamycin-mediated inhibition of pol III transcription is not due to RB, c-Myc or ERK signalling

As in yeast, mTOR is required for cell cycle progression, and inhibition of mTOR activity by rapamycin arrests cells in the G1 phase of the cell cycle (Abraham and Weiderrecht 1996; Jacinto and Hall, 2003). Expression of a rapamycin-resistant mutant of mTOR alleviates the effect of rapamycin on cell cycle progression (Hay and Sonenberg, 2004). This effect of mTOR on cell cycle progression is mediated, at least in part, by the increased translation of mRNAs encoding positive regulators of cell cycle progression, such as cyclin D1 and c-Myc, and by decreased translation of negative regulators of the cell cycle, such as $p27^{Kip1}$ (Gera *et al.*, 2004). In agreement with these findings, c-Myc levels decrease when cells are exposed to rapamycin (Fig. 4.7), and RB phosphorylation was reduced (Fig. 4.3), most likely due to a decrease in cyclin D1 levels. However, inhibition of the pol III system occurred before these effects on known regulators of pol III transcription (Fig. 3.5 and 3.7). This suggests that the initial inhibitory effects of rapamycin are independent of c-Myc and RB.

As c-Myc is a transcription factor, a possibility to consider is that its activity rather than its abundance is modulated by rapamycin treatment. Endogenous c-Myc is a regulator of pol III transcription at physiological concentrations, as demonstrated by using knock-out fibroblasts and RNAi, and occupies tRNA and 5SrRNA promoters (Felton-Edkins *et al.*, 2003b; Gomez-Roman *et al.*, 2003). However, c-Myc appears to be recruited by protein-protein interactions with TFIIIB, rather than direct DNA recognition by c-Myc (Gomez-Roman *et al.*, 2003). c-Myc's role as a regulator of pol II transcription has been well-documented, but it remains possible that c-Myc may recruit cofactors to modulate the activity of pol III, in addition to its regulation of pol III through TFIIIB. Co-factors function, in part, by affecting chromatin structure through their associated enzymatic activities, including ATPases/ helicases, histone acetyl transferases (HATs) and histone

deacetylases (HDACs) (Sudarsanam and Winston, 2000) and c-Myc dependent activation of (pol II) target genes is associated with an increase in histone H3 and histone H4 acetylation (Frank *et al.*, 2001). Therefore, further points for investigation include examining whether rapamycin treatment prevents H3 and H4 acetylation by c-Myc or stimulates the recruitment of HDACs.

6.5 Rapamycin-mediated inhibition of pol III is cell cycle-independent

In addition to data which suggested that rapamycin-mediated effects occurred before effects on the pol III and cell cycle regulators c-Myc and RB, experiments with cardiomyocytes demonstrated that rapamycin can block pol III transcription independently of the cell cycle (Fig. 4.5), as these cell types grow but do not divide. To verify this conclusion, FACS analysis could be used to ensure that fibroblast cells were cycling normally during the time points when decreased levels of pol III transcription were observed.

6.6 Regulation of pol III transcription by amino acid availability

Amino acids positively regulate mTOR signalling and activate S6K1. Conversely, amino acid deprivation impairs the activity and the phosphorylation of S6K1 (Hara *et al.*, 1998; Lynch *et al.*, 2001). Since these effects can be mimicked by rapamycin this suggests that the effects of amino acids are mediated via the mTOR pathway. The data in this thesis show that depriving cells of amino acids results in down-regulation of pol III transcription (Fig. 3.13 and 3.14), suggesting that the amino acid sensitivity of pol III transcription is mediated by mTOR. This effect was independent of RB, c-Myc and ERK signalling (Figs. 4.4, 4.6 and 4.7), and was not due to decreased abundance of TFIIIB, TFIIIC or pol III

(Fig. 3.15). Further investigation into this control may involve both looking at the interactions of the transcription factors with each other and pol III through co-immunoprecipitations and investigating the phosphorylation status of pol III and its transcription factors in response to amino acid deprivation. Also, the activity of the polymerase was not assayed and this could be a mechanism by which this negative control is exerted.

