The Regulation of RNA Polymerase III Transcription by Protein Kinase CK2

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Abstract

In order for cells to proliferate, a certain size has to be reached, which depends primarily on the rate of translation. RNA polymerase (pol) III plays a key role in protein synthesis by catalysing the production of small, untranslated RNA molecules such as transfer (tRNA) and 5S ribosomal RNA (5S rRNA). Indeed, recent evidence suggests that $tRNA_i^{Met}$ production is limiting for translation and proliferation in some cell types. Therefore, the rate of pol III transcription plays a fundamental role in cellular growth and proliferation. Regulation of pol III output is mediated via a number of different mechanisms that can alter the activities of the transcription factors which are responsible for directing pol III transcription. Work presented in this thesis aimed at investigating the mechanisms behind the regulation of pol III transcription by the protein kinase CK2.

CK2 is a highly conserved protein kinase involved in cell growth and proliferation. It has been shown to stimulate transcription of tRNA genes by pol III both in *Saccharomyces cerevisiae* and mammals. Addition of a specific CK2 inhibitor, 2-dimethylamino-4, 5, 6, 7-tetrabromo-1-H-benzimidazole (DMAT) caused a decrease in the level of pol III transcripts, confirming that CK2 exerts a potent effect on mammalian pol III transcription. Furthermore, inhibition of CK2 resulted in a decrease in the promoter occupancy of pol III and its associated factor TFIIIB. Co-immunoprecipitation and GST pull-down assays show that mammalian CK2 binds to the TFIIIB subunit Brf1, an essential subunit of TFIIIB. In addition, phosphorylation assays and the use of a phosphospecific antibody reveal that Brf1 is phosphorylated at S450 by CK2.

One of the ways by which activators of pol III transcription can enhance pol III output is by promoting transcription complex formation. Brf1 is brought to target genes through interaction with the promoter-binding factor TFIIIC. Mutation of S450 to A compromises the ability of Brf1 to bind to TFIIIC, associate with promoters and activate pol III transcription in transfected fibroblasts.

Pol III output can be elevated during virus-induced cell transformation. This thesis also examined whether the increase in pol III transcription following transformation

by the *Polyomaviridae* viruses polyoma (Py) and simian virus 40 (SV40) was accompanied by changes in phosphorylation of Brf1 S450. Phosphorylation of S450 is increased during cell transformation by these *Polyomaviridae* viruses, which suggests that this phosphorylation event could contribute to the mechanisms employed by these viruses to stimulate pol III transcription.

Nuclear RNA synthesis is repressed when eukaryotic cells enter mitosis. Reversible inhibitory phosphorylation of Brf1 was proposed to be one of the mechanisms by which transcription by pol III is repressed during mitosis. Phosphosphorylation of Brf1 S450 did not change when cells entered mitosis. However, phosphorylation of Brf1 T270 by polo-like kinase 1 compromises the binding of pol III to Brf1, contributing to the mitotic repression of pol III transcription.

In conclusion, the data demonstrate that phosphorylation of Brf1 S450 by CK2 is required for the efficient recruitment of Brf1 to pol III-transcribed genes in mammalian cells. This work has provided new insight into the molecular basis of how CK2 can stimulate the expression of pol III-transcribed genes.

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Abbreviations

4E-BP1	eIF4E-binding protein
A_{260}	absorption at 260 nm
ARPP P0	acidic ribosomal phosphoprotein P0
ATP	adenosine triphosphate
bp	base pairs
Bdp1	B double prime 1
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
BSA	bovine serum albumin
C	carboxyl
CDK	cyclin-dependent kinase
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CHO	Chinese hamster ovary
CK2	casein kinase II
CRUK	Cancer Research United Kingdom
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DSE	distal sequence element
DTT	dithiothreitol
DMAT	2-dimethylamino-4, 5, 6, 7-tetrabromo-1-H-benzimidazole
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
eIF-4B	eukaryotic translation initiation factor 4B
Erk	extracellular signal-related kinase
FBS	foetal bovine serum
FCS	foetal calf serum

GST	glutathione-S-transferase
GFP	green fluorescent protein
GTP	guanidine triphosphate
HA	haemagglutinin
HA-Brf1	HA-tagged Brf1
HAT	histone acetyltransferase
HBV	hepatitis B virus
Hdm2	human double minute 2
HPV	human papilloma virus
IAA	indole acrylic acid
IRS-1	insulin receptor substrate 1
ICR	internal control region
IE	intermediate element
IPTG	isopropyl-β-D-thiogalactopyranoside
IVTs	*in vitro* transcription assays
kb	kilobase pairs
kDa	kilodaltons
Leu	leucine
LB	Luria-Bertani (media)
M	mitosis
MAP	mitogen-activated protein
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
MOPS	3-morphilinopropanesulphonic acid
mRNA	messenger RNA
MRP	mitochondrial RNA processing
mTOR	mammalian target of rapamycin
N	amino
NB	northern blot
nt	nucleotides
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethyleneimine

PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
Plk1	polo-like kinase 1
PMSF	phenylmethylsulfonyl fluoride
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
PP2A	protein phosphatase 2A
PSE	proximal sequence element
Py	polyomavirus
RB	retinoblastoma protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNase	ribonuclease
RNP	ribonucleoprotein
RT-PCR	reverse transcriptase-polymerase chain reaction
S	serine
S6K	ribosomal protein S6 kinases
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SINEs	short interspersed elements
$SNAP_c$	snRNA activating protein complex
snRNA	small nuclear RNA
snRNP	small nuclear RNP
SV40	simian virus 40
TATA	TATA box
TAF	TBP-associated factor
TBP	TATA box-binding protein
T	threonine
TF	transcription factor
TLC	thin-layer chromatography
Tris	tris (hydroxymethyl) aminomethane
TOR	target of rapamycin

TPR	tetracopeptide repeat
tRNA	transfer RNA
Tyr	tyrosine
UV	ultraviolet
WT	wild-type

Author's declaration

I hereby declare that the thesis that follows is my own composition, that it is a record of the work done myself, and that it has not been presented in any previous application for a Higher Degree.

Louise Mitchell

1 Introduction

1.1 Eukaryotic Transcription

In order for the cell to use the biological information contained within its genome, individual genes, each of which represents a single unit of information, have to be expressed in a co-ordinated manner. This co-ordinate gene expression determines the make up of cellular ribonucleic acid (RNA), which in turn specifies the nature of the proteome and defines the activities that the cell is able to carry out (Voet and Voet, 1995).

Transcription is the process during which RNA is synthesised using specific deoxyribonucleic acid (DNA) templates and is crucial to gene expression (Voet and Voet, 1995). In eukaryotes, the task of transcribing nuclear genes is divided between three highly related complex enzymes known as RNA polymerases I, II and III (commonly referred to as pol I, pol II and pol III) (reviewed in White, 2001) and a recently discovered single polypeptide fourth nuclear RNA polymerase (pol IV) (Kravchenko et al, 2005). Each of these RNA polymerases is devoted to the transcription of specific genes. Pol I is distinctive among the nuclear RNA polymerases in transcribing only one type of gene, the large tandemly repeated, ribosomal RNA (rRNA) genes [reviewed in (Grummt, 2003) and (Russell and Zomerdijk, 2006)]. Pol II transcribes the protein-coding genes which encode the messenger RNA (mRNA), as well as some small nuclear RNA (snRNA) genes [reviewed in (Thomas and Chiang, 2006)]. The genes transcribed by pol III are referred to as class III genes and encode a variety of small stable RNA molecules, including 5S rRNA and transfer RNAs (tRNAs) [reviewed in (Dieci et al., 2007)]. Pol IV is expressed as an alternative transcript of the mitochondrial RNA polymerase and is responsible for the transcription of a number of mRNAs (Kravchenko et al, 2005).

Transcription is the first step in gene expression and is subject to a wide variety of control (Voet and Voet, 1995). It allows the cell to constantly adjust in response to environmental changes and metabolic requirements (White, 2001). The regulation of transcription is implicated in almost every biological process including growth and

development (White, 2005). It is vital to understand the regulation of transcription, since defects therein are the basis of many diseases.

This thesis focuses on transcription by pol III and its regulation by the protein kinase CK2 (formerly known as Casein Kinase II). In order to gain an appreciation for this research project, this chapter will give an overview of the various functions of the products of class III genes, the transcription machinery involved in their production and how their transcription can be regulated. The involvement of CK2 in the regulation of transcription by pol III will be touched upon in this chapter and discussed further in Chapters Three, Four, Five and Six.

1.2 Class III Genes

Pol III is responsible for approximately 10% of all nuclear transcription (White, 2002). Microscopy studies of HeLa nuclei have demonstrated that pol III transcription occurs at discrete locations (~2000 sites) within the nucleoplasm (Pombo et al., 1999). The genes transcribed by pol III encode a variety of small RNA molecules [usually shorter than 400 base pairs (bp)] that are untranslated (White, 2002). These RNA molecules have essential functions in cellular metabolism (Dieci et al., 2007). Some of the major pol III transcripts are described in the following sub-sections.

1.2.1 5S ribosomal RNA (rRNA)

Eukaryotic ribosomes are composed of two subunits, which together contain four molecules of RNA (28S, 5.8S, 5S and 18S) and approximately 80 proteins (Wool, 1979). 5S is the only rRNA to be produced by pol III, while the other three rRNAs are made by pol I. The four rRNAs are required in equal stoichiometry, each being present in one copy per ribosome (Phillips and McConkey, 1976), 5S is therefore, essential for protein synthesis. As with other class III genes, 5S is transcribed in the nucleoplasm and is transported to the nucleolus where it is processed and incorporated into the large ribosomal subunit (Lafontaine and Tollervey, 2001). Eukaryotes contain multiple copies of 5S genes, ranging from 140 in *S. cerevisiae* (Elion and Warner, 1984) up to 20,000 copies in *Xenopus laevis* (Fedoroff and Brown, 1978; Miller et al., 1978). The

human genome contains 200 to 300 5S genes and numerous dispersed pseudogenes, many of which occur in tandem array (Consortium, 2001).

1.2.2 Transfer RNAs (tRNAs)

Transcription by pol III also gives rise to the tRNA molecules (73-93 nucleotides) (Sharp et al., 1984). tRNAs function as molecular adaptors which serve to translate the genetic information carried by mRNA into a particular order of amino acid residues in a protein (Creighton, 1997). When processed, a tRNA molecule adopts an L-shaped secondary structure with a 3' terminal site for amino acid attachment (Hopper and Phizicky, 2003). It also contains a three base region called the anticodon that can base pair to the corresponding three base codon region on the mRNA nucleotide sequence (Hopper and Phizicky, 2003). This ensures the accurate synthesis of the polypeptide chain encoded by the mRNA. The human genome contains 497 nuclear genes encoding cytoplasmic tRNA molecules; there is considerable redundancy as the average copy number for each amino acid tRNA adaptor is around 10 genes (Consortium, 2001). The tRNA genes are mainly scattered throughout the genome, although some clustering can be observed (Consortium, 2001).

1.2.3 U6 small nuclear RNA (snRNA)

Small nuclear ribonucleoproteins (snRNPs) are a group of structurally related RNA-protein complexes and are found in the nuclei of eukaryotic cells (Weinberg and Penman, 1968). The most abundant snRNPs are the spliceosomes (Lerner and Steitz, 1979), which are crucial for pre-mRNA processing (Lerner et al., 1980). Spliceosomes contain five snRNA species, four of which are made by pol II whereas the smallest, U6, is made by pol III (Kunkel et al., 1986). There are multiple copies of U6 snRNA genes in the human genome and there is a high degree of U6 conservation between different organisms (Consortium, 2001).

1.2.4 Other class III genes encoding RNP components

A variety of other pol III transcripts are components of ribonucleoprotein (RNP) complexes such as 7SL, 7SK, H1 and mitochondrial RNA processing (MRP) RNAs.

The signal recognition particle (SRP) is a protein-RNA complex that recognises and delivers nascent polypeptide chains to the endoplasmic reticulum in eukaryotes. The trafficking of proteins through this organelle is critical for post-translational modifications, appropriate folding and delivery to their appropriate subcellular locations (Creighton, 1997). The eukaryotic SRP is composed of six distinct polypeptides bound to an RNA molecule (the 7SL RNA). 7SL RNA forms the structural backbone of the SRP and is extremely conserved throughout evolution (Walter and Blobel, 1982).

7SK is an abundant 330 nucleotide RNA that forms part of a 12S RNP together with eight proteins (Mattaj et al., 1993; Murphy et al., 1986). 7SK was found to act as a negative regulator of pol II transcription elongation (Nguyen et al., 2001; Yang et al., 2001). This occurs as a result of 7SK binding to the elongation factor P-TEFb; a factor responsible for phosphorylation of the C-terminal domain of the largest subunit of pol II (Nguyen et al., 2001; Yang et al., 2001).

H1 is the 369 nucleotide component of RNase P, an endoribonuclease that processes the 5' termini of pre-tRNA (Bartkiewicz, 1989; Lee and Engelke, 1989). H1 is encoded by a single copy gene in both yeast (Lee et al, 1991) and humans (Baer et al., 1990).

RNase MRP is an endoribonuclease with the ability to cleave the mitochondrial transcript to generate an RNA primer for replication of mitochondrial DNA (Chang and Clayton, 1987). Despite being identified in the mitochondrion, the majority of the RNase MRP is found in the nucleolus where it plays an important role in the processing of pre-rRNA (Clayton, 2001). RNase MRP contains a 265 nucleotide RNA generated by pol III which shows several blocks of sequence homology to H1 RNA (Gold et al., 1989).

1.2.5 Viral class III genes

A number of viruses use the cellular pol III transcription machinery to transcribe short transcription units within their genomes. The best characterised of these are the adenovirus VA1 and VA2 genes; these are transcribed by pol III at high levels during late stages of viral infection (Akusjarvi et al., 1980; Soderlund et al., 1976). The VA

RNA can undermine the host's cell's translational apparatus to allow synthesis of viral proteins (Thimmappaya et al., 1982).

The genome of Epstein-Barr virus (EBV) also contains two small adjacent genes called EBER 1 and 2 that are also transcribed by pol III (Rosa et al., 1981). These small nuclear RNAs have regions of homology to the VA genes and are 166 and 172 nucleotides long, respectively. Like VA RNAs, EBERs can subvert the translational machinery of the host cell in order to promote synthesis of viral proteins (Rosa et al., 1981).

1.2.6 Short interspersed nuclear elements (SINEs)

A variety of repetitive SINEs are pol III templates in higher organisms. The major SINE in humans is the Alu family (Jelinek et al., 1980), whereas in rodents the B1 and B2 SINE families are the most abundant (Bennett et al., 1984). B1 genes are highly homologous (~80%) to Alu genes, and both appear to have evolved from the 7SL gene (Britten, 1994; Quentin, 1992); however, the B2 genes are thought to have evolved from the tRNA genes and are essentially tRNA pseudogenes (Daniels and Deininger, 1985). SINE DNA constitute a significant proportion of mammalian genomes: over a million copies of Alu are encoded by the haploid human genome (Consortium, 2001), and approximately 384 000 copies of B1 and 328 000 copies of B2 are present per haploid rat genome (Consortium, 2004). The dispersal and amplification of SINEs throughout the genome is by retrotransposition (Rogers, 1985). Little is known about the function of their encoded transcripts, although it has been suggested that some SINE families are involved in expression of adjacent genes (Hasler and Strub, 2006), splicing, translation (Pagano et al., 2007), cell stress responses (Liu et al., 1995; Chu, 1998; Li, 1999), regulation of growth or the turnover of specific mRNAs (Hasler and Strub, 2006; Pagano et al., 2007).

The selective transcription of genes by pols I, II and III is dictated by their distinct promoters. The following section describes the promoter structures specifically recognised by the pol III transcription machinery.

1.3 Promoter structure of class III genes

Gene promoters contain specific DNA sequence elements which direct the recruitment of the appropriate transcription factors and RNA polymerase via multiple protein-protein and protein-DNA interactions [reviewed in (Schramm and Hernandez, 2002)]. This sequential recruitment is required for the transcription of a particular gene (Lassar et al., 1983). One of the distinguishing features of the majority of class III gene promoters is that crucial elements are found within the transcribed region of the gene, i.e. downstream of the transcription start site in comparison to upstream promoter elements used by pol I and pol II (Bogenhagen et al., 1980; Galli et al., 1981; Kurjan and Hall, 1982). These key elements are generally discontinuous structures composed of essential blocks separated by non-essential regions (Koski et al., 1980; Kurjan and Hall, 1982). There are three types of promoters used by pol III, known as types I, II and III.

1.3.1 Type I promoters

Type I promoters are unique to 5S rRNA genes (Figure 1.1). The promoter was initially characterised in *X. laevis* (Bogenhagen et al., 1980) and requires three internal promoter elements for efficient function: the A-block (+50 to +64 bp), an intermediate element (+67 to +72 bp) and the C-block (+80 to +97 bp) collectively known as the internal control region (ICR) (Pieler et al., 1985; Bogenhagen, 1985). The spacing between these elements is restricted and conserved. Mutations or alterations in the spacing of the elements considerably reduce transcriptional efficiency (Pieler et al., 1987). This is in contrast to the flanking regions that display little conservation and show a greater flexibility to mutations (Bogenhagen, 1985; Pieler et al., 1985; Pieler et al., 1987).

1.3.2 Type II promoters

The majority of pol III-transcribed genes, including the tRNAs and adenovirus VA genes have type II promoters (Figure 1.1). These consist of two essential and highly conserved sequence elements: an A-block and a B-block found within the transcribed region (Galli et al., 1981). The A- block of type II promoters is homologous to the A-block contained within type I promoters, and in some species these elements can be

interchangeable (Ciliberto et al., 1983), although the A-block is much closer to the start site of transcription in type II promoters. In this type of promoter the spacing is variable, the A- and B- blocks are typically separated by 30-40 bp; however, a distance of up to 365 bp can still support transcription (Baker et al., 1987; Fabrizio et al., 1987).

1.3.3 Type III promoters

A minority of pol III-transcribed genes including the 7SK, MRP and U6 genes have a type III promoter (Figure 1.1) (Murphy et al., 1987). Genes with type III promoters lack any requirement for internal promoter regions, with important sequence elements being found upstream of the start site (Schramm and Hernandez, 2002). The human U6 gene is the best characterised type III promoter. For efficient transcription of U6 genes three extragenic promoter elements are required: a TATA box (-30 to -25 bp), a proximal sequence element (-66 to -47 bp) and a distal sequence element (-244 to -214 bp) (Das et al., 1988; Kunkel and Pederson, 1988; Lobo and Hernandez, 1989).

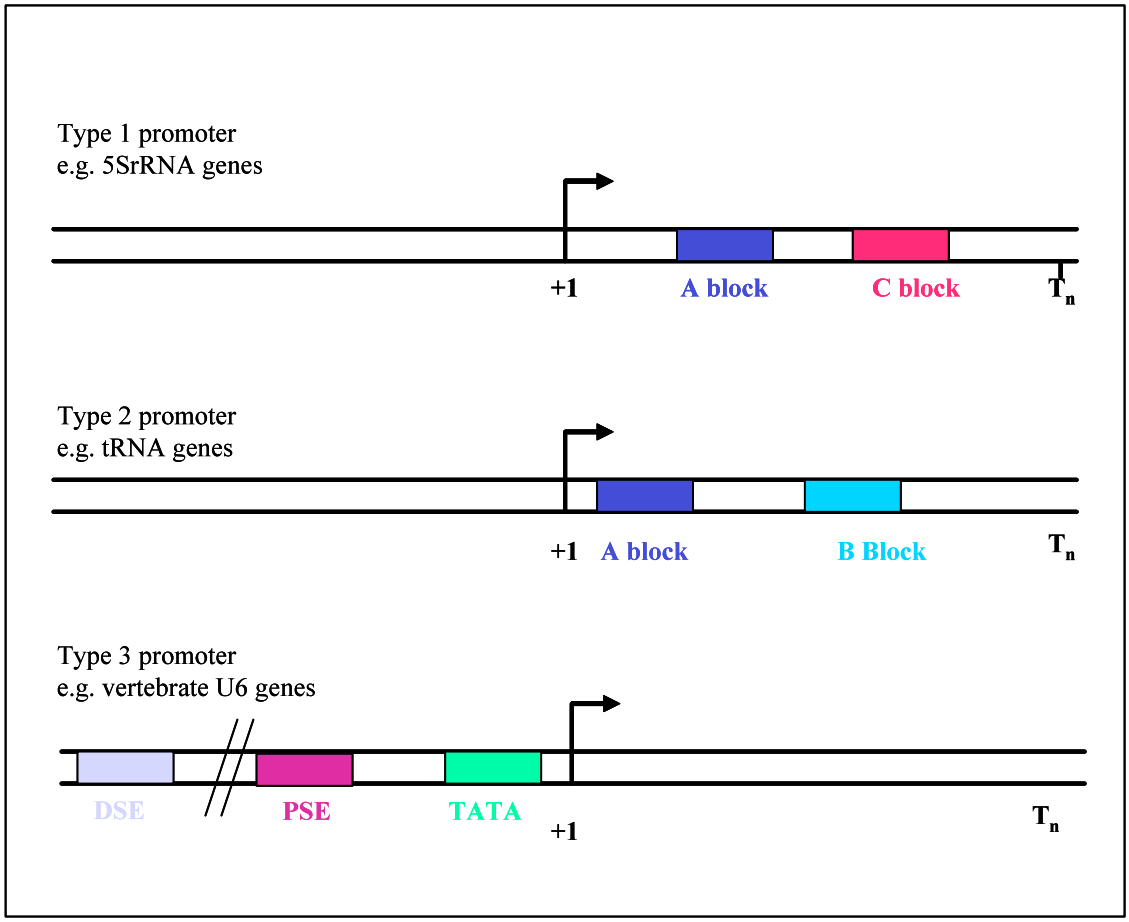


Figure 1.1 Schematic diagram of the organisation of the 3 general types of promoters used by pol III
The start site of transcription is indicated by +1 and the site of termination is indicated by T_n. Also shown are the approximate positions of various promoter elements including the intermediate element (IE), proximal sequence element (PSE) and distal sequence element (DSE) [Adapted from (White, 2001) and (Paule and White, 2000)].

All three promoter types can specify a class III gene and direct its transcription by pol III. Section 1.4 below describes the properties and functions of the various components of the transcription initiation complex required for the transcription of a class III gene.

1.4 Transcription of class III genes

1.4.1 Assembly of the transcription complex

Transcription begins with the assembly of a transcription initiation complex at the promoter. Despite the complexity of the pol III enzyme, it has little affinity for the promoter sequence and so relies on specific transcription factors which form this transcription initiation complex for its accurate recruitment. Each type of promoter has its own requirement for different transcription factors [reviewed in (Schramm and Hernandez, 2002)].

1.4.1.1 Type II promoters

The initial step in the formation of the transcription initiation complex on type II promoters is the binding of the multi-subunit pol III-specific transcription factor TFIIIC to the promoter (White, 2001). TFIIIC is one of the largest and most complex transcription factors ever studied. In *S. cerevisiae*, TFIIIC consists of six subunits TFC1 (Swanson et al., 1991), TFC3 (Lefebvre et al., 1992), TFC4 (Marck et al., 1993), TFC6 (Arrebola et al., 1998), TFC7 (Manaud et al., 1998) and TFC8 (Deprez et al., 1999). TFIIIC has an aggregate mass of more than 500 kDa (Geiduschek and Kassavetis, 2001). The subunits arrange into two globular domains capable of specifically recognising and binding directly to the A- and B-block elements of type II promoters (Schultz et al., 1989). Electron microscopy suggests that the two domains of TFIIIC can be linked by a flexible hinge which can stretch to give the appearance of a dumbbell (Conesa et al., 1993; Schultz et al., 1989). However, on promoters with long interblock separations the ability to stretch is exceeded and intervening DNA is looped out (Baker et al., 1987). Even though both A- and B-blocks are contacted by TFIIIC, the B-block is the major determinant of binding affinity (Baker et al., 1987).

Human TFIIIC is composed of six polypeptides, known as TFIIIC220, 110, 102, 90, 63 and 35 according to their molecular masses (Kovelman and Roeder, 1992; Dumay-Odelot et al., 2007). The largest subunit TFIIIC220, displays very little homology to any of the *S. cerevisiae* TFIIIC subunits and interacts specifically with the B-block (Shen et al, 1996; Yoshinaga et al., 1987). TFIIIC63 binds the A-block and strengthens

the TFIIIC-DNA interactions (Hsieh et al., 1999a). TFIIIC90 acts as the flexible linker region that bridges the two distinct sub domains formed by the other four subunits (Hsieh et al., 1999b). The more highly diverged 220, 110 and 90 kDa subunits have histone acetyltransferase (HAT) activities and may play a role in determining gene activity (Hsieh et al., 1999a; Hsieh et al., 1999b; Kundu et al., 1999).

The crucial function of TFIIIC is to recruit the initiation factor called TFIIIB, positioning this factor just upstream of the transcription start site (Teichmann and Seifart, 1995). TFIIIB consists of three polypeptides: TATA box-binding protein (TBP) and two TBP-associated factors, known as Brf1 (TFIIB-related factor 1; a 90 kDa protein named for the homology of its amino (N)-terminal half to the pol II-specific transcription initiation factor TFIIB) and Bdp1 (B double prime; a 160kDa protein which displays little homology with any known protein) (Schramm and Hernandez, 2002). Whilst TBP is needed for transcription by pols I, II and III (Cormack and Struhl, 1992), Brf1 and Bdp1 are specifically involved in the transcription of class III genes. Brf1 forms a stable complex with TBP in solution but Bdp1 is only weakly associated with this complex, if at all, in the absence of a DNA template (Huet et al., 1994; Kassavetis et al., 1991; Schramm et al., 2000). TFIIB interacts with TBP and it was expected that the TFIIB-like region in Brf1 would be responsible for interaction with TBP (Ha et al., 1993). Indeed, a weak interaction is observed at this region; however, the principal region that mediates Brf1 binding to TBP is within the C-terminal domain of Brf1 (Khoo et al., 1994).

The recruitment of TFIIIB to class III gene promoters by TFIIIC has been greatly studied in *S. cerevisiae*. The *S. cerevisiae* equivalent of human TFIIIC102 initially interacts with Brf1 (Kassavetis et al., 1992). Subsequent interactions between each of the TFIIIB subunits and various TFIIIC components are also likely to play a role in the formation of a stable pre-initiation complex (Schramm and Hernandez, 2002). In human cells, it has been established that TFIIIC90 interacts with Brf1, and that TFIIIC102 and 63 interact with both Brf1 and TBP (Hsieh et al., 1999a; Hsieh et al., 1999b). In addition, TFIIIC63 was found to interact with a pol III subunit RPC 62 (Hu et al., 2002).

All three TFIIIB subunits are required for polymerase recruitment but only Brf1 and TBP have been shown to interact directly with pol III. Of these interactions, the binding of Brf1 and the yeast pol III-specific subunit C34 (RPC39 in humans) appears to be crucial (Werner et al., 1993; Brun et al., 1997). Moreover, it has been shown that various TFIIIC subunits are capable of interacting with pol III (Hu et al., 2002). These additional interactions may help stabilise the transcription complex (Flores et al, 1999; Hsieh et al, 1999a, b).