6.7 Phosphorylation of TFIIIB and TFIIIC is due in part to the mTOR signalling pathway

Since changes in protein phosphorylation represent a mechanism that is frequently employed by cells to regulate transcription factor activity (Whitmarsh and Davis, 2000) and evidence exists that TFIIIB and TFIIIC require phosphorylation to be activated (Hoeffler *et al*, 1998, Felton–Edkins *et al*., 2003a), the phosphorylation status of these factors in response to rapamycin treatment was investigated. Phosphorylation of the Brf1 subunit of TFIIIB and TFIIIC110 was found to be due, in part, to the mTOR signalling pathway (Fig. 4.8B, 4.9B and Fig. 6.1). Since the abundance of these factors was not changed, (Fig. 3.9 and 3.11) they do not appear to be targeted for proteolytic degradation following phosphorylation by the mTOR pathway. Mass spectrometry would allow identification of the specific sites on Brf1 and TFIIIC110 which are phosphorylated by the mTOR signalling pathway. Subsequent point mutations of these residues into non-phosphorylatable forms could pinpoint whether the phosphorylation of these residues on Brf1 and/or TFIIIC110 is necessary for their function in activating pol III transcription. Future phospholabelling studies could also establish whether other subunits of TFIIIB and/or TFIIIC are phosphorylated by the mTOR signalling pathway. These approaches may provide insight into the molecular mechanism underlying mTOR-dependent regulation of pol III transcription.

It is possible that TFIIIB and TFIIIC undergo nucleo-cytoplasmic transport upon phosphorylation by the mTOR pathway. Studies in yeast show that TOR regulates subcellular localisation of transcription factors (Beck and Hall., 1999); in fact, the control of nuclear import/export appears to be a general mechanism by which TOR regulates transcription (Rohde *et al.*, 2001). The possibility of mTOR-regulated nuclear localisation of TFIIIB and/or TFIIIC could be further investigated using GFP-tagged constructs which could be transiently transfected and viewed under immuno-fluoresence in rapamycin-treated cells.

An important question is whether mTOR itself or a downstream kinase phosphorylates Brf1 and/or TFIIIC110. Since S6K1 is known to act downstream of mTOR (Pullen and Thomas, 1997) and primer extension analysis showed that increased S6K1 signalling increases pol III activity (Fig 4.10), there is a possibility that S6K1 directly phosphorylates Brf1 and/or TFIIIC110. If S6K1 activity does control pol III transcription, then a future experiment could involve expressing a dominant negative form of S6K1 in exponentially growing cells to find out if it can repress pol III transcription.

Alternatively, mTOR could indirectly control the activity of the pol III transcription factors by the inhibition of a phosphatase. Several Ser/Thr protein phosphatases have been identified as components of the mTOR signalling pathway, these include PP2A, PP4 and PP6 (Dennis *et al.*, 1999). Therefore, one of these phosphatases could dephosphorylate TFIIIB and/or TFIIIC subunits. A further experiment may be to inhibit these phosphatases specifically to find if they can protect either Brf1 and/or TFIIIC110 from inactivation by rapamycin during phospholabelling studies.