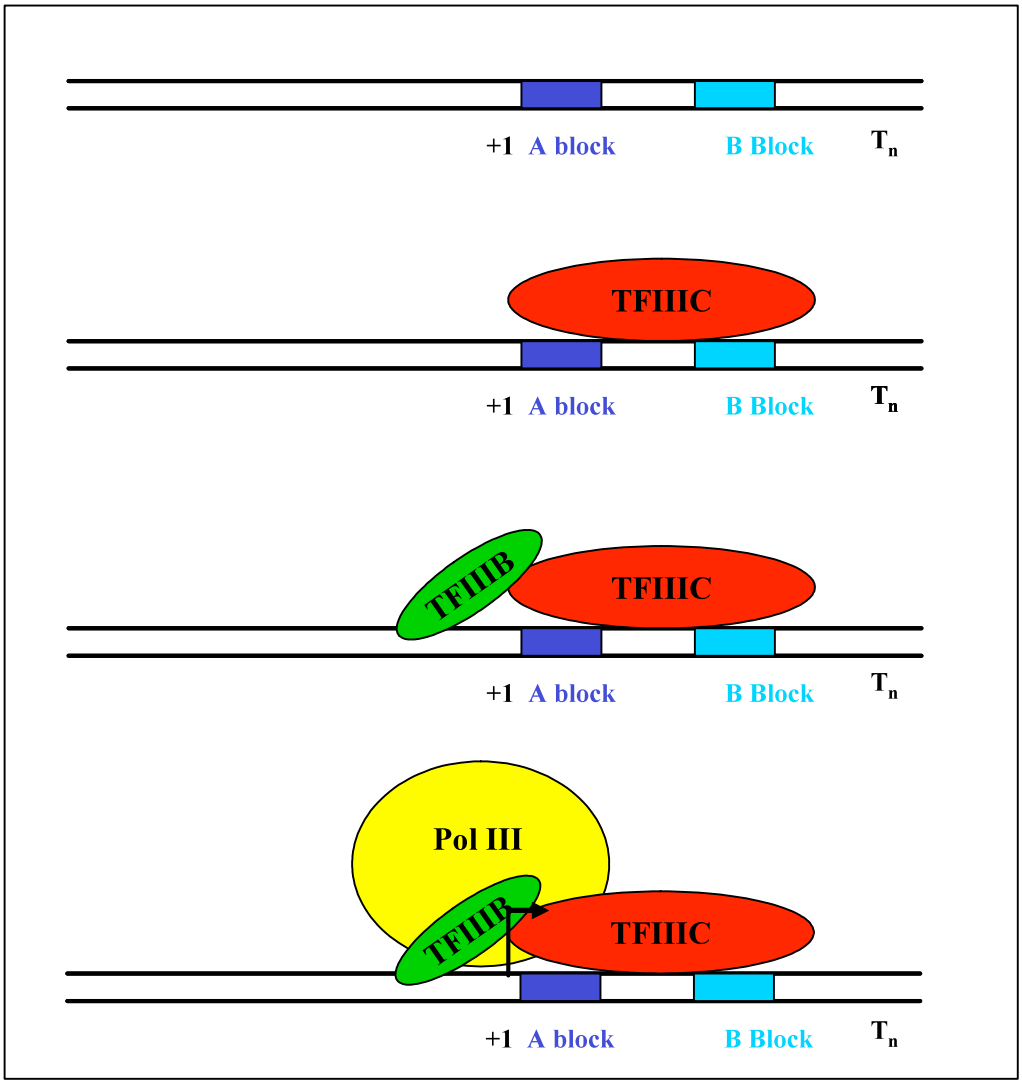


Figure 1.2 Schematic illustration of the assembly pathway used to form an initiation complex on type II promoters.
TFIIIC recognises and binds to the A and B-block promoter elements. Once TFIIIC has bound to DNA, it recruits TFIIIB via protein-protein interactions. Pol III is recruited by protein-protein interactions with TFIIIB. The transcription start site is denoted by +1 (Adapted from White, 2002).

1.4.1.2 Type I promoters

The recruitment of pol III to type I promoters also requires TFIIIB and TFIIIC. Type I promoters lack a functional B-block (Figure 1.2), which is the major determinant of DNA-binding affinity of TFIIIC. As a result, the gene-specific transcription factor TFIIIA is required for the productive association of TFIIIC with 5S rRNA genes (Engelke et al., 1980; Segall et al., 1980). TFIIIA was the first eukaryotic transcription factor to be purified to homogeneity (Engelke et al., 1980) and cloned (Ginsberg et al., 1984). TFIIIA is composed of a single polypeptide of approximately 40 kDa, and is the founding member of the C_2H_2 zinc finger family of DNA-binding transcription factors (Miller et al., 1985). It has nine zinc finger domains which bind to the A-block, intermediate element and the C-block of 5S genes (Miller et al., 1985). The N-terminal three zinc fingers specifically recognise the C-block and contribute to 95% of total DNA-binding affinity (Hanas et al., 1983). Once TFIIIA is bound to 5S genes, it is able to recruit TFIIIC although the precise manner by which this occurs remains unclear (Braun et al., 1992). TFIIIC recruitment then allows TFIIIB to bind upstream of the transcription start site, which is then followed by the recruitment of pol III (Kassavetis et al., 1995; Roberts et al., 1996)

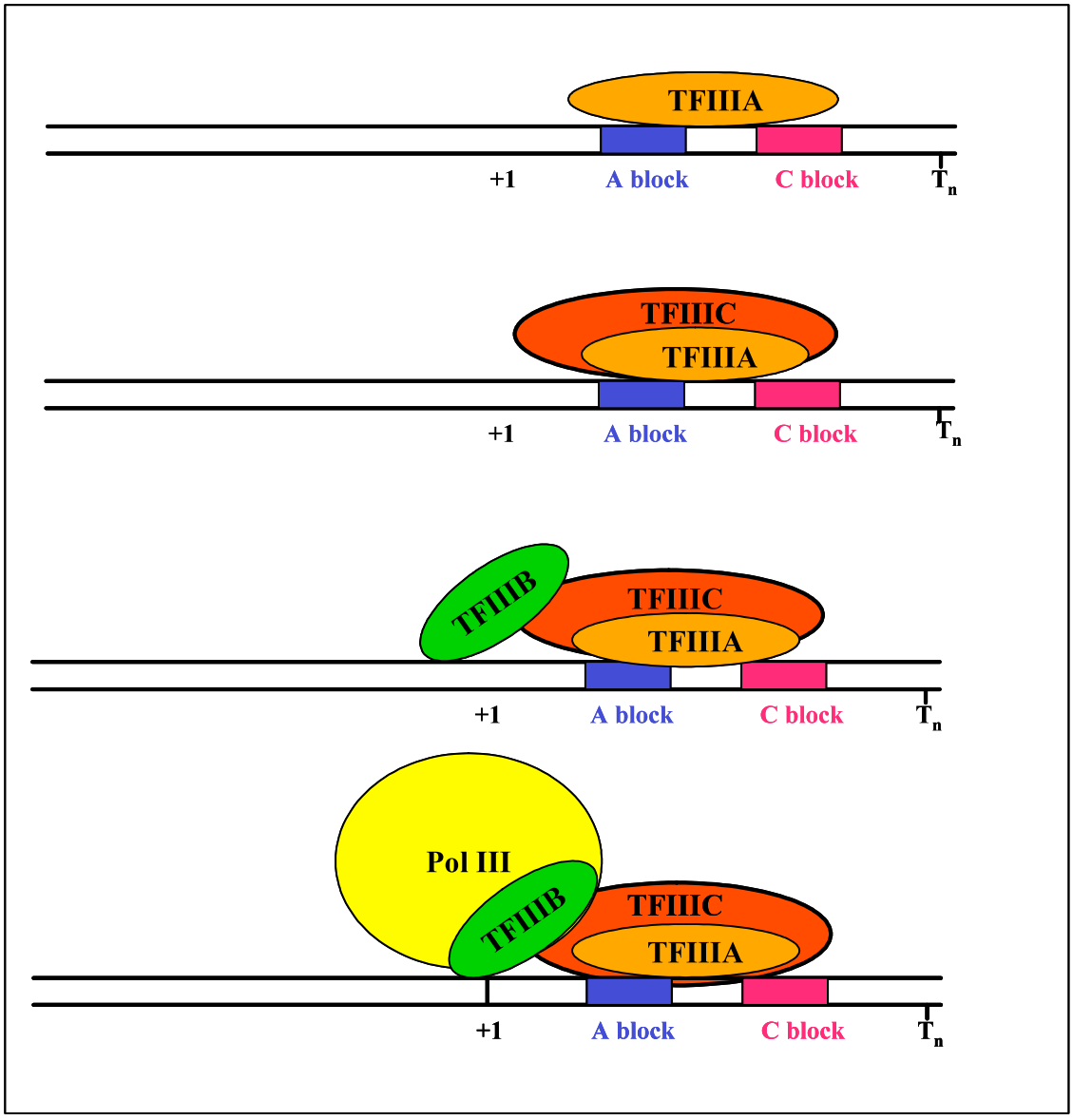


Figure 1.3 Schematic illustration of the assembly pathway used to form an initiation complex on of type I promoters.
TFIIIA binds to the internal promoter. TFIIIC is then recruited followed sequentially by TFIIIB and pol III. Once pol III has been recruited, transcription can commence.

1.4.1.3 Type III promoters

Type III promoters have distinct transcription factor requirements in comparison to types I and II promoters. TFIIIB involved in transcription from these promoters does not contain Brf1, but instead consists of a Brf1-related factor known as Brf2 as well as Bdp1 and TBP, giving rise to a specific TFIIIB-like activity (Schramm et al., 2000).

Assembly of the initiation complex begins with the binding of the PSE by a five-subunit factor known as snRNA activating complex ($SNAP_c$) (Henry et al., 1996; Wong et al., 1998) and of the TATA box by the TBP component of the specialised TFIIIB-like activity. Type III promoter occupancy is relatively slow in comparison to type I and II promoters, but can be enhanced by a series of co-operative protein-protein interactions (Schramm and Hernandez, 2002). The recruitment of $SNAP_c$ to the PSE and TFIIIB to the TATA box is enhanced by protein-protein interactions between these complexes (Mittal et al., 1996) (Mittal, 1997). An additional transcription factor, known as Oct-1, can bind to the DSE and make direct contact with $SNAP_c$ stimulating promoter occupancy through protein-protein interactions (Mittal et al., 1996; Murphy et al., 1992). However, Oct-1 is not essential for basal transcription from type III promoters *in vitro* (Hu et al., 2003). Once TFIIIB and $SNAP_c$ have assembled on the promoter, pol III can then be recruited. The interactions responsible for pol III recruitment to these promoters have yet to be determined. Figure 1.3 below represents a schematic representation of the transcription complex assembly on these promoters.

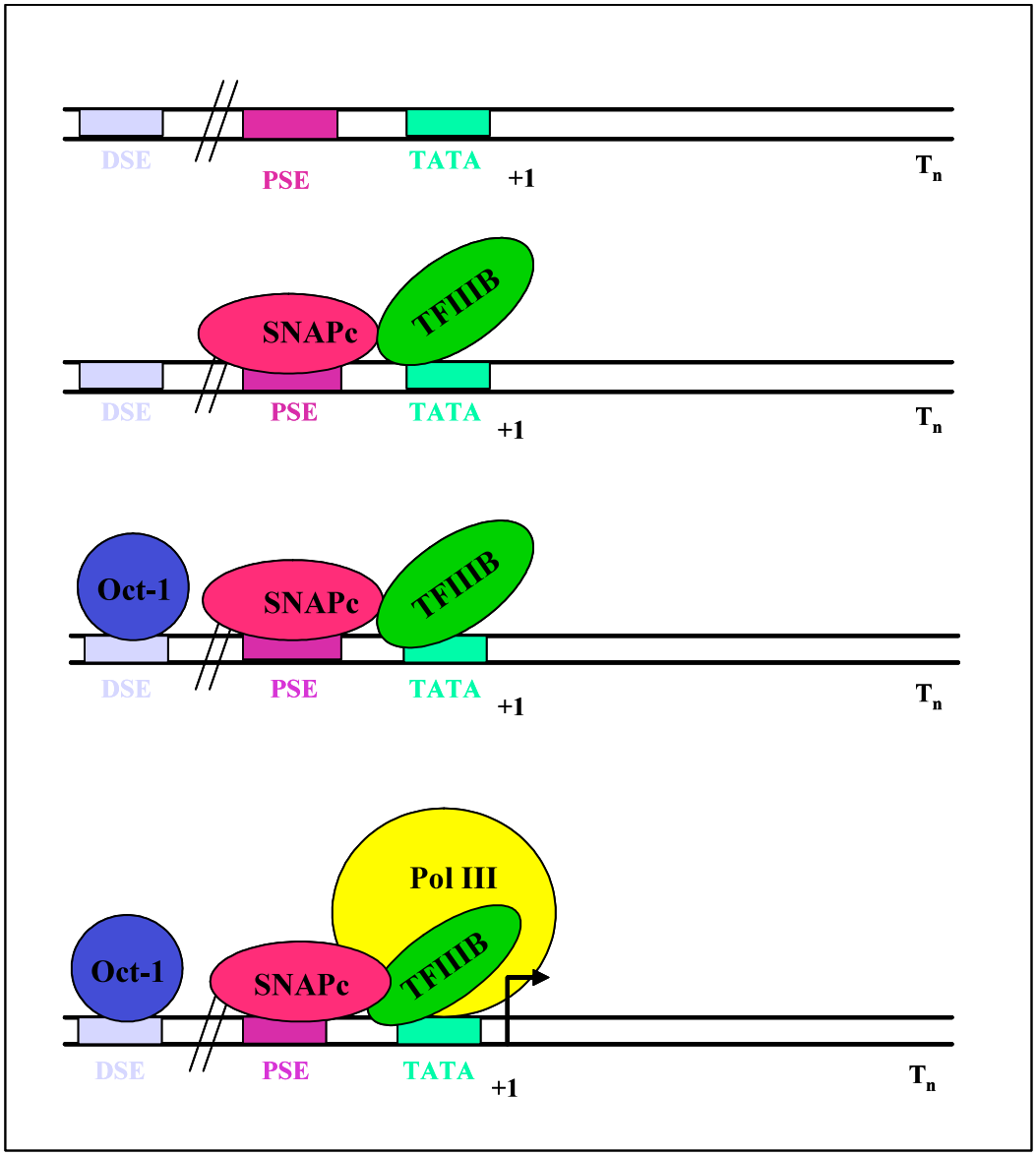


Figure 1.3 Schematic illustration of the assembly pathway used to form an initiation complex on of type III promoters.
TFIIIB and $SNAP_c$ bind cooperatively to the TATA box and PSE respectively. Binding of Oct-1 to the DSE enhances $SNAP_c$/TFIIIB recruitment. Following $SNAP_c$/TFIIIB recruitment, pol III binds and transcription can commence.

1.4.2 Pol III

RNA polymerases catalyse the covalent attachment of ribonucleotides to form an RNA chain that is complementary to the gene template being transcribed (Voet and Voet, 1995). Pol III is the largest and most complex of the eukaryotic nuclear RNA polymerases. It consists of seventeen subunits in both humans and in yeast and an aggregate mass of 600-700 kDa [reviewed in (Geiduschek and Kassavetis, 2001)]. The seventeen subunits have been cloned and shown to be essential for pol III function and

cell viability (Chedin et al., 1998; Ferri et al., 2000). This is in contrast to pol II, where several subunits have been shown to be dispensable for growth (Chedin et al., 1998). The seventeen subunits range from 10 to 160 kDa, ten of the seventeen subunits are unique to the pol III complex (known as the C subunits: C11, C17, C25, C31, C34, C37, C53, C82, C128 and C160), five are shared by all three RNA polymerases (the ABC subunits: ABC 10α, ABC 10β, ABC14.5, ABC23, ABC27), and an additional two are common to both pols I and III (the AC subunits: AC19 and AC40) (Breant et al., 1983; Buhler et al., 1980; Chedin et al., 1998). The conserved and shared subunits are thought to form the catalytic core and the unique subunits are likely to reflect their unique functional requirements such as template selection and interactions with transcription factors (Geiduschek and Kassavetis, 2001).

1.4.3 Transcription initiation, elongation and termination

Upon binding of pol III to the promoter, the double-stranded DNA is melted to allow access to the template strand (Kassavetis et al., 1990; Lofquist et al., 1993). The two strands of DNA unwind and form a small open complex referred to as the transcription bubble (Kassavetis et al., 1999). This spontaneous separation of the DNA strands is performed by pol III, although the TFIIIB subunits, Brf1 and Bdp1 have also been shown to be involved in this process (Kassavetis et al., 1998). As pol III progresses into the gene, the transcription bubble moves along with it. (Kassavetis et al., 1992) In contrast to transcription by pol I and II, no elongation factors are required, possibly due to the short length of pol III genes (White, 2001).

As RNA synthesis proceeds, pol III dissociates from the TFIIIB/DNA complex without causing any significant pausing or arrest (Kassavetis et al., 1992). TFIIIC binds DNA within the transcribed region during the pre-initiation complex assembly and remains bound during elongation (Bogenhagen, 1985). The elongation rates do not, however, appear to be affected by the presence of this transcription factor and the presence of TFIIIC delays polymerase by just 0.2 seconds at a single site upstream of the B-block making no difference in overall transcription rates (Bardeleben et al., 1994; Matsuzaki et al., 1994). It is unclear at present how the polymerase passes DNA-bound TFIIIC during transcription (Bardeleben et al., 1994). Transcription proceeds until pol III recognises a small stretch of four or more thymidine (T) residues as termination signal

(Galli et al., 1981), rather than relying on termination accessory factors like pol I and II (Cozzarelli et al., 1983). Termination signals recognition by pol III can also be influenced by the region surrounding the T cluster (Braglia et al., 2005). After the synthesis of the first transcript, pol III does not dissociate from the template and can be recycled so that multiple rounds of transcription of the same gene can take place (Jahn et al., 1987). Subsequent cycles of transcription occur more rapidly (about 35 seconds) than the first one (about 5 minutes) as the slow preliminary step of polymerase recruitment is avoided (Dieci and Sentenac, 1996). The pol III transcription factors TFIIIA, TFIIIB and TFIIIC can bend the DNA duplex and may facilitate the reinitiation of transcription by bringing the start site into close spatial orientation with the end of the gene (Jahn et al., 1987). Thus, when transcription complexes are assembled they are capable of multiple rounds of transcription (Dieci and Sentenac, 1996).

1.5 Cell growth

Cell growth (increase in cell mass) and cell proliferation (increase in cell number) are vital processes to eukaryotic life (reviewed in (Conlon and Raff, 1999). Although the terms cell growth and cell proliferation are often used interchangeably, these processes are actually distinct. It has been well documented that cells need to reach a certain size and duplicate their contents before they divide, in order to maintain a constant mean size (Johnston, 1977). Thus, cell proliferation depends on cell growth. Cell growth, on the other hand, can occur independently of proliferation. It has been shown that mutations that block cell cycle progression do not prevent cell growth (Johnston et al., 1977; Neufeld et al., 1998). Moreover, some cells such as nerve and muscle cells grow mainly after they have permanently withdrawn from the cell cycle and inactivated their cell cycle control system (Conlon and Raff, 1999).

As a high proportion of the cell's dry mass is protein, increase in cell mass requires accumulation of protein (Baxter and Stanners, 1978). In fact, cell growth is directly proportional to the rate of protein accumulation (Baxter and Stanners, 1978). The rate of protein synthesis is an important determinant of cellular growth (Baxter and Stanners, 1978). The process of translation mediates protein synthesis, where ribosomes synthesise proteins from mRNA templates. Ribosomes constitute the core of the protein synthetic machinery making ribosome content a critical determinant of protein

accumulation (Camacho et al., 1990; Zetterberg and Killander, 1965). Synthesis of rRNA is a limiting step in ribosome production, as there is little wastage of rRNA since it is totally incorporated into ribosomes (Liebhaber et al., 1978). Given its key role in determining ribosome production, rRNA gene transcription can provide a crucial control point for regulating growth rate (Liebhaber et al., 1978). Along with ribosomes, the accurate and efficient execution of translation requires many specialised pol III-produced tRNA species, which, accordingly, also impinge upon cell growth rate (Francis and Rajbhandary, 1990). Reducing levels of the initiator tRNA in yeast can influence cell growth and prolong cell doubling time (Francis and Rajbhandary, 1990) and recently it was shown that $tRNA_i^{Met}$ is limiting for translation and proliferation in mammalian cells (Marshall et al, 2008). Regulating class III gene expression clearly has the potential to influence the capacity for cell growth (reviewed in (White, 2005)). Aberrant regulation of growth is a feature of diseases such as cancer; thus, elucidating the mechanisms involved in regulating cell growth is of considerable importance (White, 2005).

1.6 Regulation of pol III transcription

Section 1.5 suggested how regulation of protein synthesis is an important aspect of growth control. Pol III plays a key role in this process and, as a consequence, pol III transcription activity is tightly linked to growth conditions. Indeed, transcription by pol III varies considerably in accordance to the metabolic requirements of the cell. Pol III transcription decreases when cells are deprived of serum or nutrients but is upregulated upon mitogenic stimulation (Clarke et al., 1996; Johnston, 1977; Mauck and Green, 1974). Control of pol III transcriptional output may be achieved through various mechanisms, mainly involving regulating its transcription factors, such as TFIIIB and TFIIIC (White, 2001). The following sub-sections describe a number of ways in which pol III transcription can be regulated.

1.6.1 Overexpression of transcription factors

Increase in pol III transcriptional output can be achieved by raising the concentration of its specific transcription factors [reviewed in (White, 2004c)]. Overexpression of transcription factors TFIIIB or TFIIIC is observed in a number of transformed cell

types. Studies of model systems revealed that levels of five subunits of TFIIIC are overexpressed at both the mRNA and protein levels following transformation by simian virus 40 (SV40), polyomavirus (Py) and Epstein Barr virus (EBV) (Felton-Edkins et al., 2006; Felton-Edkins and White, 2002; Larminie et al., 1999). TFIIIC is also specifically up-regulated in each of nine human ovarian carcinomas relative to healthy adjacent tissue and contributes to the abnormal abundance of pol III transcripts in these tumours (Winter et al., 2000).

All three subunits of TFIIIB have also been shown to increase following transformation (Felton-Edkins and White, 2002; Larminie et al., 1999). Cell culture studies have shown that TBP can be increased following infection by hepatitis B virus and Ras activation (Wang et al., 1998). The pol III-specific subunits of TFIIIB have also been shown to be overexpressed in cell culture models (Felton-Edkins and White, 2002). Cell lines transformed by either SV40 or Py were shown to overexpress the Bdp1 subunit (Larminie et al., 1999; Felton-Edkins and White, 2002). Furthermore, the Brf 1 subunit is elevated in a subset of cervical carcinomas which tested positive for the highly oncogenic human papillomavirus 16 (HPV16) (Daly et al., 2005).

1.6.2 Binding of co-repressing and co-activating proteins to transcription factors

1.6.2.1 Retinoblastoma protein

The product of the retinoblastoma susceptibility gene (Rb) is the protein RB [reviewed in (Knudsen and Knudsen, 2006)]. RB is a 105-kDa nuclear phosphoprotein that is ubiquitously expressed in normal mammalian cells. RB plays a role in controlling the mammalian cell cycle (Weinberg, 1995). Loss of RB function results in abnormal cellular proliferation and tumour formation (Sherr, 1996). When growth factors are limiting, RB regulates cell cycle progression by preventing the passage of cells into DNA synthesis (S) phase (Scott et al., 2001).

RB was found to possess a potent capacity to restrain pol III transcription (White, 1996). This is achieved by binding and repressing a variety of transcription factors such as TFIIIB (Sutcliffe et al., 2000; Scott et al., 2001). When bound to RB, TFIIIB is

unable to interact with either pol III or TFIIIC and is therefore sequestered into an inactive complex (Sutcliffe et al., 2000; Larminie et al., 1997). Only in the hypophosphorylated form, found during the G_o and early G_1 phase, can RB bind and repress TFIIIB (Scott et al., 2001). A major increase in tRNA synthesis occurs at the G_1/S phase transition, which coincides with the hyperphosphorylation of RB by cyclin D- and E-dependent kinases (cdks) (Hulleman and Boonstra, 2001; Scott et al., 2001). Hyperphosphorylated RB releases TFIIIB, allowing it to interact with TFIIIC at the promoters of class III genes and subsequently recruit pol III to these templates (Brown et al., 2000; Scott et al., 2001).

1.6.2.2 p53

p53 is a major tumour suppressor that is lost or mutated in more than half of human cancers (Hollstein et al., 1991; Vousden, 2000) and has also been found to be involved in restraining pol III transcriptional output. Like RB, endogenous p53 can interact with TFIIIB and compromise its function (Chesnokov et al., 1996; Cairns and White, 1998; Crighton et al., 2003). This interaction appears to be mediated through TBP (Crighton et al., 2003). Elevated levels of p53 are accompanied by a decrease in TFIIIB occupancy at pol III transcribed genes and co-immunoprecipitations reveal that TFIIIB can no longer bind to TFIIIC and pol III (Crighton et al., 2003). On the other hand, fibroblasts from p53-knockout mice display markedly elevated synthesis of tRNA and 5S rRNA (Cairns and White, 1998). Primary fibroblasts derived from individuals with Li-Fraumeni syndrome, who inherit a mutated p53 allele, frequently display elevated pol III transcriptional activity (Stein et al., 2002). p53 function can also be lost through the action of viral or cellular oncogenes, such as the human papilloma virus E6 or cellular human double minute (hdm) 2 oncoproteins [reviewed in (Wise-Draper and Wells, 2008)]. Both E6 and hdm2 can stimulate pol III transcription by relieving TFIIIB from p53-mediated repression (Stein et al., 2002).

1.6.2.3 c-Myc

Several oncogenic proteins activate TFIIIB indirectly by neutralising RB or p53, others such as the oncogene product of c-myc can increase pol III output by targeting TFIIIB directly. c-Myc has been shown to play an extensive role in cancer, as it can collaborate

with other oncogenes to induce cellular transformation (Dang, 1999). Chromatin immunoprecipitation (ChIP) analysis showed that c-Myc is present at pol III-transcribed genes in untransformed fibroblast cells, ovarian epithelial cells and in the transformed cervical line HeLa (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. Specific depletion of c-Myc using RNA interference reduced tRNA and 5S rRNA gene expression in HeLa cells (Felton-Edkins et al., 2003b). However, type III genes such as 7SK do not appear to be regulated by c-Myc, which suggests that c-Myc regulation of pol III transcription is type I and II promoter specific (Felton-Edkins et al., 2003b). Activation of c-Myc results in increased translation and growth, and loss of c-Myc decreases growth and protein synthesis (Dang, 1999). The growth promoting potential of c-Myc may be mediated in part through the regulation of pol III transcription.

1.6.3 Direct phosphorylation of transcription factors

Protein kinases play a global role in the regulation of transcription by catalysing reversible phosphorylation of transcription factors. Phosphorylation of transcription factors can modulate their activity by triggering conformational change, generating binding sites for interaction partners, influencing their intracellular localisation or altering their stability [reviewed in (Whitmarsh and Davis, 2000)]. Phosphorylation of transcription factors such as TFIIIB is a common mechanism of controlling activity of genes transcribed by pol III (Fairley et al., 2003; Felton-Edkins et al., 2003a; Johnston et al., 2002).