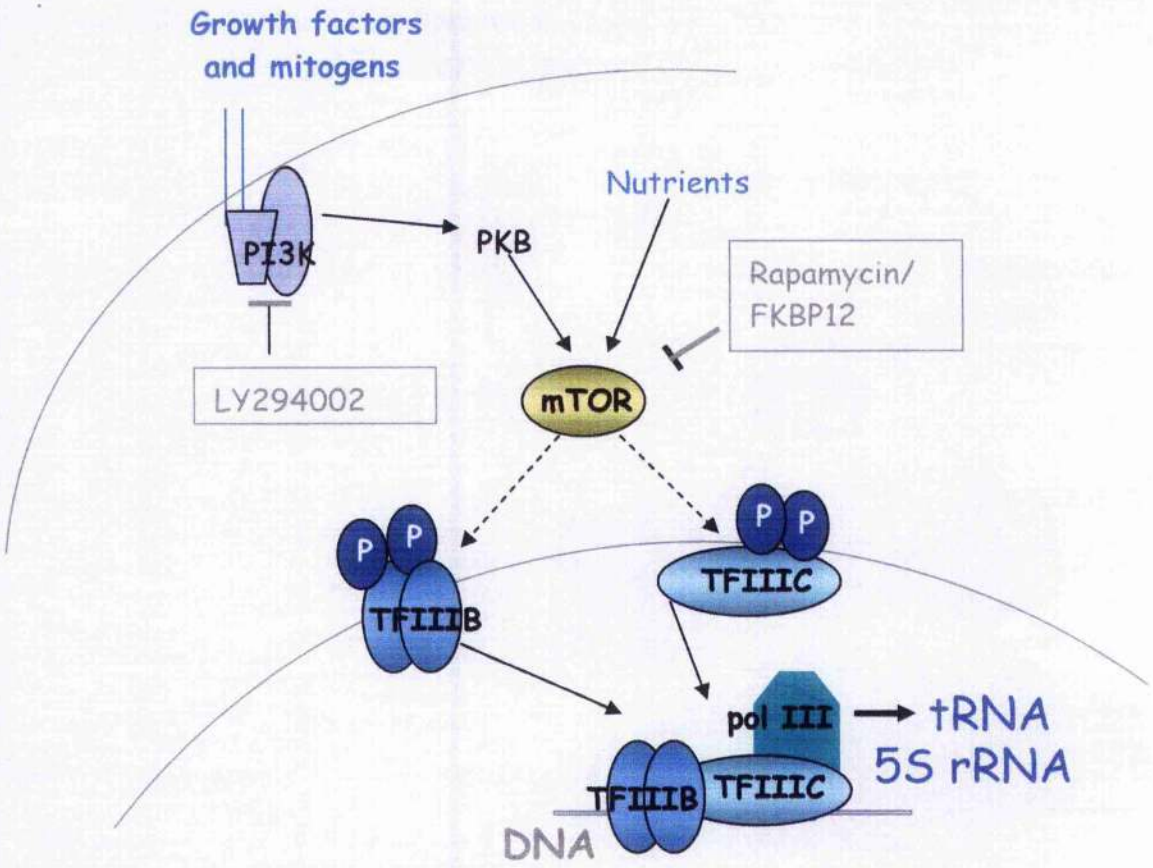


Figure 6.1: **Model of how mTOR regulates pol III transcription**

The mTOR signalling pathway in conjunction with the PI3K pathway integrates signals from both growth factors and nutrients to control pol III transcription. This may occur via the direct/indirect phosphorylation and activation of TFIIIB and TFIIIC subunits by mTOR signalling. The phosphorylation of factors may occur in the nucleus, or may occur in the cytoplasm to facilitate entry of these factors to the nucleus. Ultimately, phosphorylation may allow the interaction of these factors with each other and pol III and result in initiation of transcription at pol III templates.

6.8 The role of mTOR signalling in the modification of chromatin structure

ChIP assays show that TFIIIC, TFIIIB and pol III are excluded from pol III promoters when mTOR signalling is blocked (Fig. 4.1A/B). This may be because rapamycin-induced conformational changes of the chromatin inhibit TFIIIC's ability to occupy DNA. Bandshift analysis showed that TFIIIC retained its DNA binding affinity (Fig. 4.1D). This may suggest that the mTOR pathway can regulate pol III transcription factor binding by regulating and modifying chromatin structure. Histones are direct targets of protein kinases and are phosphorylated in response to growth factors (Mahadevan *et al.*, 1991; Thomson *et al.*, 1999). It has also been demonstrated that the activity of specific HATs can be increased or decreased by phosphorylation, whilst transcriptional repressors can participate in regulating chromatin structure through HDACs (Whitmarsh and Davis, 2000). The present study could be extended to examine HDAC promoter occupancy on pol III templates upon exposure to rapamycin. This is an attractive prospect as TOR has already been reported to regulate ribosomal gene expression by histone acetylation/deacetylation (Rohde and Cardenas, 2003). A recent genome wide study in yeast shows that an HDAC (Rpd3p) binds to the promoters of rapamycin- repressible genes following treatment with the small molecule (Humphrey *et al.*, 2004). This study is in agreement with the finding that Rpd3p is recruited to rDNA promoters to control repression following rapamycin treatment (Tsang *et al.*, 2003). These results suggest a model whereby TOR regulates gene activity via controlling the recruitment of an HDAC to the promoters of genes.

mTOR signalling was found to modulate protein-protein interactions between the basal pol III transcription complex, as rapamycin blocked the interaction between TFIIIB and TFIIIC and also between TFIIIB and pol III. Direct interaction of endogenous mTOR with the pol III apparatus was not investigated as endogenous mTOR proved difficult to coimmunoprecipitate. However, it is possible that mTOR does associate with pol III or its

regulatory factors as mTOR has been found to shuttle between the cytoplasm and the nucleus (Kim and Chen, 2000; Zang *et al.*, 2002). Nuclear shuttling of mTOR may be required for its cytoplasmic signalling to TFIIIB and/or TFIIIC. The potential downstream effects of mTOR nuclear shuttling on TFIIIB and/or TFIIIC phosphorylation could be examined if an inhibitor of nuclear export such as leptomycin B (LMB) was incorporated into a future experiment. However, it must be remembered that LMB is a general inhibitor of nuclear export and all cellular proteins undergoing Crm1-dependent nuclear export would be affected by this drug.

6.9 Rapamycin inhibition of pol III transcription may be partially mediated by S6K1 activity

The findings reported here suggest that mTOR-mediated inhibition of pol III transcription occurs in part though S6K1 signalling (Fig. 4.10). Recent studies have shown that the activity of two mammalian pol I-specific transcription factors, TIF1A and UBF, can be modulated by the mTOR inhibitor rapamycin (Claypool *et al.*, 2004; Mayer *et al.*, 2004). However, conflicting evidence exists, which reports that TIF1A is not regulated by mTOR but instead that mTOR, via S6K1, can activate rDNA transcription through phosphorylation of the pol I transcription factor UBF (Hannan *et al.*, 2003). As UBF does not appear to be a direct substrate for S6K1, the implication is that an alternative kinase exists upstream of UBF. Since it is known that S6K1 is largely cytoplasmic (Dufner and Thomas, 1999), co-immunoprecipitations may determine whether TFIIIB and/ or TFIIIC are direct substrates for S6K1 in the regulation of pol III transcription, or whether, as in the pol I system, novel kinases exist upstream of the pol III transcription factors. Emerging and older data suggest that specific members of the mTOR family, including S6K1, may themselves be targets for oncogenic activation (Sansal and Sellers, 2004). Therefore,

subversion of S6K1 would provide a direct mechanism by which an oncogene could co-ordinately increase the activity of transcription by pols I and III and translation.

6.10 Maf1; a negative effector of pol III transcription in mammalian cells

Maf1 was found to be a negative regulator of pol III transcription in mammalian cells. Both *in vitro* and *in vivo* data indicates that Maf1 represses transcription of all class III genes (Fig. 5.1 and 5.5). An addback experiment determined that this repression can be relieved by the addition of a partially-purified fraction of TFIIIB (Fig. 5.7), which suggested that an activity within the TFIIIB fraction was able to relieve Maf1-mediated repression of pol III transcription. Although the TFIIIB fraction was gradient purified on Mono Q after an initial phosphocellulose step, it undoubtedly contains proteins other than TFIIIB. Therefore, a future experiment could be to test the effect of immunodepleting the fraction, with anti-serum against individual TFIIIB components. This would find if the fraction can still counteract the repression by Maf1 and may identify the component responsible.