1.6.3.1 Extracellular signal- related kinase (Erk)

The mitogen-activated Erk cascade promotes growth in several ways, including activation of translational capacity [reviewed in (Meloche and Pouyssegur, 2007)]. It has been shown that Erk interacts with and phosphorylates the Brf1 subunit of TFIIIB *in vitro* and *in vivo*; this phosphorylation is required for the serum induction of pol III activity (Felton-Edkins et al., 2003a). As a result, a substitution in the Erk docking domain or phosphoacceptor site of Brf1 substantially reduces pol III transcription (Felton-Edkins et al., 2003a). ChIP analysis of cells treated with a specific inhibitor of

Erk showed a reduced Brf1 and pol III occupancy at 5S rRNA and tRNA genes (Felton-Edkins et al., 2003a). Felton-Edkins and co-workers (2003) suggested that Erk phosphorylation of Brf1 stimulates initiation complex assembly and enhances interactions between TFIIIB and TFIIIC, resulting in an increase in pol III recruitment.

1.6.3.2 Polo-like kinase 1

When eukaryotic cells enter mitosis, it is generally accompanied by a decrease in transcription (Prescott and Bender, 1962). Reversible phosphorylation of the basal transcriptional machinery is thought to repress nuclear transcription during mitosis. For pol III transcription, this is achieved via phosphorylation of TFIIIB (White et al., 1995). Fairley and co-workers demonstrated that Brf1 is hyperphosphorylated during mitosis, but remains associated with pol III promoters (Fairley et al., 2003). Bdp1 is selectively released from pol III promoters (Fairley et al., 2003) and as a consequence, a mitotic reduction of pol III output is observed (Fairley et al., 2003).

Polo-like kinases (Plks) are important regulators of cell cycle progression during mitosis [reviewed in (Martin and Strebhardt, 2006)]. Plks are required for numerous aspects of mitotic progression such as mitotic spindle formation and cytokinesis [reviewed in (Eckerdt and Strebhardt, 2006)]. In mammalian cells, Plk1 protein levels increase as cells approach the mitotic phase, with the peak of phosphorylation activity reached during mitosis (Golsteyn et al., 1995; Hamanaka et al., 1995). Unpublished data (J. Fairley and R.J.White) suggest that Plk1 can bind and phosphorylate Brf1. Furthermore, ChIP analysis has revealed that Plk1 is found at the promoters of pol III-transcribed genes, which supports its role in the control of transcription by pol III (J.Fairley and R.J.White).

1.6.4 Protein kinase CK2

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 (discussed further in Chapter Three), 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 tumourigenesis (Ahmed et al., 2000; Faust et al., 1996;

Munstermann et al., 1990). 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) (Johnston et al., 2002). CK2 has been shown to have a potent effect on pol III transcription in both yeast and mammalian cells (Ghavidel and Schultz, 1997; Ghavidel and Schultz, 2001; Johnston et al., 2002). This is achieved by phosphorylating TFIIIB and enhancing the assembly of the transcription initiation complex (Johnston et al., 2002).

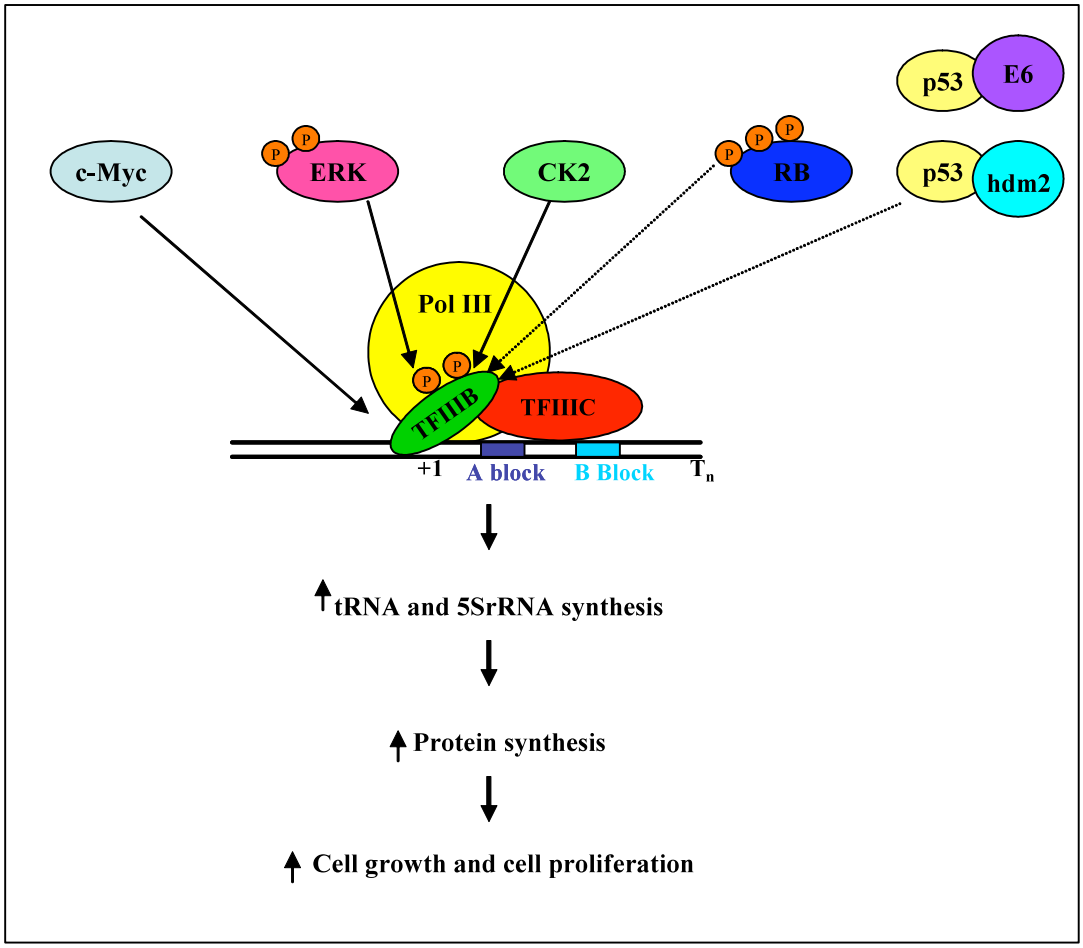


Figure 1.4 Mechanisms of regulation of pol III transcription during proliferation.
CK2, c-Myc and Erk can activate pol III transcription by targeting TFIIIB directly and aiding in transcription initiation complex assembly. Hyperphosphorylation of RB and sequestration of p53 by proteins such as hdm2 or E6 relieves TFIIIB from repression and can lead to increased pol III output. Activation of pol III transcription gives rise to increase in rRNA and tRNA involved in protein synthesis, which in turn increase cell growth and cell proliferation. Adapted from (Goodfellow and White, 2007)

1.7 Structure and cellular functions of CK2

Protein kinase CK2 (CK2) is a serine/threonine (S/T) protein kinase present in all eukaryotic cells (Pinna, 1997; Jakobi et al., 1989). CK2 exists predominantly as a tetrameric protein composed of two catalytic subunits, CK2α and/or CK2α', and two regulatory subunits: CK2β, although there is evidence that the subunits can also be found in their dissociated form (Filhol et al., 1991; Marin et al., 1997; Meggio et al., 1992). In mammals, the two catalytic isoforms CK2α (42-44kDa) and CK2α' (38kDa) are encoded by separate genes. The two isoforms show greater than 90% sequence

identity over their N-terminal 330 amino acids, which include their catalytic domains (Litchfield and Luscher, 1993). In 2001, a third catalytic isoform (designated CK2α''), that is almost identical to CK2α that the last thirty two amino acids are completely unique, was also identified (Shi et al., 2001). The various CK2 isoforms are closely related and show considerable functional overlap, but there is also evidence to support functional specialisation of the individual CK2 catalytic isoforms in yeast and mammals (Vilk et al., 1999). For example, a knockout of CK2α' shows that CK2α cannot compensate for the loss of CK2α' in spermatogenesis (Xu et al., 1999).

In contrast to the catalytic subunits, the regulatory subunit CK2β (25kDa) does not share extensive homology with any known protein, but is highly conserved among species. Indeed, its whole 215-amino acid sequence is identical between birds and mammals (Litchfield, 2003). CK2β has a number of characteristic features, such as an autophosphorylation site comprising S2, S3 and possibly S4 at its N-terminus (Boldyreff, 1993; Litchfield et al., 1991). The β subunit appears to stabilise and modulate the ability of CK2 to interact with and phosphorylate substrates (Litchfield, 2003).

CK2 is a pleiotropic and ubiquitously expressed protein kinase (Olsten and Litchfield, 2004). It has over 300 substrates which can be found in a variety of cellular compartments (mainly in the nucleus and cytoplasm) [reviewed in (Meggio and Pinna, 2003)]. Table 1.1 below represents a selected few of CK2 targets and their functions.

Substrate	Description	Reference
Cytoplasmic substrates		
IRS-1	Insulin receptor substrate 1	(Tanasijevic et al., 1993)
PP2A	Protein phosphatase 2A	(Heriche et al., 1997)
Nuclear substrates		
c-Myc	Oncogene c-Myc	(Luscher et al., 1989)
p53	Tumour suppressor p53	(Meek et al., 1990)
c-Jun	Transcription factor AP-1	(Lin et al., 1992)
c-Myb	Oncogenic transcription factor c-Myb.	(Luscher et al., 1990)

Table 1.1 Selected examples of phosphorylation targets of CK2 [Adapted from (Olsten and Litchfield, 2004)]

Due to its ability to regulate numerous substrates and its ubiquitous expression, CK2 has been implicated in a vast array of crucial cellular functions, including cell growth, proliferation and cell survival. In fact, CK2 has been demonstrated to be essential for cell viability in *S.cerevisiae* (Glover, 1998). Furthermore, disruption of the gene encoding CK2β in mice leads to failure in development (Buchou et al., 2003), as does RNA interference-mediated knockdown of CK2β in *Caenorhabditis elegans* (Fraser et al., 2000).

1.7.1 Activation of pol III transcription and CK2 activity in transformed cells

The precise regulation of pol III transcription is an important feature of normal cellular growth and proliferation. In contrast, deregulated pol III transcription is connected with the abnormal proliferation characteristic of many transformed and tumour cell types

[reviewed by (White, 2004b; White, 2004c)]. Several mechanisms have been suggested to cause the deregulation of pol III transcriptional control. As discussed above, pol III transcription can be decreased by two key tumour suppressors, p53 and RB, and activated by proto-oncogene products, including CK2 and c-Myc. Mutations of p53 and RB, and abnormal activation of proto-oncogenes, are common features of most human cancers, and can contribute to the increase of pol III transcripts observed in tumour cells (Schwartz et al., 1974; Tang et al., 2005; Winter et al., 2000). In addition, pol III transcription is activated by several viruses associated with cellular transformation and human cancers, through the activation of TFIIIB and/or TFIIIC (White, 2004b; White, 2004c). This activation of transcription factors may contribute to the deregulation of class III gene expression (Daly et al., 2005; Felton-Edkins and White, 2002; Larminie et al., 1999; Wang et al., 1995; Winter et al., 2000).

CK2 has been involved in diseases such as cancer, which is not surprising as it plays an important role in many central biological processes. Elevated CK2 activity was detected in leukaemia cells , in normal tissues with high mitotic index and in a variety of human cancers, including head and neck (Faust et al., 1996), prostate (Yenice et al., 1994), kidney (Stalter et al., 1994) breast (Landesman-Bollag et al., 2001) and lung (Daya-Makin et al., 1994). CK2 also exhibits oncogenic activity when overexpressed in transgenic mice and in cultured mammalian cells. The activation of CK2 during cell transformation could give rise to an increase in pol III transcripts and contribute to the aberrant growth of cells.

1.8 Objectives of PhD.

Regulation of class III gene expression is an important aspect of cellular function. It is crucial for the appropriate regulation of the biosynthetic capacity of cells. Protein kinase CK2 promotes growth and proliferation and increased rates of 5S rRNA and tRNA synthesis are also required for increased cell growth. In addition, CK2 has been shown to be involved in the regulation of pol I transcription.

The overall objective of this thesis was to investigate the regulation of pol III transcription by the protein kinase CK2. The first aim of the project was to confirm that the Brf1 subunit of TFIIIB is a target of phosphorylation by CK2 in mammalian cells.

This was achieved by testing the effects of specific CK2 inhibitors on pol III transcription and discussed in Chapter Three. Chapter Four investigates the potential site(s) of phosphorylation of Brf1 by CK2, *in vivo* and *in vitro*. The remainder of the investigation was concerned with uncovering the mechanisms responsible for upregulating pol III transcription by CK2 and are discussed in Chapters Five and Six.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Growth conditions

Cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents. All cell types were grown in a humidified atmosphere containing 5% CO_2 at 37°C. HeLa cells, Chinese hamster lung fibroblast (CCL 39) cells and BALB/c 3T3 (A31) cells were maintained in Dulbecco's modified eagle medium (DMEM) (Cambrex) supplemented with 10% (v/v) foetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin (all Sigma). Cells were passaged when sub confluent (approximately every 2 to 3 days) using buffered trypsin (0.05% trypsin, 0.02% EDTA; Sigma).

2.1.2 Storage of cells

Cells were stored by cryo-freezing. For cryo-freezing, trypsinised cells were resuspended in 70% DMEM (plus penicillin and streptomycin), 20% FCS and 10% dimethyl sulphoxide (DMSO) or in 90% FCS and 10% DMSO. 1 ml aliquots were transferred to cryo-tubes, and frozen overnight at -80˚C. Subsequently, the cells were transferred to liquid nitrogen for permanent storage. Thawing of cells was performed rapidly by placing cryo tubes in a water bath at 37 °C until just thawed. Cells were then diluted in fresh media, centrifuged and the supernatant was aspirated off to ensure removal of DMSO prior to resuspension in fresh media.

2.1.3 Drug treatment of cells and cell extracts

4,5,6,7-tetrabromo-1H-benzimidazole (TBB), 2-Dimethylamino-4, 5, 6, 7-tetrabromo-1H-benzimidazole (DMAT), LY290042 and rapamycin (all purchased from Calbiochem) were resuspended in dimethyl sulfoxide (DMSO). Quercetin (Sigma) was resuspended in sodium hydroxide (50 mg/ml in 1M NaOH) and this was diluted in water to give a final stock concentration of 1mM. Balb/c 3T3 (A31) and HeLa cells

were incubated in the presence of DMAT at a final concentration ranging from 5 μM to 50 μM for 4 hours. CCL39 cells were incubated for 2 hours in the presence of quercetin at a final concentration of 60 μM, with LY290042 at a final concentration of 50 μM or with rapamycin at a final concentration of 100 nM.

HeLa nuclear and whole cell extracts were treated with TBB, quercetin and DMAT at a final concentration of 10 μM for 15 minutes.

2.1.4 SV40 Transformed and Mitotic cell extracts

Cell extracts from Balb/c 3T3 A31 cells infected with SV40 of the wt830 strain were kindly donated by Dr Zoë Felton-Edkins described in (Larminie et al., 1997).

Mitotic HeLa cell extracts were generated from asynchronous HeLa cells which had been blocked using thymidine followed by nocodazole treatment as described in (White et al., 1995), and were kindly donated by Dr Jennifer Fairley.

2.2 RNA Extraction

Total cellular RNA was extracted from cells using TRI reagent (Sigma), according to the manufacturer's instructions. Media was aspirated from the cells, followed by two washes of 5ml of ice-cold phosphate buffered saline (PBS). Cells were scraped into TRI reagent and transferred to sterile 1.5ml eppendorf tubes (1 ml of TRI reagent was used per 2 to 3 wells of a 6-well plate and per 10 cm dish). The samples were incubated for 5 minutes at room temperature, to allow the complete dissociation of nucleoprotein complexes, followed by the addition of 200 μl of chloroform to each tube. Thorough mixing of chloroform and TRI reagent was ensured by vortexing each sample for 15 seconds. The samples were incubated at room temperature for a further 5 minutes and then centrifuged at 16000g for 15 minutes at 4˚C. This centrifugation separated the samples into 3 phases: a lower organic phase containing proteins, a middle interphase containing DNA, and an upper aqueous phase containing RNA. The RNA-containing phase was carefully removed, and transferred to a fresh eppendorf tube. To precipitate RNA, 0.5 ml of isopropanol was added; samples were mixed by vortexing for 10 seconds, and then incubated for 10 minutes at room temperature. Next, the samples

were centrifuged at 16000g for 10 minutes at 4˚C. Following centrifugation, the supernatant was discarded, and the remaining RNA pellet was washed using 1ml of 75% ethanol, made using diethylpyrocarbonate (DEPC)-treated H_2O (0.1% DEPC). Samples were centrifuged for a further 5 minutes at 16000g (4˚C), then the supernatant was aspirated off, and RNA pellets left to air dry for approximately 10 minutes. Once dry, RNA was resuspended in 10-30 µl of DEPC-treated H_2O. To aid resuspension, pre-warmed DEPC-treated H_2O was used, and samples were incubated at 65˚C for 15 minutes. Following resuspension, a spectrophotometer was employed to measure the absorbance of each sample at 260 nm, and the following formula was used to calculate the RNA concentration: RNA concentration (µg/ml) = absorbance at 260 nm x 40 x dilution factor. All RNA samples were stored at -80˚C.

2.3 Northern Blot Analysis

10-20 µg of RNA was diluted in DEPC-treated H_2O to give a total volume of 10 µl. Each sample was mixed with 10 µl of 2 X RNA sample buffer (1 X MOPS [20 mM MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0)], 4.4 M formaldehyde, 54% formamide) the samples were then incubated at 65˚C for 15 minutes to denature RNA secondary structure. Subsequently, samples were immediately cooled on ice to prevent any renaturation of the RNA. 2 µl of 1 mg/ml ethidium bromide and 2 µl of 10 X RNA loading dye (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) were added to each sample, prior to loading onto a denaturing formaldehyde 1% agarose gel (1% agarose, 2.2M formaldehyde, 1 X MOPS) that had been pre-run at 40V in 1 X MOPS for 30 minutes. Electrophoresis was performed for 4 to 5 hours at 40V in 1 X MOPS; afterwards the gel was visualised under a UV transilluminator to ensure effective RNA separation and equal loading.

The gel was then washed in 20 X SSC (3 M $NaCl_2$, 0.3 M sodium citrate pH 7.0) with gentle shaking for 10 to 15 minutes. For capillary transfer of the RNA, the gel was inverted and placed on a wick of Whatmann 3MM chromatography paper, which had been pre-soaked in and was fed from a reservoir of 20 X SSC. A gel-sized piece of pre-soaked Hybond N nylon membrane (Amersham), followed by 2 pieces of pre-soaked Whatmann paper, was placed on the gel ensuring no air bubbles between layers. A stack of folded paper towels was then added followed by a 0.5 kg weight, to ensure

efficient transfer of RNA to the membrane by capillary action. The transfer was allowed to proceed for 16 to 18 hours. Following transfer, the RNA was fixed to the membrane by UV crosslinking at 1200 μJ. The membrane was rinsed for 5 minutes in DEPC-treated H_2O and stored in Saran wrap until use.

To specifically detect the RNA of interest, radiolabelled complementary DNA probes were used: the B2 gene probe was prepared from a 240bp EcoRI-PstI fragment of pTB14, and the probe for acidic ribosomal phosphoprotein P0 (ARPP P0) was prepared from a 1kb EcoRI-HindIII fragment of the mouse cDNA. A Megaprime DNA Labelling Kit (Amersham) was used to label the probes by random oligonucleotide priming, according to the manufacturer's instructions. Random hexamer oligonucleotides were mixed with 25 ng of the DNA fragment to be probed, and made up to a total volume of 50 μl with DEPC-treated H_2O. This was heated at 95˚C for 5 minutes to denature the DNA. Slow cooling of the mixture to room temperature allowed the random hexamer oligonucleotides to anneal to the DNA. 10 μl of reaction buffer (dATP, dGTP, dTTP in Tris pH 7.5, β-mercaptoethanol and $MgCl_2$), 2 μl (2U) of DNA polymerase I Klenow fragment, and 50 μCi of [α-^{32}P] dCTP (Amersham) were added, and labelling was allowed to proceed at 37˚C for 1 hour. The labelled DNA was then denatured by heating at 100˚C for 5 minutes, and then stored on ice until use.

Prior to hybridising the membrane with an appropriate radiolabelled probe, it was pre-hybridised in a hybridisation oven at 45˚C for 45 minutes by rotating in 25 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% bovine serum albumin (BSA), 7% sodium dodecyl sulphate (SDS), and 45% formamide in DEPC-treated H_2O). The radiolabelled probe was then added to 25 ml of fresh hybridisation buffer, and incubated with the membrane overnight at 45˚C with rotation. The following day, the membrane was washed with rotation in hybridisation wash buffer (40 mM sodium phosphate buffer pH 7.2, 1 mM EDTA and 1% SDS) twice for 5 minutes at 65˚C, then twice for 15 minutes at 65˚C, to remove any unincorporated radioactivity. The membrane was then exposed to autoradiography film for an appropriate length of time at –80˚C. To reprobe the membrane, it was boiled in DEPC-treated H_2O for 4 minutes, then pre-hybridised and probed as before.

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

2.4.1 Preparation of cDNAs

3 µg of RNA and 200 ng of random hexanucleotide primers (Roche) were diluted in DEPC-treated H_2O to give a final volume of 24 µl. Primer annealing was carried out for 10 minutes at 80˚C. Following this, samples were transferred to ice and then 1 µl (200 U) of Superscript II reverse transcriptase (Invitrogen Life Technologies), 8 µl of 5 X First Stand Buffer (Invitrogen Life Technologies), 4 µl of 0.1 M dithiolthreitol (DTT) (Invitrogen Life Technologies) and 2 µl of a mix containing 10 mM of each of the four dNTPs (Promega) were added to each. Reverse transcription was then allowed to proceed for 1 hour at 42˚C. Heating at 70˚C for 15 minutes stopped the reaction. cDNAs were stored at –20˚C.

2.4.2 PCR

PCRs were performed using a Techgene thermal controller (TECHNE). Each reaction contained 2 µl of cDNA, 20 pmol of the appropriate primers, 0.5 U of Taq DNA polymerase (Promega), 1 X Taq DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each non-radioactive dNTP, and 1.8 µCi of [α-^{32}P] dCTP (Amersham). Table 2.1 below lists the sequences of primers used for PCR. The cycling parameters employed and product sizes obtained for each primer set are indicated in the Table 2.2 below. Reaction products were diluted 1:1 with formamide loading buffer (98% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 5 mM EDTA), and resolved on 7% polyacrylamide sequencing gels containing 7 M urea and 0.5 X TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run at 40W for 30 minutes in 0.5 X TBE prior to loading samples (1.5 µl of each). Before loading, samples were heated at 95˚C for 2 minutes, and then quenched on ice. Electrophoresis was carried out for 1 hour at 40W in 0.5 X TBE; the gels were vacuum-dried for 1 hour at 80˚C. Radiolabelled PCR products were visualised by autoradiography.

Transcript	Forward (F) and reverse (R) primers
ARPP P0	F: 5'-GCA CTG GAA GTC CAA CTA CTT C-3' R: 5'-TGA GGT CCT CCT TGG TGA ACA C-3'
$tRNA^{Leu}$	F: 5'-GTC AGG ATG GCC GAG TGG TGT AAG GCG CC-3' R: 5'-CCA CGC CTC CAT ACG GAG ACC AGA CCC-3'
$tRNA^{Tyr}$	F: 5'-CCT TCG ATA GCT CAG CTG GTA GAG CGG AGG-3' R: 5'-CGG AAT TGA ACC AGC GAC CTA AGG ATG TCC-3'
5S rRNA	F: 5'-GGC CAT ACC ACC CTG AAC GC-3' R: 5'-CAG CAC CCG GTA TTC CCA GG-3'

Table 2.1 Sequences of PCR primers used in RT.PCR.

Transcript	Cycling parameters	Product size
ARPP P0	95°C for 2 minutes, 18 to 22 cycles of [95°C for 1 minute, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 3 minutes.	265bp
$tRNA^{Leu}$	95°C for 3 minutes, 25 to 29 cycles of [95°C for 30 seconds, 68°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.	88bp
$tRNA^{Tyr}$	95°C for 3 minutes, 25 to 29 cycles of [95°C for 1 minute, 62°C for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes.	84bp
5S rRNA	95°C for 3 minutes, 18 to 22 cycles of [95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute], 72°C for 5 minutes.	107bp

Table 2.2 PCR cycling parameters and product sizes.

2.5 Storage, propagation and preparation of plasmid DNA

2.5.1 Transformation of competent cells

For plasmid storage and propagation, Escherichia coli XL-1 blue supercompetent cells (Stratagene) were transformed. These cells were stored at –80˚C and were thawed on ice prior to use, to prevent loss of transformation efficiency. 10-20 ng of plasmid DNA was added to 50 μl of thawed cells and mixed gently. The mixture was incubated on ice for 30 minutes, with occasional gentle agitation. Following this time, cells were heat-shocked for exactly 45 seconds at 42˚C, then transferred to ice for a further 2 minutes. 450 μl of SOC medium [Luria Bertani (LB) broth, 0.04% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$], which had been pre-heated to 42˚C, was then added and cells were incubated at 37˚C for 1 hour on an orbital shaker (225-250 rpm). Subsequently, 150 μl of the transformation mixture was plated on LB-agar (2% LB, 2% agar) containing 50 μg/ml of the selective antibiotic ampicillin, and then incubated at 37°C overnight to allow colony formation.

2.5.2 Preparation of plasmid DNA

An isolated bacterial colony was selected from a streaked LB-agar plate, and used to inoculate 4 ml of LB medium containing 50 μg/ml ampicillin. This mini-culture was incubated at 37˚C for approximately 6 hours on an orbital shaker (300 rpm), and then used to inoculate 250 ml of LB medium containing 50 μg/ml ampicillin. This larger culture was incubated overnight under the same conditions. The following day, plasmid DNA was purified from the bacterial cells using the QIAGEN Plasmid Maxi Kit, according to the manufacturer's instructions.

Bacterial cells were harvested by centrifugation (in Sigma Laboratory Centrifuge 4K15) at 6000g for 15 minutes at 4˚C and then resuspended in 10 ml of Buffer P1 (500 mM Tris pH 8.0, 10 mM EDTA, 100 μg/ml RNase A). Cell lysis was achieved by adding 10 ml of Buffer P2 (200 mM NaOH, 0.1% SDS). This reaction was allowed to proceed at room temperature for 5 minutes before lysates were neutralised by adding 10 ml of

chilled Buffer P3 (3 M potassium acetate, pH 5.5). Addition of Buffer P3 caused the precipitation of potassium dodecyl sulphate, SDS-denatured proteins, and chromosomal DNA and cell debris. Precipitation was enhanced by incubation on ice for 20 minutes. Plasmid DNA is circular, and therefore renatured correctly and remained in solution. Centrifugation at 20000g for 30 minutes was performed (at 4˚C) to separate precipitated debris from soluble material. Following this centrifugation, the supernatant containing plasmid DNA was promptly removed and applied to a QIAGEN-tip 500, pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). Plasmid DNA binds tightly to QIAGEN-tip resin, while the remainder of the supernatant passes through by gravity flow. The resin was then washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol), before eluting the purified plasmid DNA into a Falcon tube with 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris pH 8.5, 15% isopropanol). DNA was precipitated with 10.5 ml of isopropanol. The sample was then centrifuged at 15000g for 30 minutes at 4˚C. Following this, the supernatant was carefully decanted out leaving the pelleted plasmid DNA, which was then washed with 70% ethanol and recentrifuged. The pellet was air-dried for approximately 10 minutes, and then resuspended in an appropriate volume of sterile distilled H_2O. DNA concentration was determined by measuring absorbance at 260 nm, and using the following calculation: DNA concentration (μg/ml) = absorbance at 260 nm x 50 x dilution factor. All plasmid DNA stocks were stored at -20˚C.

2.6 Whole cell extract preparation

All steps were performed on ice or at 4˚C. Buffers, reagents and plastic-ware were kept chilled.

2.6.1 Preparation of extracts for in vitro transcription assays

Extracts for *in vitro* transcription assays (IVTs) were prepared from Balb/c 3T3 (A31) cells grown on 10 cm tissue culture dishes and treated in presence or absence of CK2 inhibitor DMAT as described in section 2.1.3. Cells were placed on ice then washed twice in phosphate-buffered saline (PBS; 170 mM NaCl, 3.4 mM KCl, 1 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.2). Cells were then scraped into PBS (5 ml/dish), and

collected in 50 ml Falcon tubes. Cells were pelleted by slow centrifugation at 500g (in a Sorvall RT 6000 D) and the PBS was then discarded. A small amount of fresh PBS (approximately 1ml) was then added to the cells to aid transfer into sterile eppendorf tubes. This PBS was then also removed following centrifugation (in an Eppendorf Centrifuge 5415R) at 12000g for 1 minute. The remaining cell pellets were gently resuspended in freshly made microextraction buffer (450 mM NaCl, 50 mM NaF, 20 mM HEPES pH 7.8, 25% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl 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). The volume of microextraction buffer used was equivalent to the volume of the cell pellet. Following resuspension, samples were immediately snap-frozen on dry ice and thawed at 30˚C. When just thawed, samples were again returned to dry ice. This freeze-thaw procedure was performed a total of 3 times to ensure optimal cell lysis. Following the final thaw, cell debris was collected by centrifugation at 12000g for 10 minutes. The supernatant was promptly aliquoted, then snap-frozen. Samples were stored at -80˚C.

2.6.2 Preparation of extracts for polyacrylamide gel electrophoresis

Extracts for polyacrylamide gel electrophoresis were prepared from cells grown on 6-well plates or 10 cm dishes. Cells were placed on ice then washed twice in PBS. Cells were then scraped directly into cell lysis buffer (20 mM HEPES (pH 7.8), 150 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, 0.5 μg/ml leupeptin, 1.0 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin and 40 μg/ml bestatin) and transferred to sterile eppendorf tubes. The cell lysates were incubated on ice for 15 minutes, and then passed through a 26-gauge needle three times. Cell debris was collected by centrifugation at 16000g for 10 minutes, and the supernatants were aliquoted, snap-frozen on dry ice, and then stored at –80˚C.

2.6.3 Determination of protein concentrations

The protein concentrations of whole cell extracts were determined using Bradford's reagent (Bio-Rad) diluted 1 in 5 with distilled H_2O. The colour change produced upon mixing this reagent with protein can be quantified by measuring absorbance at 595 nm (using a spectrophotometer), and is directly proportional to the concentration of protein in the sample. For each experiment, a standard curve was constructed by measuring the absorbance of 1, 2, 4, 6, 8, 10 and 12 μg of bovine serum albumin (BSA) in 1ml of Bradford's reagent. Whole cell extracts were diluted 1 in 10 with microextraction buffer, and then 10 μl added to 1 ml of reagent. Absorbance readings at 595 nm were performed in duplicate, and the protein concentration of each sample determined from the standard curve.

2.7 *In vitro* transcription assay (IVT)

2.7.1 Plasmids used for IVTs

Plasmids used for IVTs were pLeu (a 240bp EcoRI-HindIII fragment of human genomic DNA carrying a $tRNA^{Leu}$ gene subcloned into pAT153 (McLaren and Goddard, 1986) and pVA1 (a 221bp SalI-BalI fragment of adenovirus 2 DNA containing the VA1 gene subcloned into pUC18) (Dean and Berk, 1988).

2.7.2 Peptide substrate inhibitors used in IVTs

30μg of peptide substrate inhibitors were pre-incubated with HeLa nuclear extracts (obtained from the Computer Cell Culture Centre, Belgium) for 15 minutes at 30°C in IVTs. The peptide inhibitors were the CK2 phosphoacceptor peptide RRREEETEEE (Cancer Research UK-CRUK) or the protein kinase A (PKA) phosphoacceptor peptide LRRASLG (CRUK).

2.7.3 IVT

In vitro transcription (IVT) of class III genes was performed using 15 to 20 μg of whole cell extract (prepared as described in section 2.6.1) or HeLa nuclear extract and 250 ng of plasmid DNA containing the class III templates (section 2.7.1). Transcription was carried out at 30°C for 1 hour in a total volume of 25 μl containing 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 of each rATP, rCTP and rGTP, and 10 μCi of [α-^{32}P] UTP (400 mCi/mmol) (Amersham). Transcription was stopped by the addition of 250 μl of 1 M ammonium acetate/0.1% SDS containing 20 μg of yeast tRNA (which stabilises the newly synthesised RNA in the samples). Phenol-chloroform extraction was then performed, to remove protein and DNA, by adding 250 μl of a 25:24:1 ratio solution of phenol/chloroform/isoamyl alcohol to each sample. Samples were mixed thoroughly by vortexing, and then centrifuged at 13000g for 5 minutes. 200 μl of the resulting upper aqueous layer was then transferred to a fresh eppendorf tube containing 750 μl of ethanol. The samples were mixed by repeated inversion, and left at -20°C overnight to precipitate RNA. The following day, samples were centrifuged at 13000g for 30 minutes to pellet the precipitated RNA. The supernatant was carefully removed and discarded, then pellets were washed using 750 μl of 70% ethanol (prepared using DEPC-treated H_2O) and re-centrifuged at 13000g for 5 minutes. Again, the supernatant was discarded. RNA pellets were dried at 50°C for 5 minutes. Once dry, 4 μl of formamide loading buffer was added to each sample. Samples were then vortexed for 30 minutes to ensure complete resuspension of the RNA, and heated at 95°C for 2 minutes. Electrophoresis and autoradiography of radiolabelled transcripts was performed as described in section 2.4.2 for PCR products.

2.8 Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detection by Western blotting

2.8.1 SDS-PAGE

Proteins were resolved on denaturing sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels according to their molecular weight. Approximately 30 µg of whole cell lysate were loaded on polyacrylamide minigels (375 mM Tris pH 8.8, 0.1% SDS), with 4% polyacrylamide stacking gels (125 mM Tris pH 6.8, 0.1% SDS). Prior to loading, samples were boiled for 2 minutes in 1 X protein sample buffer (62.5 mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue). Electrophoresis was performed in 1 X SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris pH 8.3) at an initial voltage of 80V. Once the bromophenol blue dye had moved through the stacking gel and reached the resolving gel, the voltage was increased to 140V and electrophoresis was continued for 60 to 90 minutes.

2.8.2 Western blot analysis

Following resolution by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Bio-Rad) using the Bio-Rad mini trans-blot electrophoretic transfer cell system. Transfer was carried out in 1 X transfer buffer (76.8 mM glycine, 10 mM Tris pH 8.3, 16.5% methanol) at 50V for 2 hours at 4°C. Membranes were then blocked in non-fat milk buffer [125 mM Tris, 150 mM NaCl, and 0.2% Tween-20, and 5% skimmed milk powder (Marvel)] for 1 hour at room temperature, prior to incubation for 3 hours at room temperature (or overnight at 4˚C) with the relevant primary antibody diluted in milk buffer. The primary antibodies utilised for Western blotting are listed in Table 2.3. The membranes were then washed three times for 5 minutes in milk buffer, to remove excess primary antibody. Subsequently, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako), diluted 1 in 1000 in milk buffer, for 1 hour at room temperature. To ensure removal of excess secondary antibody, the blot was sequentially washed in fresh batches of milk buffer: three 10-minute washes; followed by two fifteen minute washes. Following a final 5-

minute wash in 1 X Tris buffered saline (TBS; 25 mM Tris pH 7.6, 150 mM NaCl); the bound antibodies were detected using the enhanced chemiluminescence (ECL) method (Amersham), as directed by the manufacturer.

Protein Detected	Antibody	Supplier	Dilution in milk buffer
Brf1	128	In house	1:1000
Bdp1	2663	In house	1:1000
TFIIIC110	4286	In house	1:1000
Total S6 Kinase	9202	Cell Signalling Technology	1:1000
PhosphoS6 Kinase	9202	Cell Signalling Technology	1:1000
Active Erk1 and 2	E10	Cell Signalling Technology	1:1000
Total Erk1 and 2	9102	Cell Signalling Technology	1:1000
Plk 1	F-8	Santa Cruz Biotechnology	1:1000
Haemagglutinin (HA)	F-7	Santa Cruz Biotechnology	1:1000
Actin	C11	Santa Cruz Biotechnology	1:1000

Table 2.3 Primary antibodies used for Western blot analysis

2.9 Co-Immunoprecipitation

Antibodies for immunoprecipitation were coupled with either protein A-Sepharose or protein G-Sepharose beads (Sigma). 25 µl of packed beads were used per sample and beads were washed twice with TBS prior to incubation with the appropriate antibody. 1 µg of antibodies was used in a final volume of 50 µl and incubated with the beads on a shaker for 1 hour at 4°C. Following incubation, the beads were washed twice with TBS to ensure removal of any unbound antibody. 300 µg of cell extract mixed with the beads containing bound antibody at 4°C for 3 hours on an orbital shaker. After the incubation, the beads were pelleted by gentle pulse centrifugation and the supernatant discarded. The beads were then subjected to three washes with TBS 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 Western blotting.

2.10 Transient transfection

2.10.1 Plasmids used for transient transfection.

Plasmids used for transient transfection of CCL39 or HeLa cells were pVA1 which contains the adenovirus VA1 gene, peGFP (Promega), $pcDNA_3$ expression vector (Invitrogen Life Technologies) and $pcDNA_3$-HABrf1, which encodes wild type Brf1 (Sutcliffe et al., 2000). Plasmids bearing mutations (T270A, T270D, S450A and S450D) that were introduced into $pcDNA_3$-HABrf1 by PCR-mediated site-directed mutagenesis (as described in section 2.18) were also used for transfection.

2.10.2 Transfection using lipofectamine

HeLa and CCL39 cells were transfected using the Lipofectamine reagent (Invitrogen Life Technologies). Transient transfection with lipofectamine required cells to reach a confluency of ~75% at the time of transfection. Two wells of a 6 well plate were transfected per treatment. Each set of wells were transfected with 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 contents of the two tubes were pooled and 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 37ºC. After the incubation period, 1 ml of the DNA-OptiMEM-Lipofectamine 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 37ºC in an incubator. The DNA-OptiMEM-Lipofectamine mix was then removed by aspiration from each well and replaced with 3 ml of fresh growth 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 diluted in 1.5 ml of OptiMEM and 24 µl Lipofectamine per dish. The DNA-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 ml of OptiMEM and 4.5 ml of OptiMEM was added until the end of the 45 minute incubation. At this point, the DNA-OptiMEM-Lipofectamine 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.

2.11 Primer Extension

Expression levels of the transfected pol III template VA1 (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. VA1 (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 80ºC for 10 minutes with 9 µl of First Strand Buffer (Invitrogen Life Technologies) and 1 µl of the relevant labelled probe to act as a primer. Samples were immediately transferred to a hot block for two hours incubation. 30 µl of an elongation mix containing 1 M DTT, 5 µl 5 mM dNTP mix (in DEPC- dH_2O), 4 mg/ml actinomycin D, 100 U of Superscript II Reverse Transcriptase (Invitrogen Life Technologies), was then added to the samples to initiate reverse transcription and the reaction was allowed to proceed for 1 hour at 42ºC. Reaction products were ethanol precipitated overnight, as described in section 2.8. 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 as described in section 2.4.2. Quantification of results was achieved by densitometry (Total Lab v1.11).

2.12 Chromatin immunoprecipitation (ChIP) assay

A31 and HeLa cells were grown on 10 cm tissue culture dishes for ChIP assays. One to three dishes were used per immunoprecipitation. To crosslink cellular DNA and proteins, 1% formaldehyde was added to each dish. Crosslinking was allowed to proceed for 10 minutes at 37˚C, then excess glycine was added at a final concentration of 0.125 M to stop the crosslinking, and plates were transferred to ice for harvesting. Cells were then harvested in the media/formaldehyde/glycine solution, and transferred to 50 ml Falcon tubes. Tubes were centrifuged (in a Sorvall RT 6000 D) at 500g for 5 minutes at 4˚C. Subsequently the cells were washed twice by resuspension in ice-cold PBS, followed by centrifugation at 500g for 5 minutes. The cell pellets were then washed with ice-cold PBS/0.5% NP-40, and then centrifuged at 500 g for 5 hours at 4°C. Following removal of supernatant after the final wash, cells were resuspended in 40 ml of high salt buffer (0.5% NP-40/PBS, 1 M NaCl) and incubated on ice for 30 minutes. Cells were then centrifuged at 500g for 5 minutes, and washed once in 0.5% NP-40/PBS. Subsequently, hypotonic disruption was performed by resuspending cells in 40 ml of low salt buffer (0.1% NP-40, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl) and incubating on ice for 30 minutes. Following this, samples were centrifuged at 500g for 5 minutes at 4˚C, and the resulting pellets resuspended in 1 ml of low salt buffer. To obtain nuclei, samples were then passed through a 26-gauge needle three times. The samples were re-centrifuged as before, but this time the pellets were resuspended in 2.7 ml of low salt buffer, then lysed with 300 µl of 20% sarkosyl. Subsequently, lysed nuclei were transferred to a sucrose cushion (40 ml low salt buffer/100 mM sucrose) and centrifuged at 4000g for 10 minutes at 4˚C. The supernatant was discarded, and the pellet was resuspended in 3 ml of TE (10 mM Tris pH 8.0, 1 mM EDTA). This 3 ml was then applied to a second sucrose cushion and the centrifugation process repeated. The final pellet containing genomic DNA was resuspended in 2 ml of TE, and then the DNA was sheared into smaller fragments (1kb on average) by sonication (Branson Sonifier 250, 10 X for 10 second intervals, and 30% duty cycle). 0.2 ml of 11 X NET buffer (1.65 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM Tris pH 7.4) was added to 2 ml of sonicated material, then this was transferred to eppendorf tubes for centrifugation at 13000g for 5 minutes. The supernatants were then aliquoted evenly into eppendorf tubes. Each aliquot was incubated in the presence

of 5 µg (25 µl) of an appropriate antibody overnight at 4˚C on a rotating wheel. Antibodies used for ChIP analysis are listed in Table 2.4. As a negative control, one aliquot was incubated in the absence of antibody. 10% of the aliquot volume was retained for use as an input control.

The following day, Protein-A-Sepharose beads (Sigma) were added for a further 2 hours of incubation, and then recovered on polypropylene columns (Pierce). Columns were washed twice in 10 ml of RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice in 10 ml of LiCl buffer (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA pH 8.0) and twice in TE. Beads were then transferred to 1.5 ml eppendorf tubes and immunoprecipitated material eluted by incubating the beads with 200 µl of 1% SDS/TE on a rotating wheel for 10 minutes at room temperature. This was performed twice, and each time the supernatant was collected following centrifugation at 13000g for 1 minute. The pooled supernatants along with the inputs were incubated overnight at 42˚C with 0.125 mg/ml Proteinase K (Promega) to degrade the antibodies and any other proteins present. Subsequently, genomic DNA was extracted twice using 400 µl phenol/chloroform/isoamylalcohol (25:24:1) and once using chloroform alone. Ethanol precipitation of DNA was achieved by adding 1 ml of ethanol and 40 µl of sodium acetate to each sample; the tubes were then mixed and stored at -20˚C overnight. The immunoprecipitated DNA was then resuspended in 40 µl TE, and quantified by PCR. 1 µl of immunoprecipitated or input DNA was used per PCR reaction following the protocol outlined in section 2.4.2. PCR primers and cycling parameters are indicated in Tables 2.1 and 2.2. Table 2.4 below indicates the antibodies used in the ChIP assays.

Protein recognised	Antibody	Supplier
Brf1	128	In house
Largest pol III subunit (RPC 155)	1900	In house
TFIIIC110	4286	In house
CK2β	C40420	BD Transduction Laboratory
CK2	06-873	Upstate Biotechnology
Haemagglutinin (HA)	F-7	Santa Cruz Biotechnology
c-Myc	9E10	Santa Cruz Biotechnology
TFIIA-γ	FL-109	Santa Cruz Biotechnology

Table 2.4 Antibodies used for ChIP assays

2.13 Expression of GST fusion proteins

2.13.1 Plasmids used for expression of proteins

Full length CK2α cDNA subcloned into the pGEX-2T vector (Amersham) was kindly donated by Dr Yves Goldberg. Oligonucleotide primers were designed by Dr N. Kenneth and Dr J. Fairley to amplify portions of Brf1 (described in table 2.5 below). The PCR products were inserted between Bam HI/Xho I sites in the pGEX-4T3 vector (Amersham) for bacterial expression. Furthermore the pGEX-4T3 vector containing the Brf1 H2 region fragment (table 2.5) was used as a template for site-directed mutagenesis as described in section 2.18. The GST fusion proteins generated are indicated in table 2.6 below.

Brf1 region	Primer sequence
Zn-Ribbon domain	F: 5'-GCC GAG GAT CCA TGA CGG GCC GCG TGT GCC-3' R: 5'-GGC TTA CTC GAG TCA GAT GTG GCG CCT CCC ATT CTG-3'
H1 region	F: 5'- CAA GGA TCC CAC CAC CTG GGG AAC CAG CTG-3' R: 5'-CGT CGT CTC GAG CTA GAA CTC ATC AAT GGT CCA AC-3'
H2 region	F: 5'- GCA GGA TCC GAA GCA GCA GGA AGC C-3' R: 5'-GTG CTC GAG TCA CCG TCG CTT GCA AGA CTT C-3'

Table 2.5 PCR primers used for amplifying portions of Brf1.

2.13.2 Protein expression

Escherichia coli BL21 Rosetta competent cells (Invitrogen Life Technologies) were used for expression of recombinant native and mutated portions of Brf1 and for expression of recombinant CK2α. A single colony of transformed bacteria from the appropriate plasmid was grown overnight in 10 ml of LB broth with 100 μg /ml of ampicillin and incubated at 37°C in an orbital shaker. A total of 500 ml of fresh LB medium, containing 100 μg/ml of ampicillin was inoculated with 10 ml of the overnight pre-culture and incubated at 37°C. Optimal protein production was achieved by induction with 0.1 mM IPTG (isopropyl-β-d-thiogalactopyranoside) when the cultures had reached an optical density at 600 nm of 0.8. Cells were harvested by centrifugation, at 5000g for 10 minutes, washed once with phosphate-buffered saline, resuspended in 20 ml of chilled lysis buffer (20 mM HEPES (pH 7)), and broken by three passages of 30 seconds through the sonicator. Cell extracts were cleared by centrifugation (10,000 × g, 15 min, 4°C), and supernatants were divided into aliquots and stored at −80°C until they were used. GST and GST fusion proteins were coupled to glutathione agarose

beads (Sigma) according to the manufacturer's instructions prior to use for kinase assays or pull down experiments.

Brf1 portion	Residues (amino acids)	GST fusion protein
Zn ribbon domain	1-93	GST-ZR
H1 region	281-397	GST-H1
H2 region	393-513	GST-H2
H2 region bearing mutation at S410	393-513 (S410>A)	GST-H2S410A
H2 region bearing mutation at S426	393-513 (S426>A)	GST-H2S426A
H2 region bearing mutation at S450	393-513 (S450>A)	GST-H2S450A

Table 2.6 GST-Brf1 fusion proteins expressed.

2.14 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 Whatmann 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_2O 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.

2.15 Phosphorylation assays

2.15.1 In vitro kinase assay using GST fusion proteins

2 μg of recombinant GST protein alone and portions of recombinant Brf1 tagged with GST (table 2.6) were bound to glutathione agarose beads (20 μl). These were incubated for 10 minutes at 30ºC in the presence of 50 μg of HeLa cell extract treated with either vehicle (DMSO), or with 10 μM CK2 inhibitors: TBB, DMAT or quercetin. Alternatively, the recombinant proteins were incubated with 250 ng of recombinant CK2α (New England Bio Labs). Samples were incubated for 15 minutes at 30ºC in a master mix containing 20 mM Tris-HCl pH 7.4, 20 μM ATP, 10 mM $MgCl_2$, 1 mM DTT and 5 μCi of [γ-^{32}P] ATP. The bound fusion proteins were then washed once with 300 μl TBS/0.1 % (v/v) Triton X-100 (Sigma) and then washed a further twice in 300 μl TBS. The samples were then re-suspended in 2x protein sample buffer, and analysed by SDS-PAGE. The amount of ^{32}P incorporated into GST-Brf1 fragments was assessed by autoradiography.

2.15.2 In vitro kinase assay using synthetic peptides and thin layer chromatography.

Polyetheneimine (PEI)-cellulose thin layer chromatography (TLC) was used to separate ^{32}P labelled synthetic substrate peptides (generated by CRUK). PKA peptide (LRRASLG), CK2 peptide (RRREEETEEE), Brf1 peptide consisting of residues 446 to 455 (ELDLSGIDDL) and Brf1 peptide consisting of residues 554 to 563 (PHREDAQPEH) were reconstituted in distilled H_2O. 50mM concentrations of the peptides were incubated at 30°C for 30 minutes, in presence of 750 ng of recombinant CK2 (New England Bio Labs) and 15 μl of kinase buffer (20 mM Tris-HCl pH7.4, 20 μM ATP, 10 mM MgCl2, 1 mM DTT and 15 μCi of [γ-^{32}P] ATP). After incubation, the reaction was stopped by adding 20 μl of 150 mM phosphoric acid. 5 μl of the samples were then spotted onto the anion-exchange TLC plates (Machery-Nagel) that had been previously soaked with 0.5 M NaCl for about 5 minutes and rinsed with water. The loaded TLC plates were then air-dried for about 10 minutes. Chromatography was performed using 15 mM phosphoric acid and 0.25 M NaCl as the mobile phase. The

samples were allowed to migrate for an hour. The TLC plates were allowed to dry and subsequently exposed to X-ray film. The ^{32}P-labelled compounds were visualised by autoradiography.

2.15.3 In vitro kinase assay using peptide array

The Brf1 sequence from amino acids 1 to 677 was divided into 200 overlapping peptide fragments, each consisting of 16 amino acids with an offset of 2 amino acids. The peptides were synthesised by CRUK as an array of spots on a cellulose membrane and remained covalently attached to the membrane via their carboxyl termini. The entire set of peptide mixtures was incubated with radiolabelled 10 μCi [γ- ^{32}P] ATP and kinase buffer (20 mM Tris-HCl pH7.4, 50 μM ATP, 10 mM $MgCl_2$, 1 mM DTT), in the absence or presence of 750 ng of recombinant CK2α (NE Biolabs). After the incubation, the membrane was washed five times in 75 mM phosphoric acid for 15 minutes and twice in ethanol for 2 minutes. The membrane was dried at room temperature and subjected to autoradiography.

2.15.4 CK2 activity assay

CK2 activity was assayed using synthetic peptide [RRRDDDSDDD (Upstate)] and nuclear or cell extracts which have been subjected to the various treatments (as indicated in the figure legends). Phosphorylation assays were performed in a 30 μl reaction volume containing 20 mM Tris-HCl pH 7.4, 20 μM ATP, 10 mM $MgCl_2$, 1 mM DTT, 5 μCi of [γ-^{32}P] ATP, 1 mM peptide and 5 μg of protein. After incubation for 15 min at 30°C, reaction mixtures were spotted onto Whatmann P81 ion-exchange papers (Fischer Scientific). P81 filters were then washed three times with 75 mM phosphoric acid and once with acetone. The filters were allowed to air dry and radioactivity was quantitated with a liquid scintillation counter. To determine the specific ^{32}P-incorporation into the substrate peptide, the incorporation of label into proteins obtained in the absence of the peptide was subtracted from the total ^{32}P-incorporation. ^{32}P incorporated into the sample of interest was expressed as a fold change in incorporation relative to the control samples, which were denoted as 1.

2.15.5 Phosphate labelling in vivo

Labelling was carried out by incubating CCL39 cells with 0.5 mCi/ml [^{32}P] orthophosphate for 3 hours in phosphate- free DMEM (Gibco). After incubation, cells were washed twice in 5 ml 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 26 gauge needle three times before centrifuging at 13, 000g for 10 minutes at 4ºC to pellet the cell debris. An aliquot of the supernatant was then used in a Bradford protein assay to determine protein concentration and 250-500 µg of protein was used in subsequent immunoprecipitation as described in section 2.10.

2.16 *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 30ºC for 20 minutes. Translation was carried out by adding 30 µl of STP3 Translation Mix and 4 µl (40 µCi) ^{35}S-labelled Met, made up to a final reaction volume of 50 µl with nuclease-free water. Reactions were gently mixed and incubated at 30ºC for 1 hour. 5 - 10 µl of each sample was analysed by SDS-PAGE. The gel was then incubated with 20 ml of amplify (Amersham) for 2 hours. Subsequently the gel was, Coomassie stained, destained, dried and visualised by autoradiography. The plasmids used for *in vitro* transcription-translation were: pCDNA$_3$HABrf1 described in section 2.10.1 and pCDNA$_3$HATBP (kindly donated by Dr. Stefan Roberts); both contained the T7 promoter.

2.17 GST pull- downs

Glutathione S-transferase (GST) fusion proteins were expressed in bacteria and purified on glutathione-agarose beads (Sigma) as described in section 2.14. Equal amounts of immobilised GST and GST-fusion proteins (GST-CK2α, GST-H2 and GST-H2S450A; described in section 2.13.2) were used in the pull down assays. 25 µl of packed glutathione beads bearing the GST-fusion proteins or GST alone were incubated with 100 µg of HeLa nuclear extract in 500 µl of TBS or with 250 µl reticulocyte lysate containing either ^{35}S-labelled Brf1 or TBP. Samples were incubated on a rotating wheel for 1 hour at 4ºC. Beads were then washed once in 500 µl of TBS/0.25mM NaCl/ 0.5 % (v/v) Triton X-100 and followed by a further 4 washes with TBS. Bound material was resolved by SDS-PAGE and either Western blotting or autoradiography was used to detect interacting proteins.

2.18 Site-directed mutagenesis.

Mutant forms of Brf1were prepared by using the plasmid pGEX-4T3 H2 (Brf1) and/or pcDNA$_3$HA Brf1 as the double stranded DNA templates. PCR-based mutagenesis (Quik-Change site-directed mutagenesis kit; Stratagene) was used for all mutations. For each mutant, synthetic oligonucleotide primers were designed containing the desired mutation. The primers used to create mutants of Brf1 are described in table 2.7 below.