TFIIIB has previously been identified as a likely target of repression in rapamycin-treated cells in yeast (Zaragoza *et al.*, 1998; Desai *et al.*, 2004). Indeed, Maf1 was found to be necessary for rapamycin-induced inhibition of pol III transcription (Upadhya *et al.*, 2002; Desai *et al.*, 2004), implying that Maf1 lies downstream of the mTOR kinases. In the present study, phospho-labelling and *in vitro* kinase assays determined that Maf1 can act as a phospho-acceptor (Fig. 5.8 and Fig. 5.9). Moreover, the phosphorylation of Maf1 is reduced in mammalian cells where the mTOR signalling pathway is blocked (Fig 5.9), suggesting that mTOR controls the phosphorylation of Maf1. This is in agreement with the finding in yeast that Maf1 is downstream of TOR. Serum-starvation also led to reduced

Maf1 phosphorylation and decreased occupancy of Maf1, pol III and Brf1 on $tRNA^{Leu}$ promoters. Since mTOR is considered a nutrient sensor, it is possible that the downstream effects of serum starvation on Maf1 phosphorylation were transduced through changes in the activity of mTOR.

Maf1 was found to inhibit pol III complex assembly whether added before, during or after complex assembly (Fig. 5.6), and *in vitro* experiments demonstrated that titrating in a TFIIIB component could relieve this inhibitory effect of Maf1 on pol III transcription (Fig. 5.7). Further studies showed that Maf1 could interact with Brf1 (Fig. 5.10) and with pol III (Fig. 5.9C). These data, together with the finding that phosphorylation of Maf1 is inhibited by both serum-starvation and rapamycin-treatment of cells, suggests that Maf1 lies at the end of a nutrient sensing signalling pathway. Therefore, it is possible that Maf1 receives signals from the mTOR signalling pathway to regulate transcription by pol III thus providing a means to co-ordinate nutrient availability with the growth capacity of the cell (see Figure 6.2).