Enzyme mutation	Primer
S410A	5'- CCGGCCCTGGGGGCCCTGCTGCACCC-3'
S426A	5'- GGCATCTCAGACGCCATCCGGGAATGC-3'
S450A	5'- AGCTGGACCTCGCTGGCATTGATGACCTG-3'
S450D	5'- AGCTGGACCTCGATGGCATTGATGACCTG-3'
T270A	5'- CGGAATTTGAAGACGCCCCCACCAGTCAGTTG 3'
T270D	5'- CCGAATTTGAAGACGACCCCACCAGTCAGTTG-3'

Table 2.7 Primers used in PCR to obtain each of the desired mutations

Thermal cycling was used to extend the oligonucleotide primers. The mutant strand synthesis reactions for thermal cycling were in a final volume of 25 µl and contained 2.5 µl of 10X Quik-Change ™ Multi reaction buffer, 0.75 µl QuikSolution, 1µl (stock 100ng/µl) of double stranded-DNA template, 1 µl of mutagenic primers, 1 µl of dNTP mix and 1 µl of Quik-Change ™ Multi enzyme blend. The cycling parameters were 95ºC for 1 minute and then 30 cycles of 95ºC for 1 minute, 55ºC for 1 minute, and 65ºC for 10 minutes. Following the temperature cycling, the reaction was placed on ice for 2 minutes to cool the reaction to below 37ºC. Dpn 1 restriction enzyme (10U/µl) was added to each amplification reaction to digest the parental (non-mutated) DNA template and immediately incubated at 37ºC for 1 hour. All recombinant plasmids were propagated into XL-10 Gold ® ultracompetent cells (Stratagene). The entire gene was sequenced to confirm the desired mutation and to check PCR fidelity.

2.18.1 Specificity of phosphorylation site specific antibody

2.18.2 Antibody production

Two antibodies were raised by immunising rabbits with an 11-amino-acid synthetic peptide of human Brf1 containing residues 444 to 460 coupled to keyhole limpet

haemocyanin (Eurogentec, Belgium). One antibody was specific to the non-phosphorylated peptide sequence (non-phosphoS450Brf1) and the other was specific to the peptide in which the S at position 450 was converted to phosphoS (phosphoS450Brf1)

2.18.3 Peptide competition assay

100 mM of phosphorylated and non-phosphorylated peptides [SHYD (p) SDGDKSD] and an equal volume water control were pre-incubated with aliquots of 0.5 µg/ml of the phosphoBrf1S450 specific antibody prior to use in the immunoassay procedures. The tubes were incubated for 30 minutes at room temperature with gentle rocking. In the meantime HeLa nuclear extracts were resolved by SDS-PAGE and transferred onto nitrocellulose (as described in section 2.9). The pre-incubated antibody in each of the three tubes was then ready for use. The contents of each tube were pipetted onto the three identical nitrocellulose strips. The strips were then subjected to Western blotting as described in section 2.9.2. This procedure was repeated using total Brf1 (antiserum 128-4; table 2.3).

2.18.4 Alkaline phosphatase treatment

Cell lysates from A31 and HeLa cells were treated with a final concentration of 1 U/µl of alkaline phosphatase (Roche) and subjected to Western blotting.

3 RNA Polymerase III Transcription is Activated by Protein Kinase CK2

3.1 Introduction

CK2 has been implicated in a number of essential cellular processes such as cell growth and proliferation [reviewed in (Guerra and Issinger, 1999)]. The significance of CK2 in cell growth is highlighted by an elevation in CK2 activity in some transformed cells compared to normal cells (Prowald et al, 1984; Rose et al, 1981). Furthermore, in response to stimulation by insulin, epidermal growth factor (Klarlund and Czech, 1988; Sommercorn et al, 1987) or to serum (Orlandini et al, 1998, Carroll and Marshak, 1989), mammalian cells showed an increase in the activity of CK2. In mammalian cells the use of antisense oligonucleotides (Pepperkok et al, 1991), microinjected antibodies that partially disrupt function of CK2 (Lorenz et al, 1993) and expression of kinase deficient CK2α' (Vilk et al, 1999) all give rise to an inhibition of proliferation.

The growth-related functions of CK2 are supported by its participation in the phosphorylation of a large number of substrates in the cell, many of which are proteins found in the nucleus which are involved in gene expression and cell growth [reviewed in Olsten and Litchfield, 2004]. The pol III machinery has been also been shown to be a target of phosphorylation by CK2 (Johnston et al, 2002; Ghavidel and Schultz, 1997; Hockman et al, 1996). In *S. cerevisiae*, a biochemical complementation approach using conditionally CK2-deficient mutants was used to show that when a yeast strain carrying a temperature sensitive version of CK2 α' is shifted to the non-permissive temperature, both growth and pol III transcription are selectively impaired (Hockman et al, 1996; Ghavidel et al, 1999). The addition of TFIIIB was sufficient to rescue transcription in CK2α' mutant cell extracts and dephosphorylation of TFIIIB by phosphatase treatment greatly reduced this effect (Ghavidel and Schultz, 1997). Ghavidel and co-workers (1997) identified the TBP subunit of TFIIIB as the target of phosphorylation by CK2. The phosphorylation of TBP by CK2 is required for efficient association of TFIIIB with TFIIIC on type II promoters during the assembly of the pre-initiation complex on the promoter in yeast (Ghavidel and Schultz, 1997; Ghavidel et al, 1999).

The TAF components of TFIIIB, Brf1 (Johnston et al., 2002) and Bdp1 (Hu et al., 2004) have also been shown to be phosphorylated by CK2 in mammalian cells. In 2002, Johnston and co-workers showed that CK2 could activate pol III transcription by binding and phosphorylating TFIIIB (Johnston et al., 2002). In contrast, phosphorylation of Bdp1 has been shown to be inhibitory to pol III transcription (Hu et al., 2003 and Hu et al., 2004). Hu and co-workers (2003) used a minimal pol III transcription system composed of purified pol III enzyme and recombinant transcription factors $SNAP_c$, Brf2-TFIIIB and TFIIIC which are required for transcription of genes with type III promoters (e.g. human U6 gene) to investigate the role of CK2 in regulating transcription by pol III at these promoters. Using this system they demonstrated that both the pol III enzyme complex and Brf2-TFIIIB were targets of CK2 phosphorylation (Hu et al., 2003). In 2004, Hu and co-workers established that the Bdp1 component of Brf2-TFIIIB is phosphorylated by CK2 during mitosis, and that this is accompanied by Bdp1 dissociation from the U6 promoter, giving rise to transcription repression. However, they also revealed that despite having an inhibitory role in mitosis, CK2 can stimulate transcription by pol III in S phase by phosphorylating pol III itself. They suggested that CK2 can be aimed 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) (discussed further in Chapter Six).

This thesis aims to further characterise the role of CK2 in the activation of genes transcribed by pol III using type I and II promoters. Work done in this chapter will not only reinforce observations made by Johnston and co-workers, but with the use of a newly developed CK2 inhibitor, 2-dimethylamino-4,5,6,7-tetrabromo-1-H-benzimidazole (DMAT), will demonstrate a more specific role for CK2 in the activation of transcription by pol III. The results in this chapter demonstrate that CK2 does activate pol III transcription in human cells and murine fibroblasts that Brf1 is phosphorylated by CK2 *in vivo*, and that CK2 binds to TFIIIB.

3.2 Results

3.2.1 CK2 activates pol III transcription in vitro

To explore the role of CK2 in the regulation of transcription by pol III, it was necessary to verify that reducing CK2 activity would have an effect on pol III transcription. Johnston et al (2002) and Hu et al (2003; 2004) demonstrated a role for CK2 on pol III transcription, based on the use of kinase inhibitors such as quercetin and LY290042 respectively. Since the publication of these studies, more efficient inhibitors both in terms of potency and selectivity 4, 5, 6, 7-tetrabromo-1*H*-benzotriazole (TBB) and the TBB derivative DMAT have become commercially available. These inhibitors are cell permeable, can bind to the hydrophobic pocket adjacent to the ATP/GTP binding site of CK2 and have been tested against a panel of ~30 kinases (Sarno et al, 2002; Pagano et al, 2004; Sarno et al, 2005). This chapter presents data obtained using these two inhibitors, as well as data obtained using quercetin and LY290042.

In the first instance, CK2 activity was repressed using a specific inhibitor DMAT (Figure 3.1). Indeed, Figure 3.1A shows that when 10 μM DMAT was pre-incubated with HeLa nuclear extracts for 15 minutes, it significantly reduced CK2 activity compared to the control DMSO-treated nuclear extracts. An *in vitro* transcription (IVT) assay was performed and Figure 3.1B shows that the addition of 10 μM DMAT to HeLa nuclear extracts dramatically decreased transcription of a $tRNA^{Leucine}$ ($tRNA^{Leu}$) gene and the adenoviral VA1 gene (lane 2) compared to the control samples (lane 1).

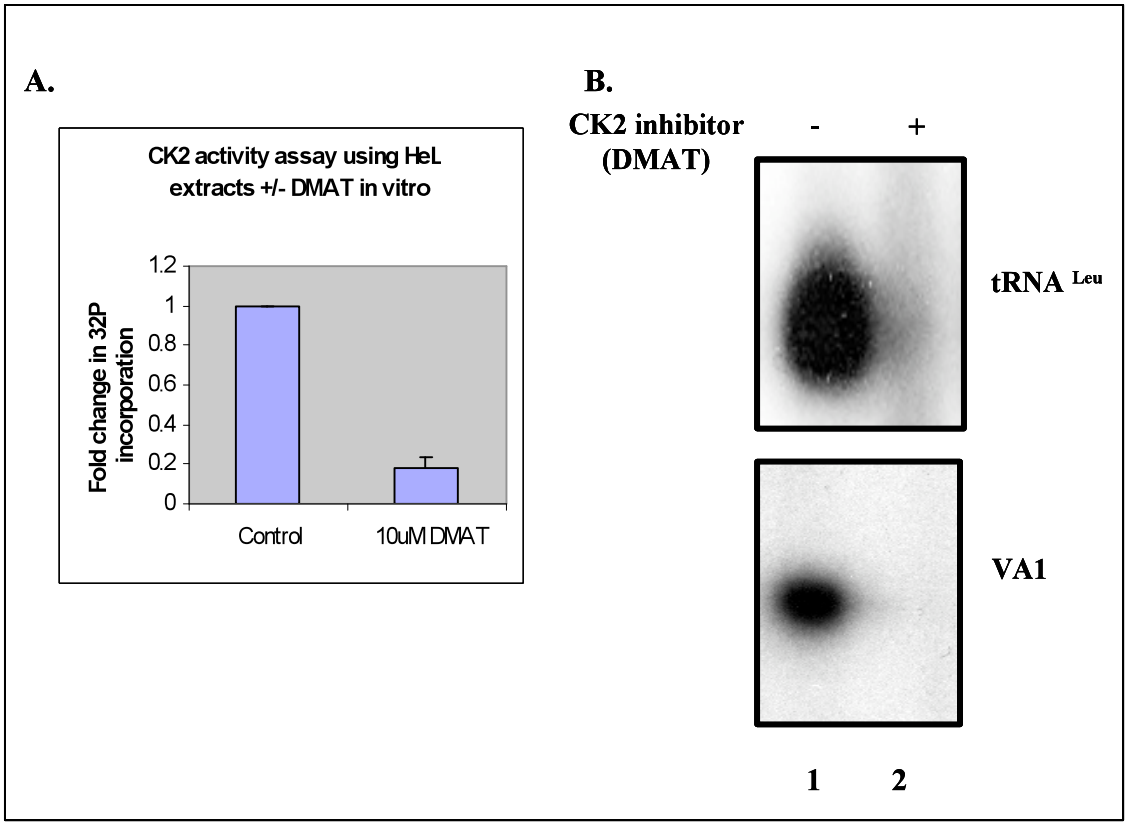


Figure 3.1. DMAT blocks VA1 and tRNA expression *in vitro*
A: CK2 activity assay of HeLa extracts in absence or presence of 10 µM DMAT. The activity of the control extracts was normalised to 100% and the activity of the treated extracts as a percentage of the control (bar graph, standard deviation of 3 determinations). B: Transcription using HeLa cell nuclear extract (15 µg) and a pLeu template (250 ng) or with pVA1 template (250 ng) after preincubation for 15 min at 30°C with DMSO (lane 1), or with 10 µM DMAT (lane 2).

An alternative manner in which CK2 activity was specifically reduced was by using a CK2 phosphoacceptor competitive substrate peptide. The CK2 activity assay shown in Figure 3.2A confirmed that CK2 phosphorylates efficiently the CK2 substrate peptide and but not the control protein kinase A (PKA) peptide. Figure 3.2B shows that pol III transcription of the VA1 gene and $tRNA^{Leu}$ gene templates were repressed after preincubation of HeLa nuclear extracts with the CK2 phosphoacceptor peptide (Figure 3.2B, lane 3). This effect is highly specific, since expression of both pol III templates was virtually unaffected by equal amounts of peptides bearing consensus phosphoacceptor sites for PKA (Figure 3.2B, lane 2). From Figures 3.1 and 3.2, it can be concluded that in human cell extracts CK2 exerts a strong effect on pol III transcription *in vitro*.

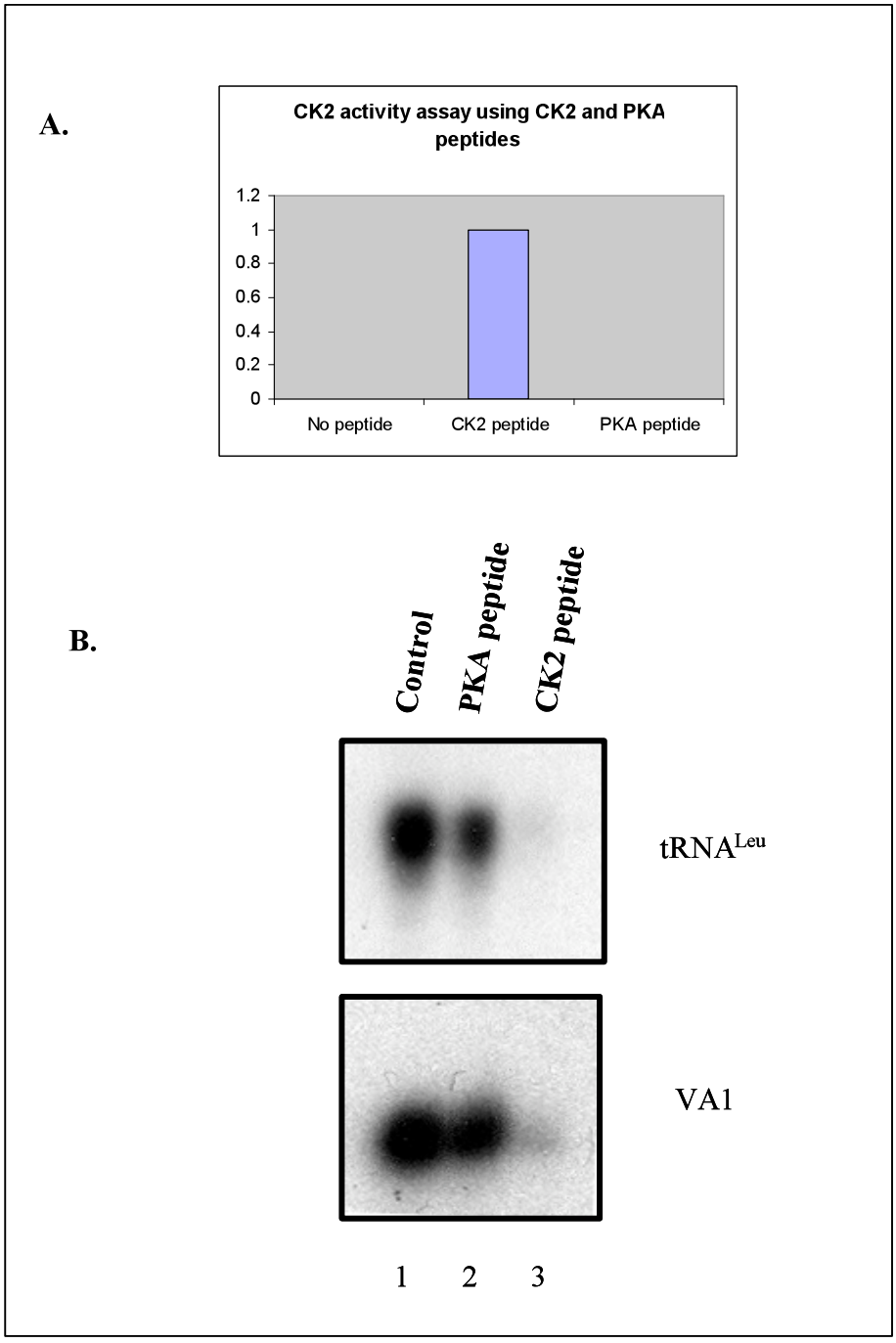


Figure 3.2. CK2 phosphopeptide blocks VA1 and tRNA expression *in vitro*
A: CK2 activity assay using: no peptide, CK2 peptide or PKA peptide. B: Transcription using HeLa nuclear extract (15 µg) a pVA1 template (250 ng) or pLeu template (250 ng) after preincubation for 15minutes at 30°C with buffer (lane 1) or with 30 µg of PKA phosphoacceptor peptide (lane 2) or CK2 phosphoacceptor peptide (lane 3).

3.2.2 CK2 activates pol III transcription in cultured mammalian cells

To determine whether CK2 exerts an effect *in vivo*, several techniques were used to demonstrate that the inhibition of CK2 by DMAT results in a decrease in the level of class III transcripts. In the first instance, Northern blotting was performed using RNA obtained from cells, which had been treated with increasing amounts of DMAT (5 to 50 μM) or DMSO alone (control). The experiment revealed that expression of the pol III transcript derived from the B2 middle repetitive gene family is reduced with addition of DMAT (Figure 3.3, lanes 2-5) in comparison to the control (Figure 3.3, lane 1). This effect is specific, since the level of acidic ribosomal phosphoprotein P0 (ARPP P0) mRNA control is relatively unaffected by the treatment. B2 RNAs have very short half-lives (less than 5 minutes) (Bladon et al, 1990), and therefore an increase in their abundance is indicative of the rate of ongoing transcription by pol III.

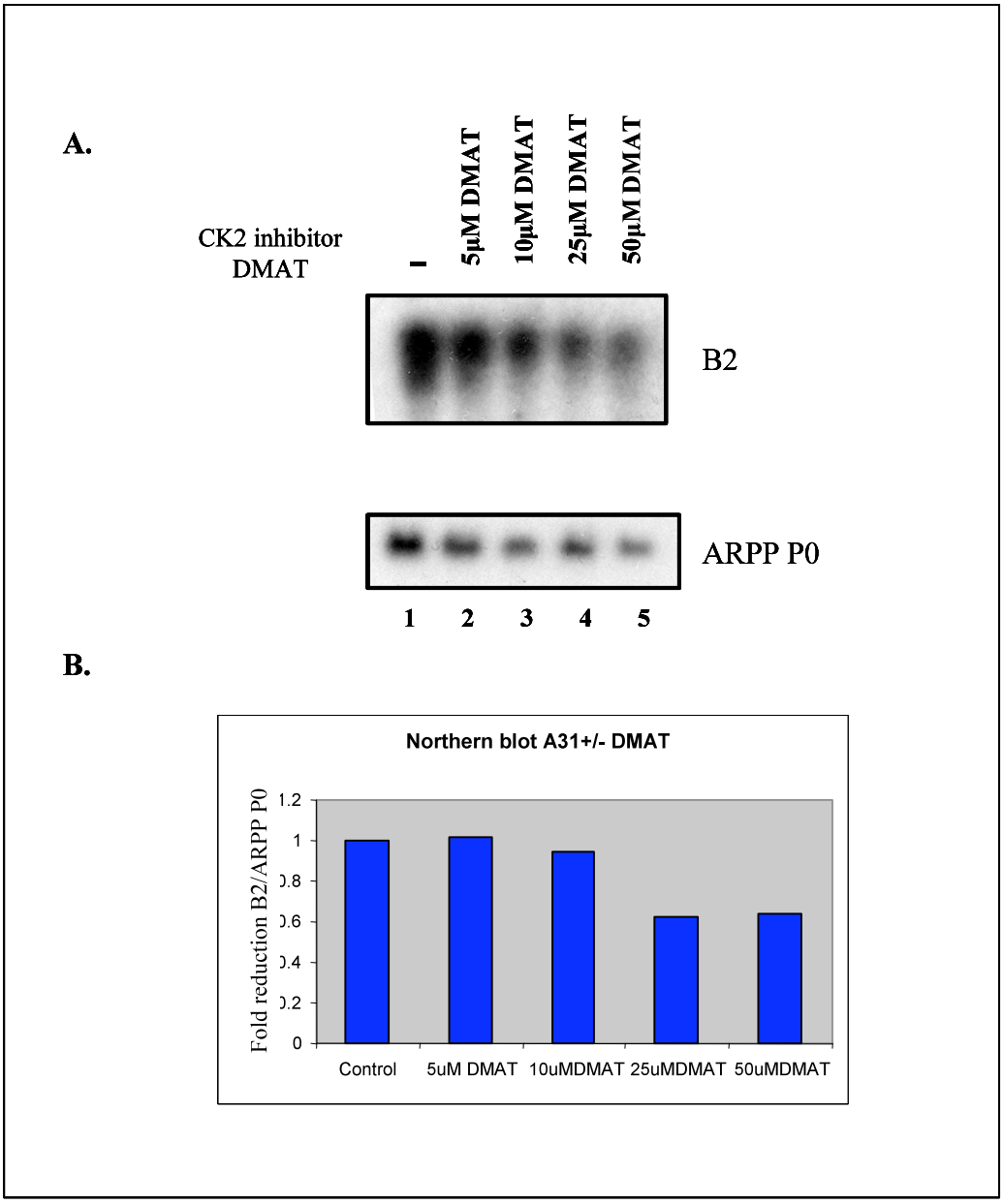


Figure 3.3. DMAT reduces the expression of the B2 pol III transcript in proliferating murine fibroblasts.
A: Northern blot analysis of total RNA (10 µg) from A31 cells cultured in 10% serum and treated with 5 µM, 10 µM, 25 µM and 50 µM DMAT (lanes 2, 3, 4 and 5 respectively) or DMSO alone (lane 1). The upper panel shows the blot probed with a B2 gene; the lower panel shows the same blot that has been stripped and reprobed with an ARPP P0 gene. B: The B2 and ARPP P0 levels were quantified by densitometry and B2 levels were normalised to ARPP P0.

As described in Chapter One, pol III transcribes a variety of genes in addition to B2. To assess the effect of DMAT on the expression of a range of other pol III-transcribed genes, semi-quantitative RT-PCR analysis was used. As previously, cells were treated with DMAT. Figure 3.4A shows a decrease of around 45-50% in CK2 activity

following treatment with 50 µM DMAT. The RNA was extracted from the treated and untreated cells and then analysed by RT-PCR using primers specific for various pol III templates. This experiment revealed a decrease in tRNAs tyrosine (tRNA ^{Tyr}) and Leucine (tRNA^{Leu}) (Figure 3.4B, lane 2) in the cells treated with 50µM DMAT compared to the control cells (DMSO) (Figure 3.4B, lane 1). The primers used to measure RNA levels were designed to hybridise to rapidly processed introns within the nascent tRNA transcripts. The levels of tRNA^{Tyr} and tRNA^{Leu} provide a direct measure of transcriptional output (Winter et al, 2000).

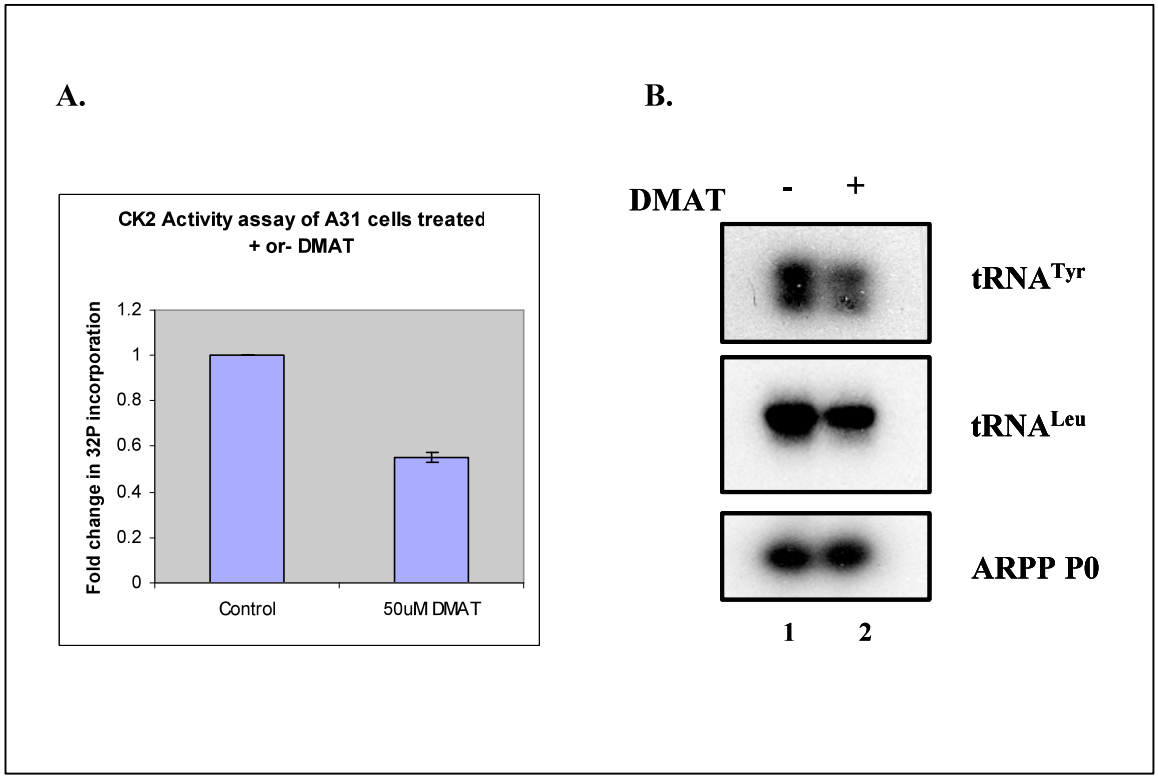


Figure 3.4. DMAT reduces the levels of tRNA^{Leu} and tRNA^{Tyr} *in vivo*.
A: CK2 activity assay of A31 cells treated with 50 µM DMAT. B: Specific primers for the transcripts indicated were used to PCR amplify cDNAs generated from total RNA of cultured cells treated with 50 µM DMAT.

The decrease in various pol III transcripts following CK2 inhibition as determined by Northern blotting and RT-PCR analysis indicates that pol III transcription is activated by CK2. In particular, the increase in intron-containing tRNAs directly demonstrated an induction of transcription by pol III. This may have been due to an increase in the intrinsic activity of pol III transcription machinery. To directly test whether the pol III machinery was more active following CK2 inhibition, IVTs were performed. Whole cell extracts were prepared from DMAT-treated cells (Figure 3.5, lanes 2-5) and control

DMSO-treated cells (Figure 3.5, lane 1) and were incubated in presence of the a $tRNA^{Leu}$ template for 1 hour at 30 ºC. RNA produced was resolved on a 7% acrylamide gel and *in vitro* transcription of pol III template $tRNA^{Leu}$ was examined.

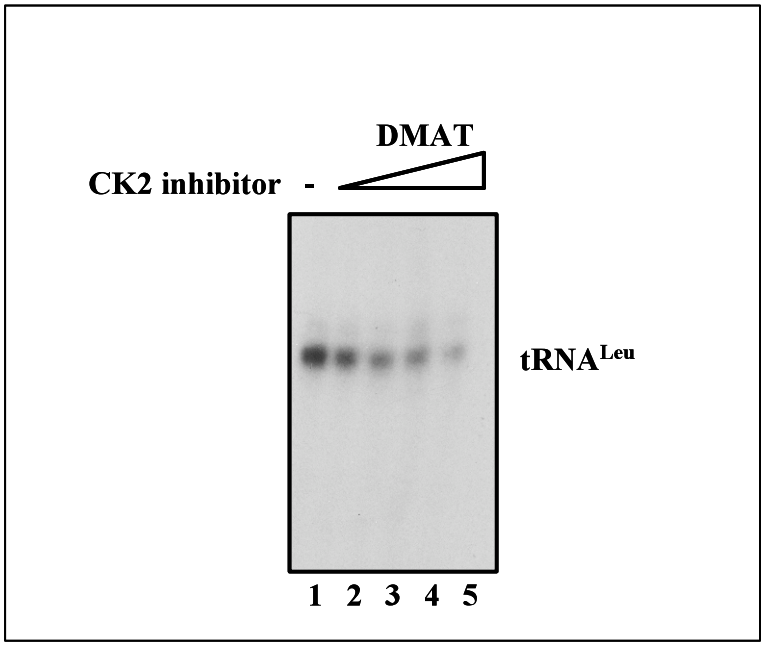


Figure 3.5. DMAT reduces pol III activity in proliferating murine fibroblasts. *In vitro* transcription assay using extracts (15 µg) from A31 cells cultured in 10% serum and treated with 5 µM, 10 µM, 25 µM and 50 µM DMAT (lanes 2, 3, 4 and 5 respectively) and a pLeu template (250 ng).

This experiment showed that pol III transcriptional activity was higher in extracts derived from control cells (Figure 3.5, lane 1) compared to the DMAT-treated cells (Figure 3.5, lanes 2-5), this supports the conclusion that pol III transcription is activated by CK2.

3.2.3 Brf1 is phosphorylated by CK2

The data indicates that CK2 kinase activity has a strong stimulatory effect on pol III transcription both *in vitro* and *in vivo*. The next set of experiments was to test whether any components of the pol III machinery are phosphorylated by CK2. Brf1 has been shown to be limiting for pol III transcription in yeast (Sethy-Coraci et al, 1998). Indeed, Johnston and co-workers (2002) demonstrated that Brf1 is phosphorylated *in vivo* in Chinese hamster ovary (CHO) cells. Figure 3.7 below investigates whether Brf1 is phosphorylated in Chinese hamster lung fibroblast (CCL 39) cells. To examine

whether Brf1 is subject to phosphorylation *in vivo*, CCL39 cells were transfected with a vector encoding HA-tagged Brf1 (pBrf1). The cells were cultured in the presence or absence of the CK2 inhibitor quercetin. The cells were then labelled with [^{32}P] orthophosphate, harvested and subjected to immunoprecipitation with an anti-HA antibody. The precipitated material was resolved by 7.8% SDS-PAGE and the experiment revealed that Brf1 is phosphorylated *in vivo* in CCL39 cells. Western blotting confirmed that equal amounts of Brf1 were immunoprecipitated in each instance.

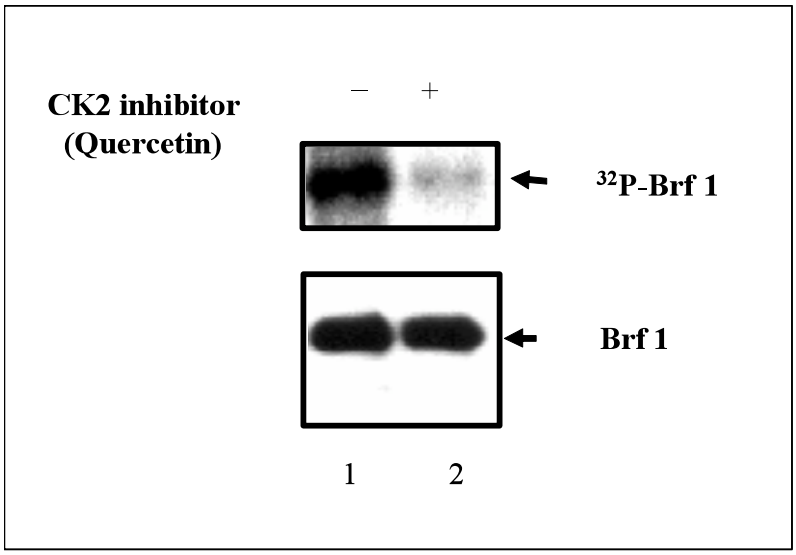


Figure 3.6. Brf1 is phosphorylated *in vivo*.
CCL 39 cells growing in 10% FBS were transiently transfected with pBrf1 and labelled 48 hours later with [^{32}P] orthophosphate for 2 hours in absence or presence of 60 µM quercetin. Cell extracts were prepared and Brf1 was immunoprecipitated with anti-HA antibody F-7 transferred to nitrocellulose and visualised by autoradiography (top panel) or by Western blotting (lower panel).

These findings confirm that CK2 contributes to the phosphorylation of Brf1 *in vivo* and that this phosphorylation is not restricted to CHO cells.

The Target of Rapamycin (TOR) kinases, have also been shown to activate pol I and pol III transcription in yeast (James et al, 2004 and Zaragoza et al, 1998) and addition of the specific macrolide inhibitor of mTOR rapamycin to mammalian cells has been shown to decrease pol III transcription (Unpublished data, E. Graham, B. Ramsbottom and R.J. White). The TOR kinases can co-ordinate nutrient availability and the protein biosynthetic capacity of the cell, Figure 3.8 below show a simplified diagram of the

mTOR pathway, whereby it can regulate the synthesis of ribosomal proteins and expression of ribosomal RNA.

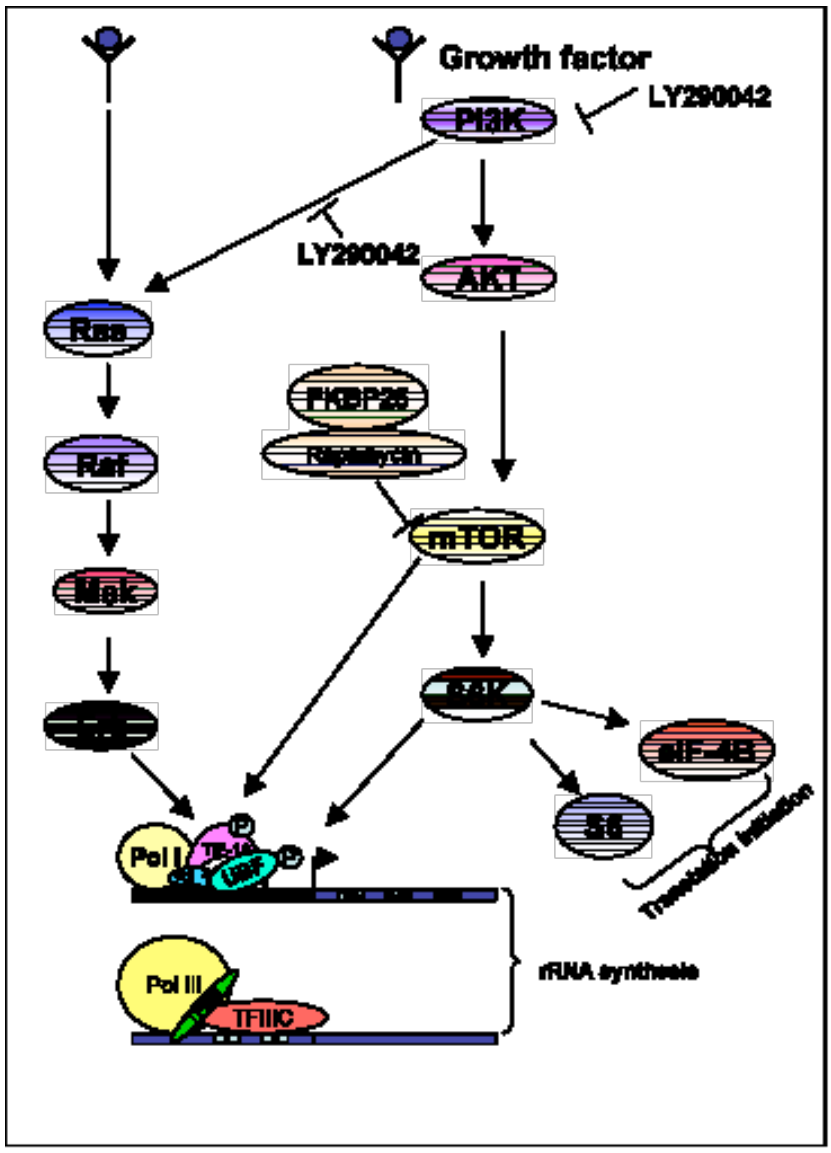


Figure 3.7. The mammalian target of rapamycin (mTOR) signalling pathway activates pol III and pol I transcription.

Activation of phosphatidylinositol 3-kinase (PI3K) by growth factors signals the cell to grow and proliferate; this signal is transmitted through the cell by Akt/PKB to mTOR. Activation of the PI3K/Akt/mTOR pathway by growth factors controls protein synthesis at the level of translation initiation and ribosome biogenesis. Upon stimulation, mTOR activates proteins via phosphorylation involved in translation such as ribosomal S6 kinase (S6K). In this simplified cartoon Akt modulates the activity of transcription factors important for pol I and pol III activity and rRNA synthesis, thereby regulating ribosome biogenesis and global protein synthesis. Rapamycin specifically inhibits mTOR and LY290042 inhibits PI3K (Adapted from Mayer and Grummt, 2006).

In vivo labelling experiments were performed to check whether mTOR could also influence Brf1 phosphorylation. To test this an *in vivo* labelling experiment was performed whereby CCL39 cells were transfected with mammalian expression vector (pBrf1) and cultured in the presence or absence of rapamycin (Figure 3.9A, lane 3) or with LY290042 (Figure 3.9A, lane 4). The cells were subsequently labelled with [^{32}P] orthophosphate, harvested and subjected to immunoprecipitation with an anti-HA antibody. Figure 3.8A shows that Brf1 does not appear to be phosphorylated by mTOR, since no change in ^{32}P incorporation can be detected with the addition of rapamycin, which specifically inhibits mTOR, nor with LY290042. This suggests that mTOR could influence pol III transcription via a different target of the pol III machinery.

In yeast the FKBP binding protein FKBP25 (Figure 3.7) can be found in complex with the CK2 and CK2 can phosphorylate one member of the FKBP family (Wilson, 1997 and Litchfield, 1993), this evidence suggested the possibility that rapamycin could influence pol III transcription in a CK2-dependent manner. Figure 3.8C shows a CK2 activity assay to test whether extracts from rapamycin treated cells have reduced CK2 activity. The results show that CK2 activity is almost identical in extracts from control and treated cells, arguing that rapamycin does not exert its effect on pol III transcription by reducing CK2 activity.

LY290042 has been suggested to be a potential CK2 inhibitor (Davies et al, 2000), in this experiment the activity assay in Figure 3.7C demonstrates that there is little or no effect on the CK2 activity when cells had been treated with LY290042. The Western blots in Figure 3.8B show that both the rapamycin and LY290042 were active since the phosphorylation of S6 Kinase was ablated with the addition of LY290042 and with the addition of rapamycin (Figure 3.8B, top panel), although the levels of total S6 Kinase were essentially unaffected (Figure 3.8B, second panel).

Since the PI3K has been linked to the Erk pathway, the levels of phosphorylation of Erk were also verified for any variations as it has been shown that Erk can phosphorylate Brf1 (Felton-Edkins, 2003). Figure 3.8B (third panel) shows that the levels of phosphoErk in lane 2 are decreased slightly with the addition of LY290042 and that rapamycin does not affect the levels of phosphoErk in lane 3. The bottom panel shows no change in the levels of total Erk.

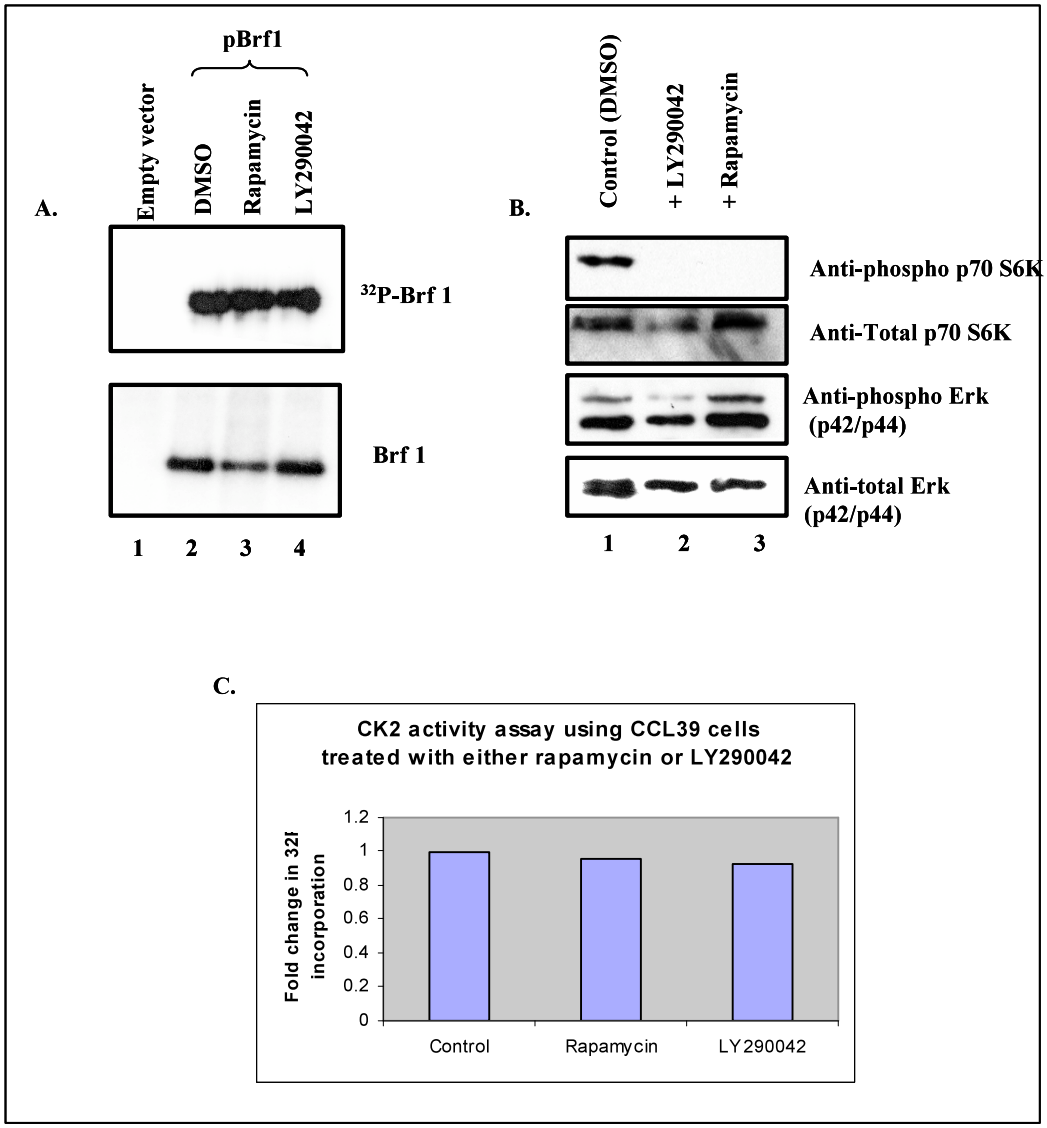


Figure 3.8. Brf1 is not phosphorylated *in vivo* by mTOR.
(A) CCL 39 cells growing in 10% FBS were transiently transfected with pBrf1 and labelled 48 hours later with [^{32}P] orthophosphate for 2 hours in absence or presence of rapamycin. Cell extracts were prepared and Brf1 was immunoprecipitated with an anti-HA antibody F-7, transferred to nitrocellulose and visualised by autoradiography (top panel) or by Western blotting (lower panel). (B) Cell extracts were prepared and also subjected to Western blotting with an antibody against phospho S6 kinase and total S6 kinase and against phospho-Erk (p42/44) and total Erk (p42/44). (C) Cell extracts were also subjected to a CK2 activity assay following treatment with either DMSO alone, rapamycin or LY290042.

3.2.4 CK2 binds TFIIIB

To investigate whether CK2α interacts with TFIIIB, GST pull down assays were performed, whereby *in vitro* translated full length Brf1 and TBP were tested for binding to a GST-CK2α fusion protein (Figure 3. 9). This experiment revealed that CK2α can directly interact with full length Brf1 and TBP (Figure 3.9, lane 3) but not to GST alone (Figure 3.9, lane 2). Having established that CK2 interacts with TFIIIB *in vitro*, the interaction between native TFIIIB and CK2 was further analysed.

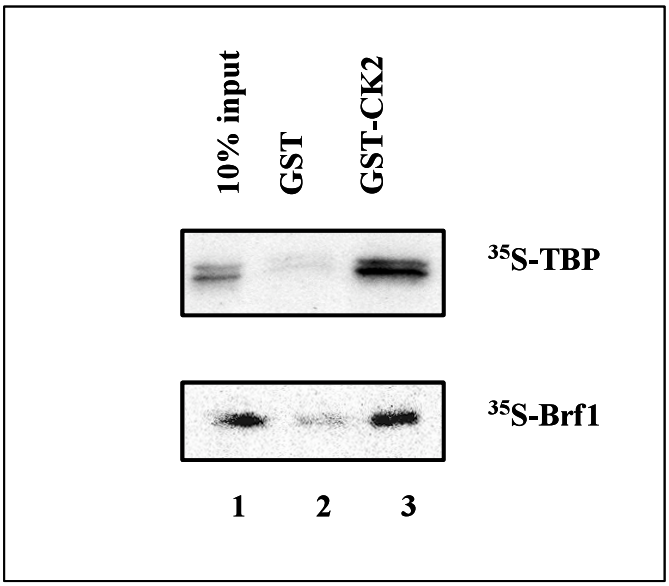


Figure 3.9. Interaction of TFIIIB and CK2α *in vitro*.
Aliquots of 5 µg of purified GST or GST-CK2α were incubated with 40 µl of glutathione Sepharose beads as described in Chapter Two. The TFIIIB subunits TBP and Brf1 were produced in reticulocyte lysate using the TnT T7 *in vitro* transcription/translation system (Promega). The newly synthesised proteins were labelled with [^{35}S] methionine and 5 µl of lysate were used in each reaction. The precipitated proteins were applied to SDS–PAGE; 0.5 µl of lysate was used as input shown above.

HeLa nuclear extract were immunoprecipitated with an anti-CK2α antibody and an anti-TFIIA antibody. The beads were then washed as described in Chapter Two and the bound material was analysed by Western blotting using an anti-Brf1 antibody. Figure 3.10 revealed that Brf1 can specifically interact with CK2α (Figure 3.10, lane 2) whereas Brf1 was not detected in lane 3, where anti-TFIIA was used as a negative control.

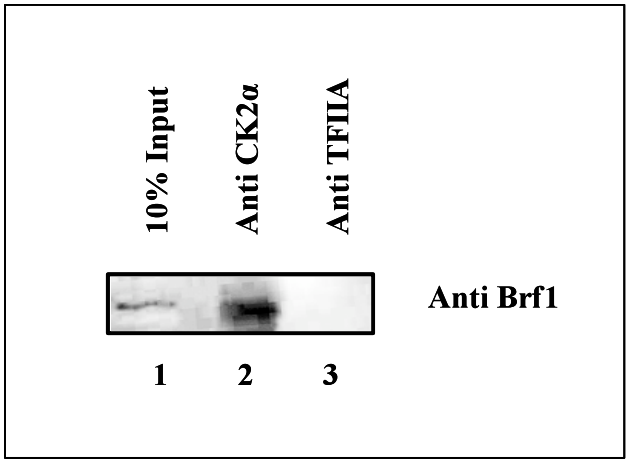


Figure 3.9. Interaction of endogenous TFIIIB with CK2α.

HeLa nuclear extract (250µg) was immunoprecipitated with an anti-CK2α antibody and a negative control antibody against TFIIA. The precipitated material was resolved by SDS- PAGE and analysed by Western blotting using an anti-Brf1 antibody.

From the data in Figures 3.9 and 3.10 it can be concluded that CK2α can bind to isolated TFIIIB subunits Brf1 and TBP *in vitro* and to endogenous TFIIIB.

3.3 Discussion

This chapter supports the findings that the protein kinase CK2 can activate transcription of genes by pol III in mammalian cells. Indeed, several research groups have shown that CK2 activity is required for efficient transcription by pol III in *S. cerevisiae* and in mammals (Hockman et al, 1996; Johnston et al, 2002 and Hu et al, 2003). Disruption of CK2 causes the synthesis of tRNA and 5S rRNA to decline 80-90% in yeast (Hockman et al, 1996). With the use of a very specific cell permeable inhibitor of CK2, DMAT (Pagano et al, 2004), Northern blotting, RT-PCR analysis and IVTs presented in this chapter, revealed that specific inhibition of CK2 by DMAT is accompanied by a decrease in pol III transcription. CK2 is a highly conserved protein and regulates pol III transcription in both yeast and mammals. The conservation of this control suggests that CK2 may be a crucial regulator of pol III transcription.

This study shows that Brf1 is subject to phosphorylation *in vivo*, when CCL39 cells were treated with the CK2 inhibitor quercetin there was a decrease in phosphorylation of Brf1. This supports *in vivo* labelling experiments done in CHO cells by Johnston and co-workers (2002) and also demonstrates that it is not cell type-specific. One common theme that emerges from the data is that CK2 can phosphorylate and regulate TFIIIB complexes in both yeast and mammals. In *S. cerevisiae*, only TBP is phosphorylated by CK2 *in vitro* and *in vivo*, and it was concluded that the phosphorylation of TBP is responsible for the activation of TFIIIB by CK2. Ghavidel and Schultz identified S128 which is found on the surface of TBP, as a potential CK2 target. Although a serine is found at the corresponding position of human TBP (S222), its surrounding residues do not match the CK2 consensus (S/Txxacidic where n=3 is the acidic residue). Other residues in TBP might be phosphorylated by CK2 in humans; however, *in vitro* kinase assays from Hu et al (2004) showed that mammalian TBP does not seem to be target for phosphorylation by CK2, despite the presence of CK2 phosphoacceptor sites found within TBP. Transcription of the human U6 gene (which has a type III promoter) by pol III can be activated by CK2 following phosphorylation of the pol III enzyme complex itself (Hu et al, 2003). These studies were performed using purified pol III and were done *in vitro* only, and it remains to be verified whether this applies to all class III genes and whether the pol III complex is phosphorylated *in vivo*.

The TOR kinases can stimulate pol III transcription in yeast (Zaragoza et al, 1998), and there is evidence that this signalling pathway can also influence pol III activity in mammals (unpublished data, E. Graham, B. Ramsbottom and R.J.White). In this study Figure 3.9 shows that Brf1 does not appear to be subject to phosphorylation by mTOR, since there is no significant change in ^{32}P incorporation of Brf1 with the addition of rapamycin. Their data have revealed that it is in fact the TFIIIC component of the pol III machinery which is subject to phosphorylation by mTOR. Although CK2 has been found to bind to FKBP25 (Pinna et al, 1995), the CK2 activity assay in Figure 3.9 does not show considerable variations in activity when rapamycin is added which implies that the effects of rapamycin on pol III transcription appear to be CK2-independent. LY290042 has been used as a CK2 inhibitor (Davies et al, 2000 and Hu et al, 2003), but in this study no significant changes in CK2 activity were observed when LY290042 was added to proliferating cells. In this experiment (Figure 3.9), LY290042 does not appear to be an efficient CK2 inhibitor, even though it is able to inhibit the PI3K/mTOR pathway as shown in the Western blots.

The next sets of experiments performed were to verify the associations of CK2 and TFIIIB. CK2 and Brf1 were found to interact, which is consistent with previous findings (Johnston et al, 2002). *In vitro*, CK2 was also shown to bind to TBP, consistent with yeast studies (Ghavidel et al, 1997).

Pol I and pol III together are responsible for ~80% of RNA synthesis in cells and are often subject to tight co-regulation. CK2 has been implicated in activation of transcription by pol I both in mammals and in yeast (Johnston et al, 2002; Hockman et al, 1996). The fact that CK2 can regulate transcription by pol I and pol III suggests that CK2 plays an important role in controlling the overall biosynthetic capacity of the cell.

Having confirmed that pol III transcription is activated by CK2 in mammalian cells, the next aims of the project were to determine which particular residue(s) of Brf1 are phosphorylated by CK2, which will be investigated in the following chapter.

4 Serine 450 of Brf1 is Phosphorylated by CK2 *in vitro* and *in vivo.*

4.1 Introduction

CK2 is rather unusual among the protein kinases in that it can use both ATP and GTP as phosphate donors (Allende and Allende, 1995). The minimum consensus sequence for phosphorylation by CK2 is denoted by: S/T-X-X-D/E where X represents any residue and glutamate (E) or aspartate (D) can be found at the n + 3 position (Allende and Allende, 1995; Meggio et al., 1994). However, the E or D at the third position can however be replaced by phosphoserine (Litchfield et al., 1990; Meggio et al., 1992) or phosphotyrosine (Meggio et al., 1991) but not phosphothreonine (Litchfield et al., 1990; Meggio et al., 1991). There are thirteen consensus sites for CK2 in Brf1 which are T12, S30, T245, T265, T276, S323, S351, T365, S410, S426, S450, T520, and S580 (Figure 4.1).

Brf1 is a subunit of TFIIIB which is required for the recruitment of the pol III to the promoter. It consists of 677 amino acids and has a predicted molecular weight of around 90 kDa (Wang and Roeder, 1995). Figure 4.1 shows a simplified linear representation of Brf1 and highlights its key domains. The amino (N)-terminal Zinc (Zn) binding domain which contains a sequence motif of Cys-X_2-Cys-$X_{15/17}$-Cys-X_2-Cys (where X= any residue and Cys= Cysteine) and adopts a Zn ribbon structure (Colbert and Hahn, 1992). The "core domain" consists of two imperfect repeats, which can bind to TBP (Juo et al, 2003). The carboxyl (C)- terminal half of Brf1which contains three homology regions designated H1, H2 and H3, which are also well conserved and found in yeast (White, 2002). The H2 region of Brf1 has also been shown to bind to TBP (Juo et al., 2003).