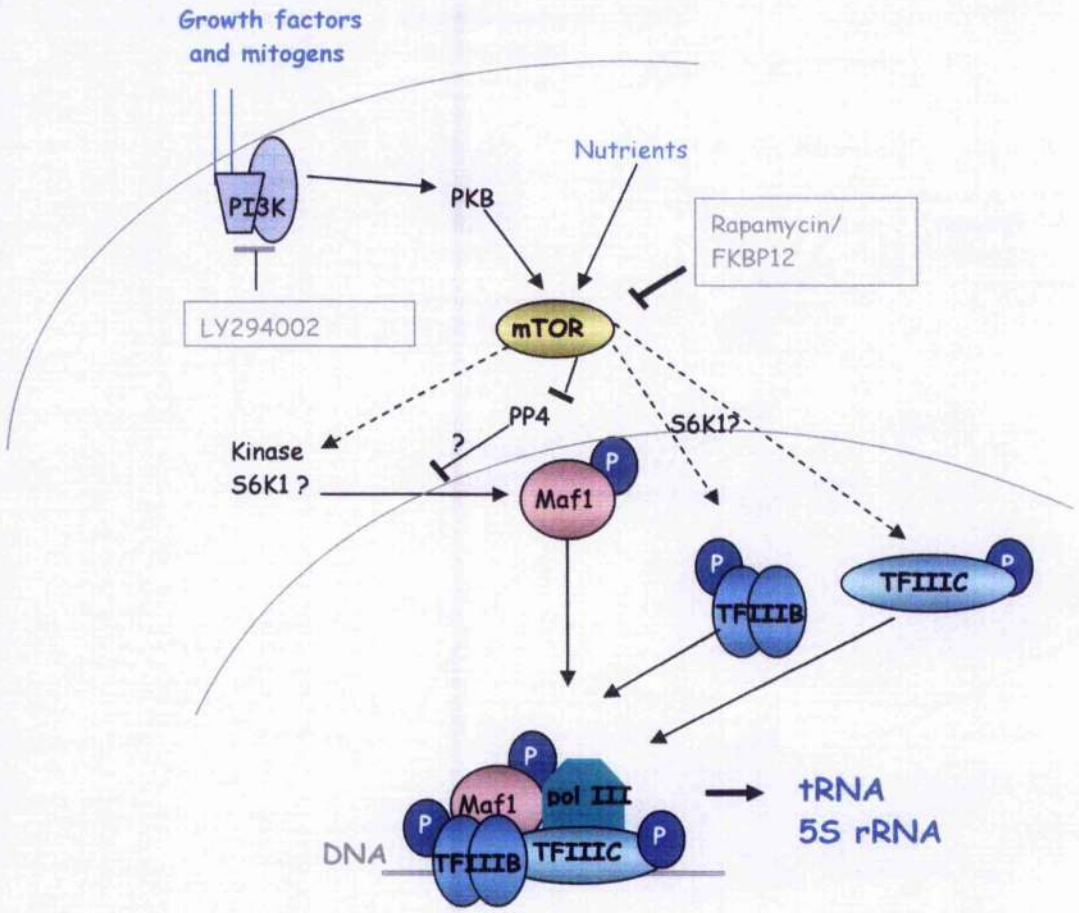


Figure 6.2: **Model of how mTOR regulates pol III transcription through Maf1**

The mTOR signalling pathway in conjunction with the PI3K pathway integrates signals from both growth factors and nutrients to control pol III transcription. This may occur via the direct/indirect phosphorylation and activation of TFIIIB and TFIIIC subunits by mTOR signalling along with the phosphorylation and inactivation of the repressor Maf1. S6K1, a downstream effector of mTOR signalling may phosphorylate Maf1 and/or the pol III transcription factors TFIIIB and TFIIIC. A phosphatase could oppose this phosphorylation but will be held inactive by mTOR signalling so providing dual control. Upon phosphorylation by the mTOR pathway, TFIIIB becomes active and Maf1 becomes inactive but the two remain associated at pol III promoters. Upon nutrient deprivation, an active phosphatase may dephosphorylate Maf1 and inhibit TFIIIB, resulting in neither of them being bound at pol III promoters. The phosphorylation of factors may occur in the nucleus, or, may occur in the cytoplasm to facilitate entry of these factors into the nucleus. Ultimately phosphorylation may allow the interaction of these factors with each other and pol III and result in initiation of transcription at pol III templates.

6.11 Importance of these findings; how mTOR signalling and pol III transcription are implicated in cancer

Historically, researchers searching for cancer therapeutics have focused on the proteins that control how a cell divides and multiplies, as opposed to those proteins involved in the regulation of cell size. This is understandable as, at a superficial level, cancer is a problem of uncontrollable cell proliferation. It has long been known that cell proliferation is dependent on cell growth but not *vice versa* (Hartwell, 1971; Nurse, 1975; Johnston, 1977). Consequently, it is widely accepted that mutations in signalling proteins regulating cell growth, in addition to those regulating cell cycle progression, contribute to the formation of tumours (Vogt, 2001).

High rates of pol III transcription are necessary for cells to sustain growth (White, 2004b), and pol III is targeted directly by transforming agents (Brown *et al.*, 2000) and the tumour suppressors p53 and RB (White, 2004b). Therefore, it is not surprising that abnormal pol III activity is a feature of cancer cells (White, 2004a). Since mTOR controls the activity of pol III transcription (Chapter 3 and 4), and components of the mTOR signalling pathway have been identified as targets for oncogenic activation (Shamji *et al.*, 2003), we can see how deregulation of mTOR signalling to the pol III transcription apparatus could be one mechanism for increasing the growth potential of the cell. This is especially relevant given that the regulation of transcription of pol I and III by mTOR is due, in part, to signalling through S6K1 (Hannan *et al.*, 2003; Mayer *et al.*, 2004; Fig. 4.10), and that the activity of S6K1 is markedly up-regulated in tumours that carry mutations in PTEN and TSC1/2 (reviewed in Ruggero and Pandolfi, 2003).

Preclinical data indicate that rapamycin and its derivatives inhibit proliferation and growth of many tumour cell lines in culture (Huang and Houghton, 2003). Moreover, mTOR inhibitors (CCI-779 and RAD001) are now considered as promising anti-cancer drugs, and

are being explored in phase II and III clinical trials. Recent reports have demonstrated their remarkable ability to suppress tumour growth (Hidalgo and Rowinsky, 2000; Neshat *et al.*, 2001; Podsypanina *et al.*, 2001). It stands to reason that without cell growth there will be no sustained proliferation, therefore, it is possible that mTOR encourages proliferation indirectly by promoting cell growth via the co-ordinate regulation of translation and transcription by pols I and III.

Appendices

Appendix 1: List of Suppliers

All materials and reagents used were of the highest quality available and were obtained from the following suppliers:

Ambion Inc. Austin, USA

siPORT Lipid Transfection Agent

siRNA oligonucleotides

Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK

α-[^{32}P] dATP

α-[^{32}P] dCTP

α-[^{32}P] UTP

[^{32}P] Orthophosphoric acid in water

ECL Western blotting detection reagents

Horseradish peroxidase (HRP)-conjugated donkey anti rabbit IgG antibody

HRP-conjugated donkey anti mouse IgG antibody

Megaprime DNA Labelling System RPN 1604/5/6/7

Protein A Sepharose

Recombinant Protein Molecular Weight Markers (Range 100000-250000)

Ultrapure NTP set (100mM).

Anachem Ltd, Luton, Bedfordshire, UK

30% acrylamide/bisacrylamide

Biomol Research Laboratories Inc. Plymouth Meeting, USA

PKB substrate peptide inhibitor (GRPRTSSFAEG)

Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK

Bradfords Reagent

Poly-Prep Chromatography Columns

BDH Laboratory Supplies, Poole, Dorset, UK

Acetic acid

Calcium chloride

Ethanol

Magnesium acetate

Magnesium chloride hexahydrate

Magnesium sulphate heptahydrate

Methanol

Potassium chloride

Sodium acetate 3-hydrate

Sodium dihydrogen orthophosphate dihydrate

Calbiochem (CN Bio-sciences) Beeston, Nottingham, UK

Hepes

LY294002

MOPS

Rapamycin

Cell Signalling Technology Inc. Beverly, USA

Antibodies (codes in Table 2.2)

Dako, Glostrup, Denmark

Peroxidase-Conjugated Swine anti-rabbit Immunoglobulins

Peroxidase-Conjugated Rabbit anti-mouse Immunoglobulins

Eurogentec, Seraing, Belgium

Antibody (code in Table 2.2)

Fisher Scientific, Loughborough, Leicestershire, UK

Ammonium acetate

Chloroform

Dimethyl sulphoxide (DMSO)

Glycine

Hydrochloric acid

Potassium hydroxide

Sodium dodecyl sulphate (SDS)

Ethylenediaminetetraacetic acid (EDTA)

Sodium Hydroxide

Fisons Chemicals, Leicestershire, UK

OptiFlow Safe1 scintillation fluid

Gibco BRL

1 x amino acids mix

dialysed Fetal Bovine Serum (dFBS)

Earle's Balanced Salt Solution (EBSS)

L-Glutamine

Optimem

Phosphate-free Dulbecco's Modified Eagle Medium (DMEM)

T4 DNA ligase and 10x ligase buffer

Life Technologies Inc.