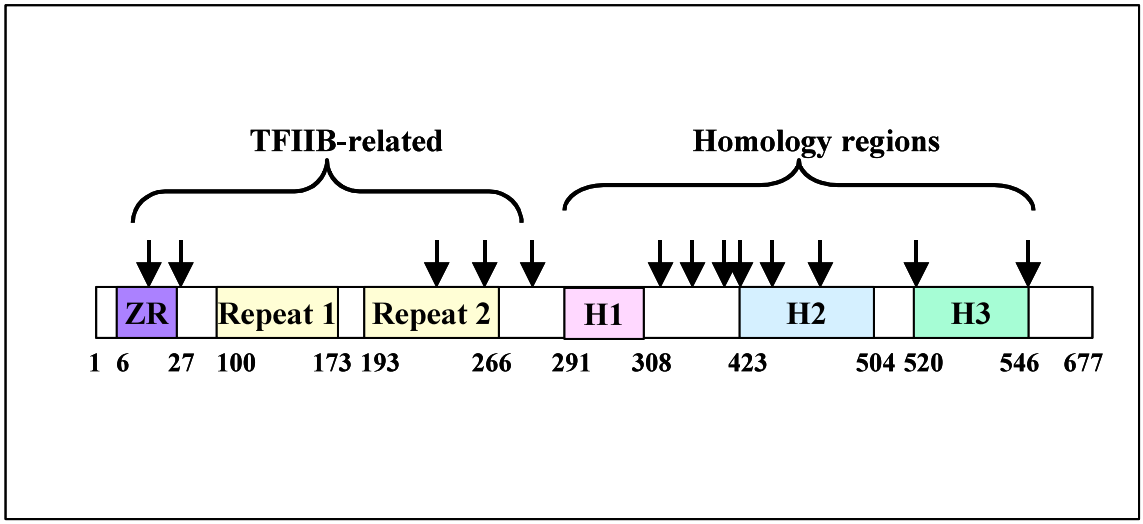


Figure 4.1. Schematic linear illustration of the structure of mammalian Brf1. The regions of Brf1 are the Zn binding motif (ZR, residues 6-27), repeats 1 and 2 (residues: 100-173; 193-266), the homology regions: H1 (residues: 291-308), H2 (residues: 423-504), H3 (residues: 520-546). The arrows indicate the 13 possible CK2 targets within Brf1.

The MAP kinase Erk can induce pol III transcription by binding and phosphorylating Brf1 (Felton-Edkins et al., 2003a). This enhances the interaction of TFIIIB with both TFIIIC and pol III, resulting in an increase in pol III transcription (Felton-Edkins et al., 2003a). In mitosis, Brf1 is phosphorylated (Fairley et al., 2003) and unpublished data (J.Fairley and R.J.White) suggest that Plk1 is the kinase responsible. Mitotic phosphorylation of Brf1 results in a weakened interaction with Bdp1, which can compromise the recruitment of pol III to the promoter and inhibit class III gene expression (Fairley et al., 2003).

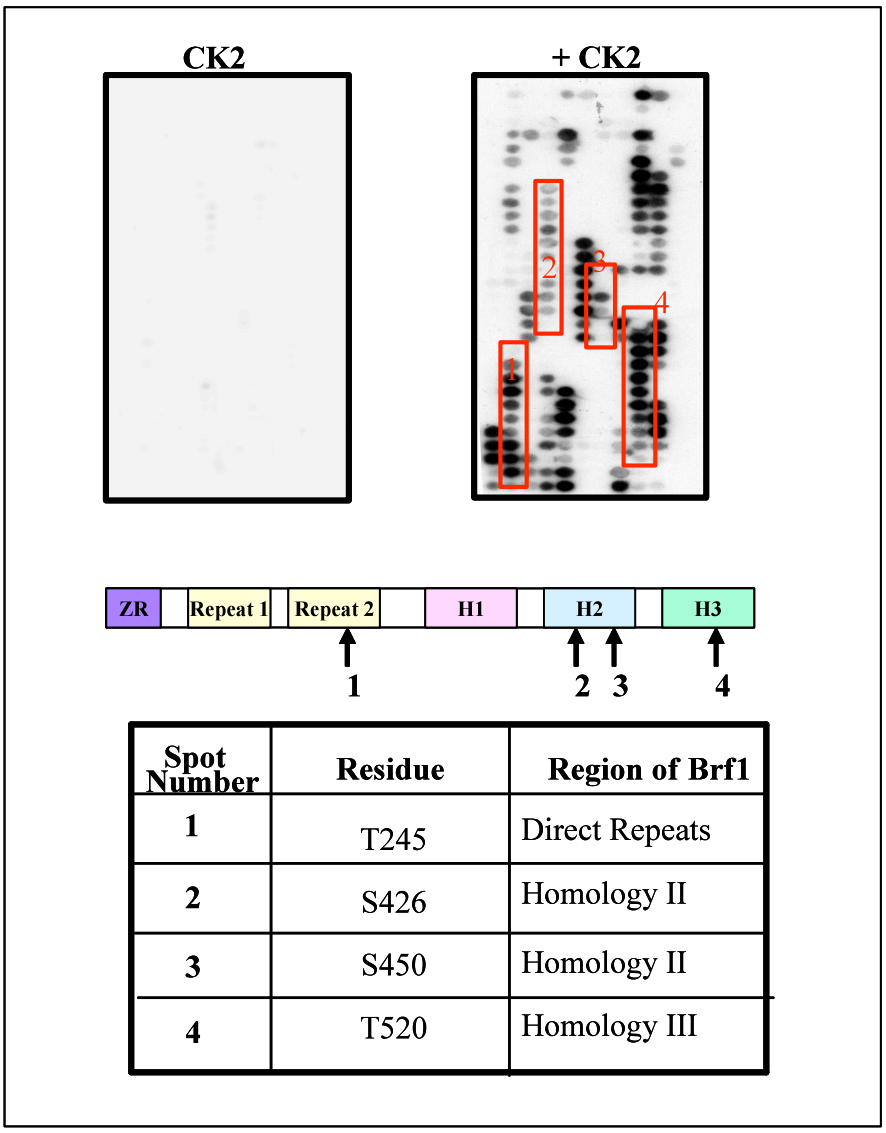
To better understand the functional significance of the phosphorylation of Brf1 by CK2, it is important to identify the phosphorylation sites involved. The aim of the work presented in this chapter was to identify the residue(s) phosphorylated by CK2 using several approaches, such as peptide array, glutathione-S-transferase (GST) fusion proteins, small synthetic Brf1 peptides, site directed mutagenesis and a phosphospecific antibody. The data presented demonstrate that the Brf1 residue S450 found in the H2 region of Brf1 is a target of phosphorylation by CK2.

4.2 Results

4.2.1 S450 is phosphorylated by CK2 in vitro

In an attempt to map the phosphorylation sites of CK2 on Brf1, a rapid and general method was initially employed. The Brf1 sequence from amino acids 1 to 677 was divided into 200 overlapping peptide fragments, each consisting of 15 amino acids with an offset of 2 amino acids. The peptides were synthesised as an array of spots on a cellulose membrane, and remained covalently attached to the membrane via their C-termini. These membranes were then used in a kinase assay. The entire set of peptides was incubated with radiolabelled [γ- ^{32}P] ATP and a kinase buffer, in the absence or presence of recombinant CK2. After extensive washing, the membrane was dried and exposed to film. The results shown in Figure 4.2 revealed that several peptides were phosphorylated. However, out of the phosphorylated peptides, only four contained specific CK2 consensus sites. The amino acids that were positively implicated were T245, S426, S450 and T520.

This method was used to try and narrow down which particular residue(s) of Brf1 could be phosphorylated. The method was not very specific, as many unspecific peptides were phosphorylated *in vitro* as shown in Figure 4.2. However, it does not rule out the possibility that the residues T245, S426, S450 and T520 could be phosphorylated by CK2.



Spot Number	Residue	Region of Brf1
1	T245	Direct Repeats
2	S426	Homology II
3	S450	Homology II
4	T520	Homology III

Figure 4.2. Determination of phosphorylation sites for CK2 on Brf1 by peptide array.
The top left panel shows the negative control Brf1 peptide array following incubation with radiolabelled [γ- ^{32}P] ATP and kinase assay buffers minus recombinant CK2. The top right panel shows the peptide array following phosphorylation by recombinant CK2. The boxed spots indicate peptides which have been phosphorylated by recombinant CK2 and bear consensus CK2 sequences. The linear schematic representation of Brf1 in the middle indicates the relative positions of the phosphorylated sequences on Brf1. The table at the bottom represents the precise position of the phosphorylated residues and their relative domain position.

In addition to the peptide arrays, an alternative method was also used to identify the Brf1 site(s) of CK2 phosphorylation. Individual Brf1 domains were isolated from

bacteria as fusion proteins with GST (Figure 4.3) and subsequently subjected to *in vitro* kinase assays.

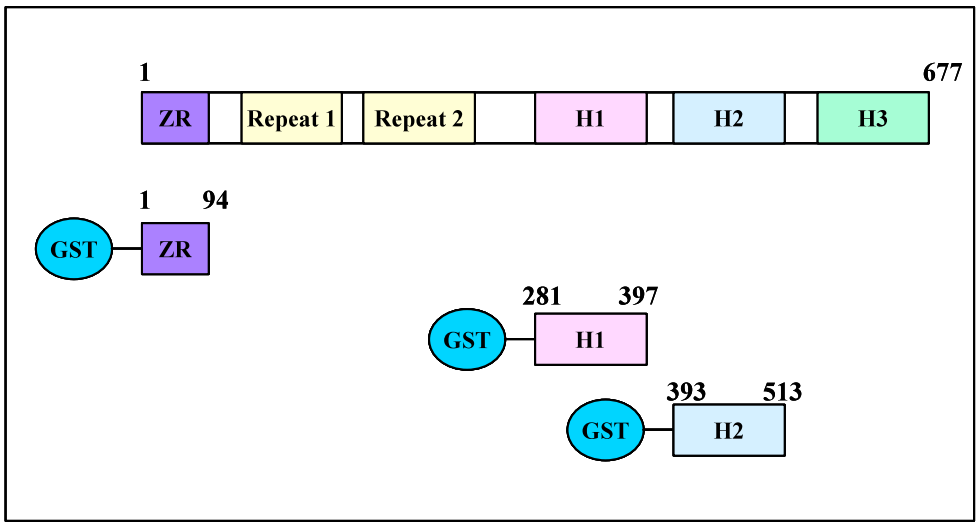


Figure 4.3. Schematic illustration of the GST fusion proteins encompassing individual Brf1 domains.
GST fusion proteins were generated from Brf1 represented at the top. Individual fusion proteins were made incorporating different domains of Brf1. The Zn ribbon domain (GST-ZR), the H1 region (GST-H1) and the H2 region (GST-H2).

As shown in Figure 4.3, only three fragment proteins of Brf1 that were successfully expressed: The GST-fusion proteins incorporating the direct repeats and the H3 region could not be obtained in spite of several attempts at expression. In the first instance, equal amounts of GST fusion proteins, as confirmed by Coomassie staining (Figure 4.4, bottom panel) were incubated with HeLa nuclear extracts which had been treated with chemical CK2 inhibitors including DMAT (Figure 4.3, top panel), quercetin (Figure 4.4, second panel) and TBB (Figure 4.4, third panel) or with DMSO only (negative control). Phosphorylation of the H1 and H2 fusion proteins appear to be reduced with the addition of CK2 inhibitors (lanes 6 and 8) compared to the control samples (lanes 7 and 9). In contrast, the GST-ZR fusion protein does not appear to be phosphorylated under these conditions (lanes 3 and 4), and the negative control GST protein alone was not phosphorylated, as expected. These data show that phosphorylation in this system has specificity. For both H1 and H2, the CK2 inhibitors block phosphorylation to a considerable degree, which suggests that CK2 is the predominant kinase for these domains under the conditions of this assay. The inhibition of phosphorylation appeared to be greater for the H2 fusion protein.

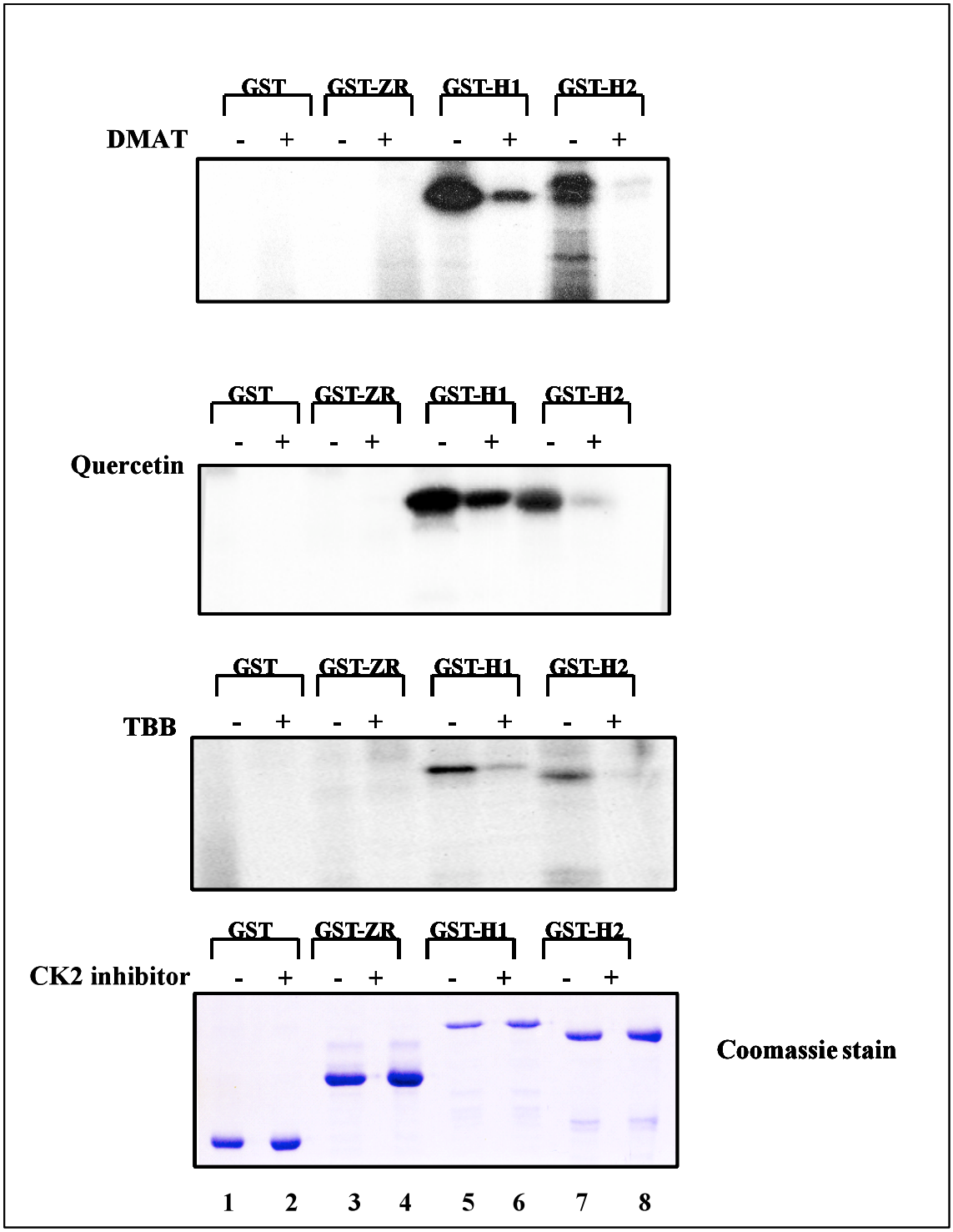


Figure 4.4. The H1 and H2 regions of Brf1 are phosphorylated by CK2 *in vitro*. Equal amounts (bottom panel) of the fusion proteins of GST and individual Brf1 domains were isolated from bacteria and subjected to *in vitro* kinase assay using HeLa extract in absence or presence of the CK2 inhibitors, DMAT (top panel), quercetin (second panel) and TBB (third panel).

The GST fusion proteins were also incubated in the presence of recombinant CK2, kinase buffer and radiolabelled ATP (Figure 4.5). The results demonstrate that the

GST-H2 fusion protein only is phosphorylated when low doses of recombinant CK2 are added. In contrast, the GST-H1 fusion protein is not phosphorylated, which could be due to a requirement by CK2 for another protein to be present in cell lysates in order to enable phosphorylation of GST-H1. Another possibility could be that GST-H1 could be phosphorylated by another kinase which can be inhibited by the chemical inhibitors used in Figure 4.4.

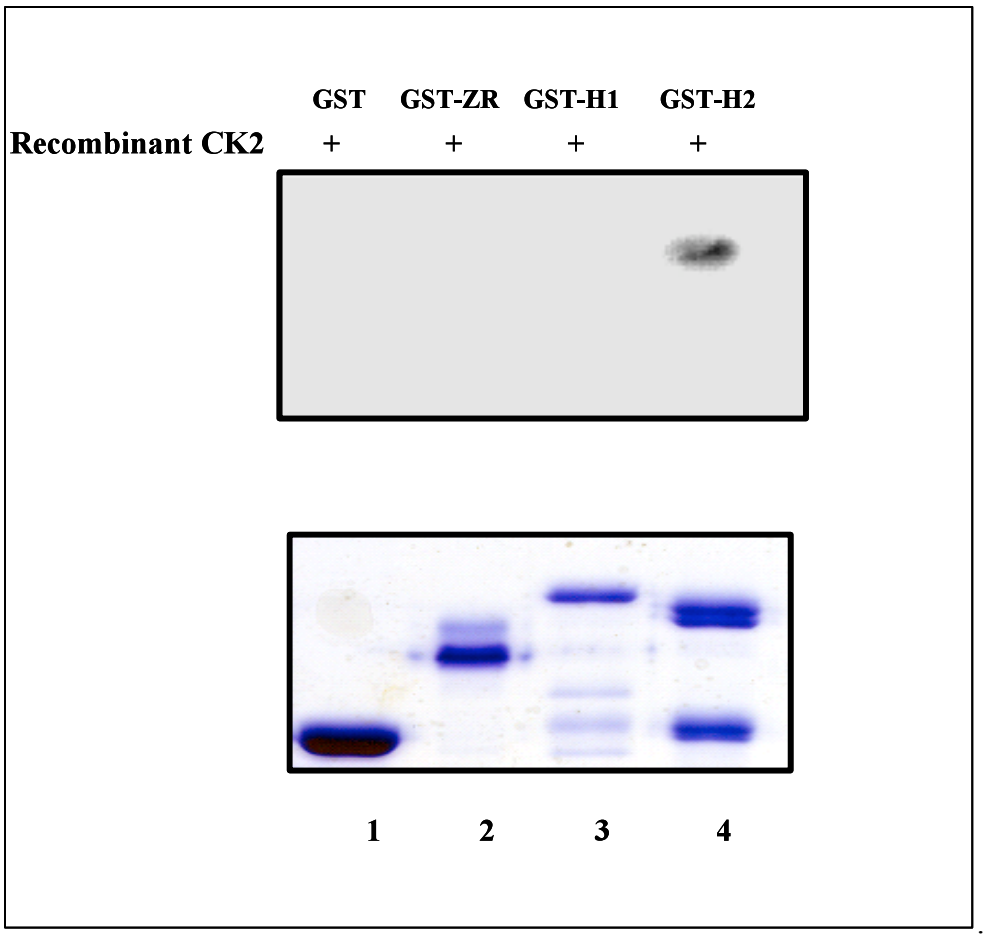


Figure 4.5 GST-H2 fusion proteins are phosphorylated by recombinant CK2 *in vitro*.
The indicated fusion proteins of GST and individual Brf1 domains were isolated from bacteria and subjected to an *in vitro* kinase assay in the presence of recombinant CK2 and radiolabelled ATP. The samples were resolved by SDS-PAGE before staining with Coomassie brilliant blue (bottom panel). Gels were dried and subjected to autoradiography (top panel).

The combined data from the peptide array and the *in vitro* kinases assays point towards the H2 region of Brf1 as a potential target of phosphorylation by CK2. The experiments described in the rest of this chapter focused on characterising phosphorylation targets within this region of Brf1.

The H2 region of Brf1 contains three potential sites of phosphorylation by CK2: S410, S426 and S450. Data from the peptide array suggested that the latter two residues could be targets of CK2 phosphorylation. In order to explore this possibility further, site-directed mutagenesis of S410, S426 and S450 to non-phosphorylatable alanines was performed, and the mutants were expressed as GST fusion proteins. The GST fusion proteins bearing the mutations were then used in an *in vitro* kinase assay. Equal amounts of the GST fusion proteins as shown in Figure 4.6 (bottom panel) were incubated in the presence of CK2, kinase buffer and radiolabelled ATP. The results showed that the third mutation, S450A, resulted in a dramatic decrease in phosphorylation of the H2 region. This indicates that Brf1 S450 is phosphorylated *in vitro* by recombinant CK2.

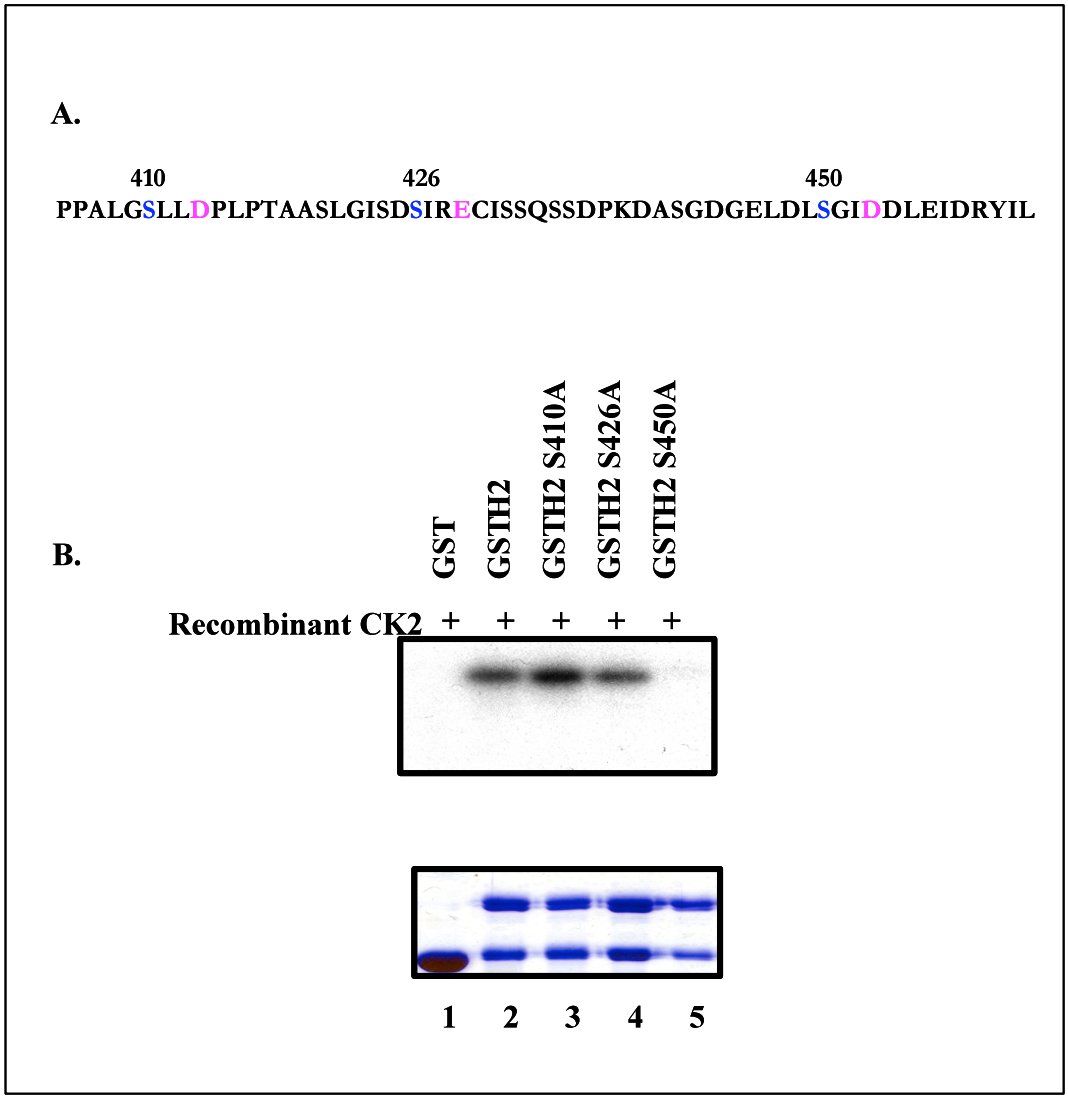


Figure 4.6 S450 is phosphorylated *in vitro*.
The indicated fusion proteins of GST and either wild-type H2 or mutated forms of H2: GST-H2 S410A, GST-H2 S426A, GST- H2 S450A, were subjected to an *in vitro* kinase assay in the presence of recombinant CK2, followed by Coomassie staining and autoradiography.

Studies using small synthetic peptides as substrates can help delineate substrate specificity. To further investigate whether the S450 residue is phosphorylated, a Brf1 synthetic peptide bearing this residue (sequence: ELDLSGIDDL) was generated. Small acidic peptides cannot bind to the cation-exchange P81 phosphocellulose paper as used previously in CK2 activity assays. To circumvent this, multiple lysine or arginine residues can be added to the peptide. However, this could have effects on the conformation and specificity of the peptide substrate. It has been reported that polyetheneimine (PEI)-cellulose thin layer chromatography (TLC) can be used to

separate ^{32}P labelled acidic, neutral and basic substrate peptides. Therefore, an *in vitro* kinase assay was therefore performed with no peptide control (lane 1), PKA peptide (lane 2), CK2 peptide (lane 3), Brf1 peptide bearing the S450 (lane 4) and Brf1 peptide consisting of residues 554 to 563 (lane 5) and were resolved using this TLC system. The results confirmed that S450 is phosphorylated *in vitro* by CK2.