Lipofectamine

Invitrogen, Groningen,The Netherlands

5 x First Strand Buffer

Superscript II Reverse Transcriptase

Konica Europe, GmbH, Hohenbrunn, Germany

Konica medical film

MWG Biotech, Germany

Oligonucleotide primers

New England Biolabs (UK) Ltd, Hitchin, Herfordshire, UK

Prestained protein marker, broad range (6-175)

Novagen, Madison, USA

Single Tube Protein System 3, T7 Kit

Oxoid Ltd, Hampshire, UK

Bacteriological Agar

Premier Brands UK, Knighton Adbaston, Staffordshire, UK

Marvel powdered milk

Promega, Southampton, UK

All restriction enzymes

Alkaline phosphatase

Deoxynucleotide triphosphates (dNTPs)

Klenow

Nuclease-free water

RNasin Ribonucleotide Inhibitor

T4 polynucleotide Kinase (PNK) and 10 x PNK buffer

Taq polymerase

Roche Diagnostics, GmbH, Mannheim, Germany

Creatine phosphate

Hexanucleotide mix

Yeast tRNA

Stratagene

E.Coli XL-1 Blue Supercompetent Cells

Qiagen Ltd, Crawley, West Sussex, UK

Plasmid maxi kit

QIAprep gel extraction kit

QIAprep spin miniprep kit

Santa Cruz Biotechnology Inc. Santa Cruz, California, USA

Antibodies (codes in Table 2.2)

Schleicher & Schuell UK Ltd, Brunswick Park, London, UK

Nitrocellulose membrane (0.45 μM)

Sigma Chemical Company Ltd., Poole, Dorset, U

Actinomycin D

Adenosine 5'-triphosphate (ATP)

Adenosine monophosphate (AMP)

Agarose

α-Amanitin

Ammonium sulphate

Ampicillin

Amplify

Aprotinin from bovine lung

Bestatin hydrochloride

B-glycerophosphate

Boric acid

Bovine serum albumin (BSA)

Bromophenol blue

Coomassie Brilliant Blue R-250

Ethidium bromide

Ethylenediamine-N,N,N',N'-tetra acetic acid (EDTA)

D-Glucose

Diethyl pyrocarbonate (DEPC)

Dithiothreitol

Dulbecco's Modified Eagle Medium (DMEM)

Dulbecco's Modified Eagle Medium/Medium 199 (DMEM/M199)

Dulbecco's Phosphate Buffered Saline (D-PBS)

Fetal Bovine Serum (FBS)

Formaldehyde

Formamide

G418 Sulphate

Gelatin

Glutathione-Agarose

Glycerol

Glycogen

Horse Serum (HS)

Igepal (CA-630)

Imidazole

Isoamyl alcohol

Isopropanol

N-lauroylsarcosine

Lithium chloride

Leupeptin trifluoroacetate salt

B-Mercaptoethanol

Monoclonal anti-HA agarose conjugate

Penicillin

Pepstatin A

Phenol solution

Phenylmethylsulfonyl fluoride

Proteinase K

Protein A Sepharose

Sodium acetate

Sodium chloride

Sodium citrate (dihydrate)

Sodium deoxycholate

Sodium fluoride

Sodium orthovanadate

Sucrose

Streptomycin

N, N, N', N',-tetramethylethylenediamine (TEMED)

TRI Reagent

Tris acetate

Tris base

Triton X-100

Trypsin-EDTA

Trypsin Inhibitor from glycine max (soybean)

Tween 20

Urea

Xylene cyanol FF

Upstate

PKA phospho-acceptor peptide (LRRASLG)

Whatman International Ltd., Maidstone, Kent, UK

Whatman 3mm filter paper

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