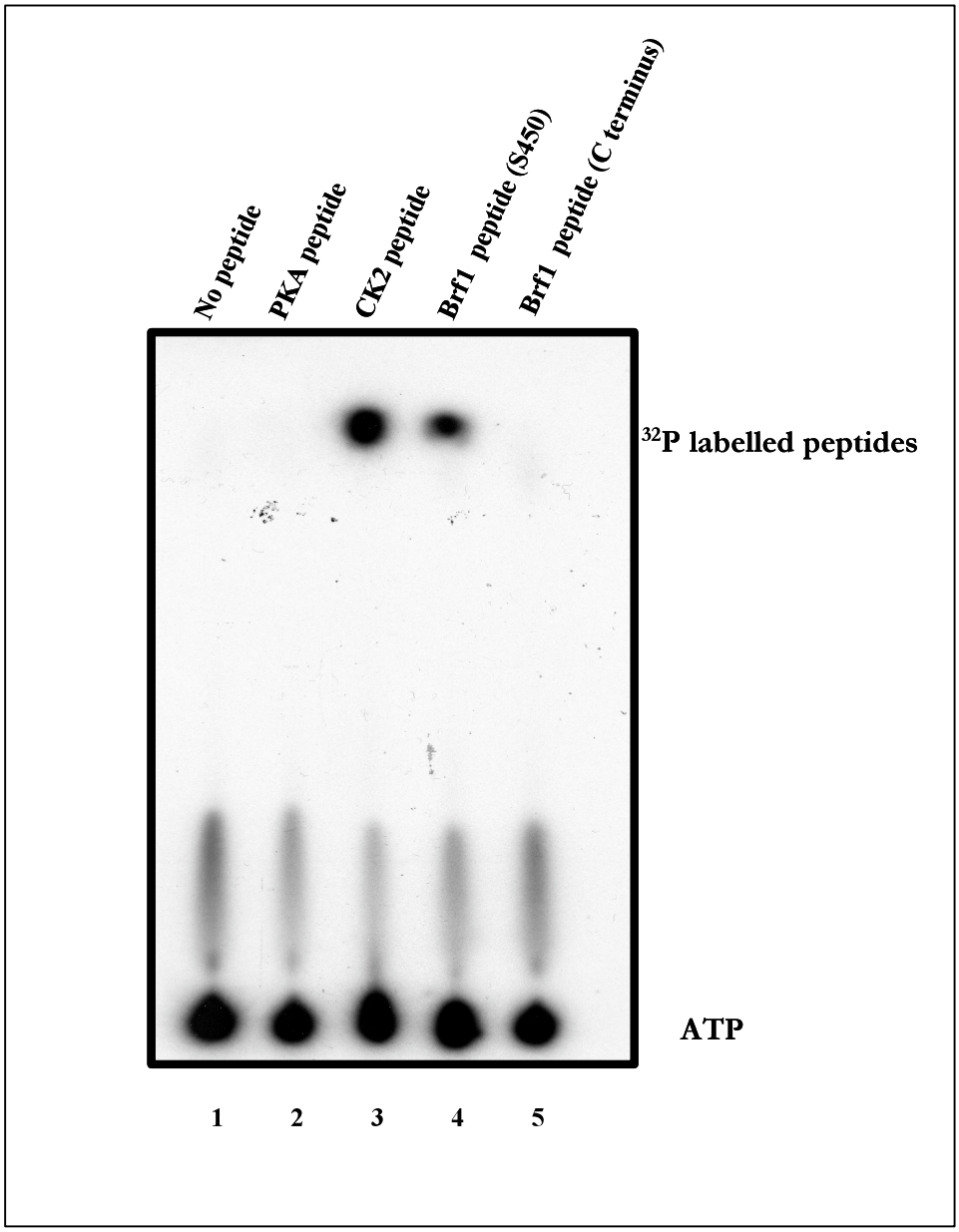


Figure 4.7. S450 is phosphorylated by recombinant CK2.
Autoradiogram of TLC separating the ^{32}P phosphopeptides from unincorporated [γ-^{32}P] ATP. *In vitro* kinase assay using no peptide control (lane 1), PKA peptide (lane 2), CK2 peptide (lane 3), Brf1 peptide bearing the S450 (lane 4) and Brf1 peptide consisting of residues 554 to 563 (lane 5).

4.2.2 Characterisation of the anti- phosphoS450Brf1 antibody

To determine whether S450 of Brf1 is phosphorylated *in vivo*, an antibody was produced against a peptide from Brf1 [SHYD (p) SDGDKSD] phosphorylated at that residue (phosphoS450Brf1). The antibody was affinity purified to reduce the possibility of the antibodies binding to epitopes not found in the original peptide. Characterisation of the antibody was required to demonstrate its specificity to the phosphorylated residue S450.

To begin with, the antibody was tested to determine whether it recognised a phosphorylated form of Brf1. Pre-treatment of A31 cells and HeLa cells with alkaline phosphatase reduced the recognition of the endogenous Brf1 proteins by the antibody (compare lanes 1 and 2). This experiment confirmed that the antibody recognises a phosphorylated form of Brf1.

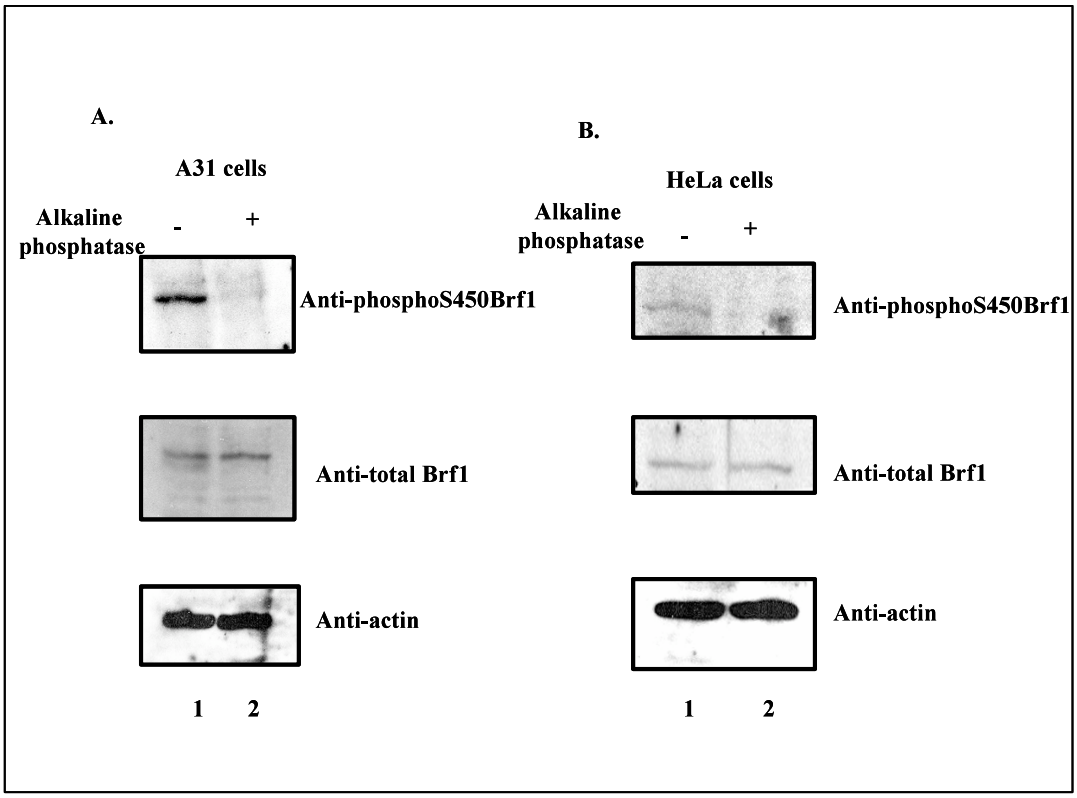


Figure 4.8 The anti-phosphoS450Brf1 antibody recognises phosphorylated Brf1.
Lysates from A31 and HeLa cells were pre-treated with alkaline phosphatase and subjected to Western blotting with anti- phosphoS450Brf1 (top panels), with anti-Brf1 antibody (middle panels) and with anti-actin antibody (bottom panels) respectively.

It was also necessary to determine whether the antibody recognised specifically Brf1 phosphorylated on S450. The final bleed antibody (top panel) and the phospho-peptide affinity purified antibody (third panel) did not recognise bacterially expressed GST or the GST-H2 fusion protein (Figure 4.9, lanes 1-3). In contrast, the antibody reacted strongly with the same GST-H2 fusion protein which had been phosphorylated *in vitro* by recombinant CK2 (Figure 4.9, lane 4). All fusion proteins were expressed at equivalent levels as shown by the GST blot (bottom panel). Moreover, the second panel shows that if the antibody was affinity purified against unphosphorylated peptide, there is no difference in immunoreactivity with GST-H2 or CK2 phosphorylated GST-H2 (second panel, lanes 3 and 4). These findings show that the anti-phosphoS450Brf1 antibody is specific to the H2 region phosphorylated at S450.

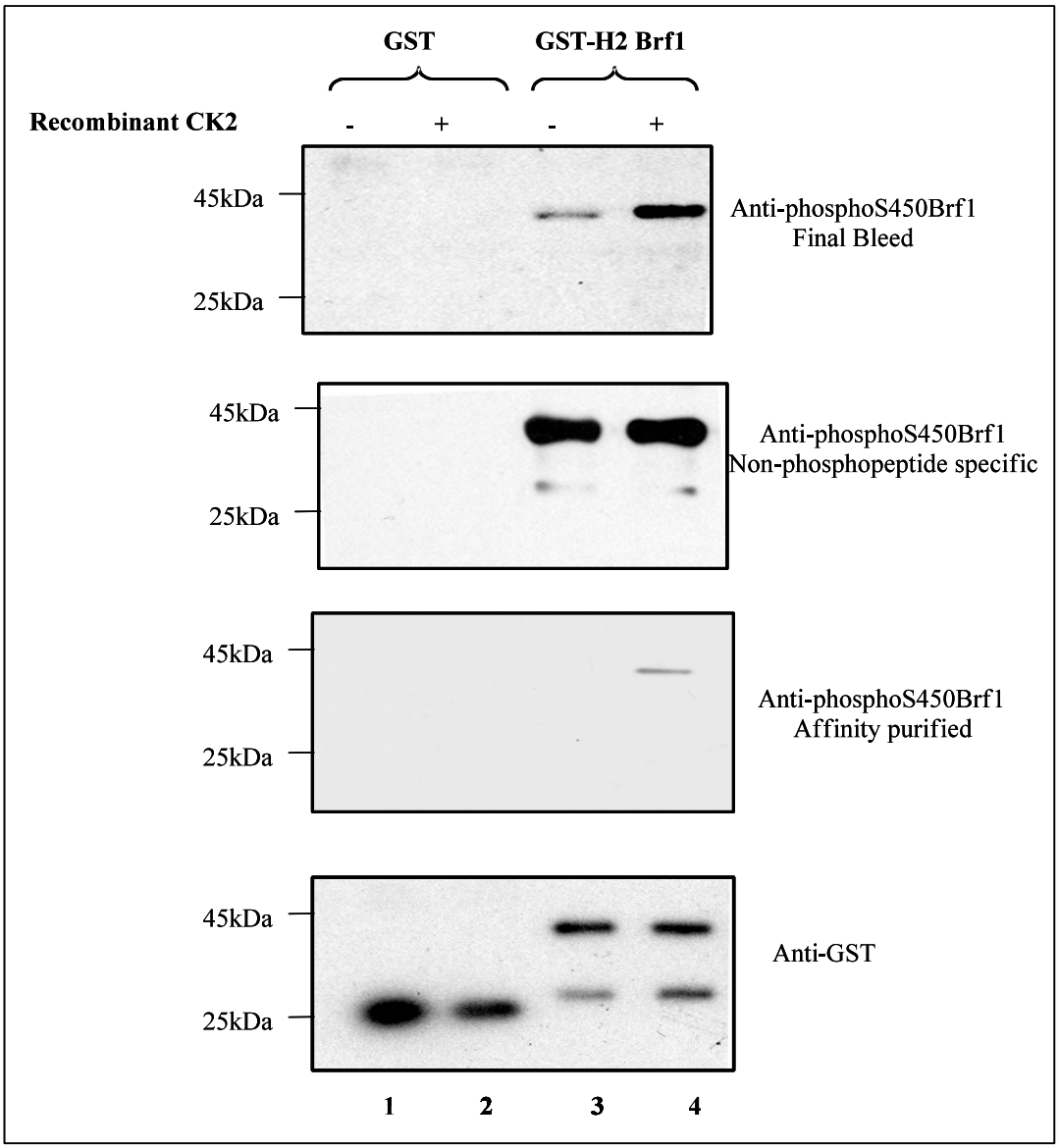


Figure 4.9. The anti-phosphoS450Brf1 antibody is specific to phosphorylated Brf1 H2.
GST (lanes 1 and 2) and GST H2 (lanes 3 and 4) fusion protein were incubated in absence or presence of recombinant CK2 and subjected to an *in vitro* kinase assay. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose before being subjected to Western blotting with final bleed phospho-S450Brf1 antibody (top panel), non-phosphopeptide specific S450Brf1 antibody (second panel), affinity purified phosphoS450Brf1 antibody (third panel) or GST antibody (bottom panel).

It was then assessed whether the phosphospecific antibody would recognise the residue S450, the GST fusion proteins bearing mutations at S410, S426 and S450 were used in kinase assays, as previously, but in this instance resolved proteins were transferred to nitrocellulose and probed with the anti-phosphoS450Brf1 antibody. This experiment

revealed that the antibody did not recognise the GST fusion protein bearing the S450A mutation, indicating that it can specifically recognise this residue.

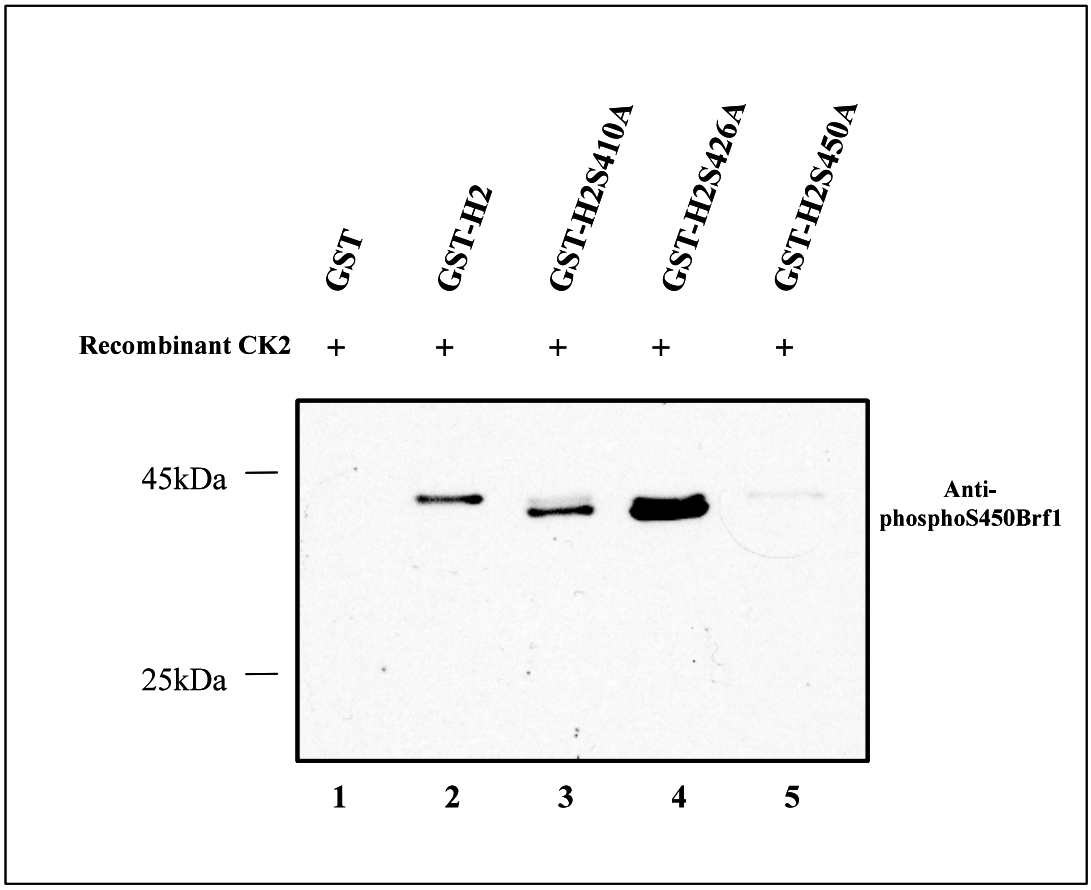


Figure 4.10. The anti-phosphoS450Brf1 antibody does not recognise the S450A mutant.
Equal amounts of GST fusion proteins were used in an *in vitro* kinase assay. Proteins were resolved by SDS-PAGE and Western blotting with the anti-phosphoS450Brf1 antibody.

Further confirmation that the antibody recognises phospho-S450 is provided in Figure 4.11. A peptide competition assay was performed using one Brf1 peptide phosphorylated at S450 and a second peptide which consists of the same sequence but not phosphorylated. Excess amounts of the peptides were incubated individually with the antibody prior to use in the Western blot shown in Figure 4.11.

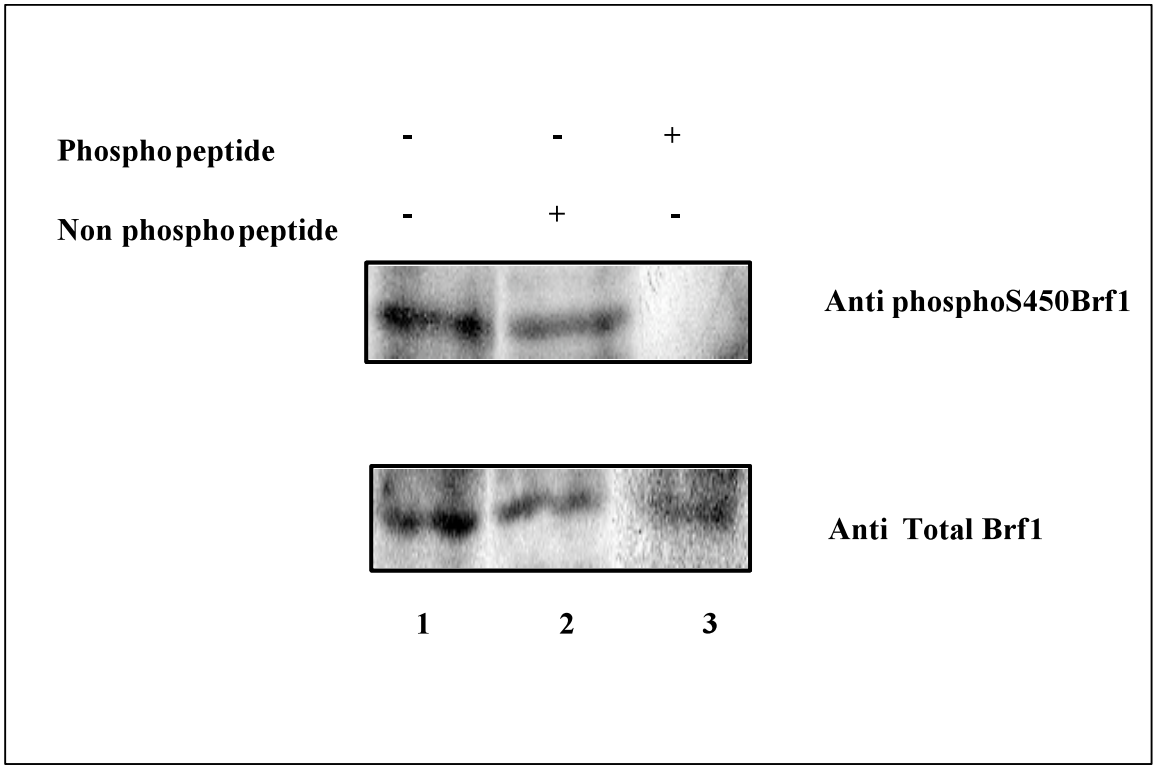


Figure 4.11. The anti-phosphoS450Brf1 antibody does not recognise the non-phosho S450.
Cell lysates from HeLa cells were subjected to Western blotting with either anti-phosphoS450Brf1 or total Brf1 antibodies in the absence (lane 1) or presence of the indicated peptides (lanes 2 and 3 respectively).

Band detection by the anti-phosphoS450Brf1 antibody was abolished in the presence of the phosphorylated peptide, but not its unphosphorylated equivalent. This confirms that the antibody specifically recognises the phosphorylated version of Brf1 S450 from cells.

4.2.3 S450 is phosphorylated by CK2 in vivo

To determine whether CK2 is involved in the phosphorylation of S450 *in vivo*, A31 cells were cultured in the presence or absence of DMAT. As disruption of CK2 activity is lethal to both embryos and cultured cells (REF), the assays were performed under conditions of only partial CK2 inhibition to preserve cell viability. Lysates from cells treated with either DMAT or DMSO alone were subjected to Western blot analysis with the anti-phosphoS450Brf1 antibody.

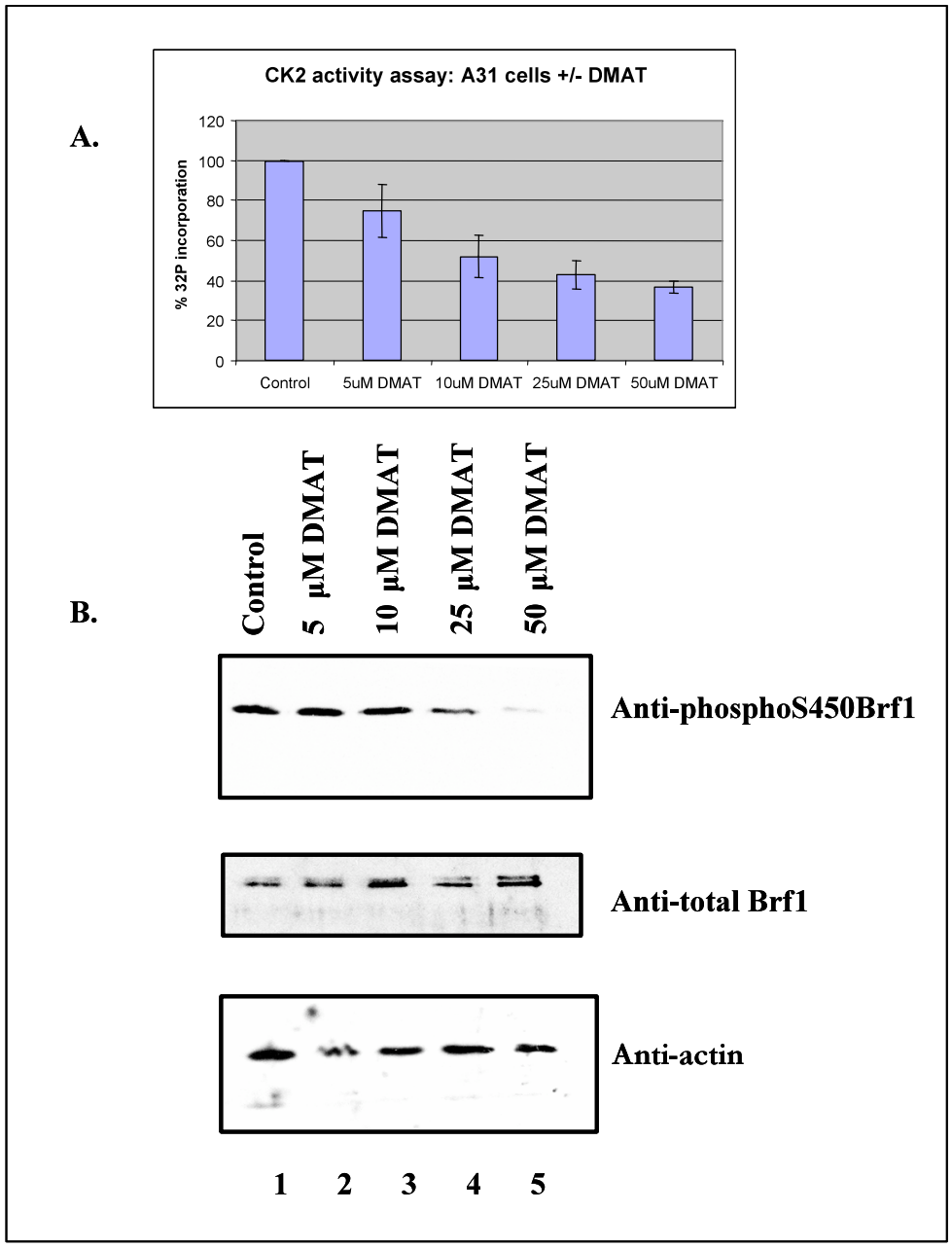


Figure 4.12 Effect of DMAT on S450 phosphorylation *in vivo*.
A31 cells were treated with the indicated concentrations of CK2 inhibitor DMAT for 4 hours. A. CK2 activity was measured in the cell extracts treated with DMSO or 5 µM, 10 µM, 25 µM and 50 µM DMAT respectively. B. Whole cell lysates were subjected to Western blotting with either anti-phosphoS450Brf1 (top panel), anti-total Brf1 (middle panel) or anti-actin (bottom panel).

Exposure to DMAT resulted in a significant decrease in the phosphorylation of S450 without affecting the levels of Brf1. The combined results show that Brf1 is phosphorylated *in vitro* and *in vivo* by CK2 at S450.

4.3 Discussion

The aim of this chapter was to investigate which residue(s) of Brf1 is (are) phosphorylated by CK2 using several different approaches. Initially, *in vitro* kinase assays using GST fusion proteins of various Brf1 fragments were performed. These assays revealed that the H2 domain of Brf1 is a target of phosphorylation by CK2 (Figure 4.2, Figure 4.4 and Figure 4.5). However, data also indicated that possible CK2 targets may also be found within the H1 region, since a decrease in phosphorylation was observed in the presence of selective CK2 inhibitors, as shown in Figure 4.4.

Because the combined data from the peptide array and the kinase assays using the GST fusion proteins indicated that the H2 region was a strong candidate target for phosphorylation by CK2, the second part of the chapter focused on this H2 region. Furthermore, the H2 region of Brf1 has 29% sequence identity between yeast and humans and is a region where TBP can bind (Juo et al., 2003). Mutagenesis studies of the H2 region revealed that out of the three potential CK2 phosphorylation sites only one of them (S450) appeared to be a target of phosphorylation by CK2. The residue S450 was shown to be phosphorylated by CK2 *in vitro*, as shown in Figure 4.6 and Figure 4.7.

Although the H2 region is conserved between yeast and mammals, sequence alignment of yeast Brf1 and human Brf1 revealed that human residue S450 is equivalent to residue E463 in S.*cerevisiae* as shown in Figure 4.13 below.

Figure 4.13 Sequence alignment of residues in the H2 region of yBrf1 and hBrf1. Sequence shows part of the H2 of Brf1 in *S. cerevisiae*, and *Homo sapiens.* The arrow indicates E463 in yeast and S450 in human.

The finding that S450 is not evolutionarily conserved is very interesting since this region is well conserved from yeast to humans. The divergence between yeast and that of higher organisms may reflect the differences in the regulation of transcription between lower and higher eukaryotes. The residue has evolved from being an E in

lower eukaryotes to an S in higher eukaryotes. The CK2-mediated phosphorylation of this region could be an important regulatory event in the functions of higher eukaryotic Brf1 proteins and could highlight the fact that Brf1 might require to be negatively charged at this residue to be active. As CK2 is ubiquitous and active, it remains to be determined if this phosphorylation is a constitutive event or whether it can vary depending on the physiological status of the cell and this will be considered in Chapter Six.

Although it was clear that S450 is phosphorylated *in vitro*, it remained to be determined whether it was phosphorylated *in vivo*. A phosphospecific antibody against that residue was generated and characterised (Figures 4.8 to 4.11). Using this antibody, it was demonstrated that the addition of DMAT to proliferating fibroblasts decreased phosphorylation of S450 in a dose-dependent manner (Figure 4.12). This led to the conclusion that S450 is phosphorylated *in vivo* by CK2.

A 2.95 Å resolution crystal structure of the yeast Brf1-TBP-DNA ternary complex is available and shown in Figure 4.14 below. This structure shows part of the H2 domain (residues 437-506) (Juo et al., 2003). Figure 4.14 also shows where the E463 (equivalent to human S450) lies within this complex. In addition, the position of the residue equivalent to human S426, a site which was shown not to be phosphorylated by CK2 (Figure 4.6), is indicated. As predicted from the crystal structure of yeast Brf1, the equivalent residue of S450 is solvent exposed and the side chain is readily available for modification. This makes it an excellent site for CK2 phosphorylation, as opposed to the equivalent residue of S426, which is buried thus potentially explaining why it is not phosphorylated by CK2 in Figure 4.6.

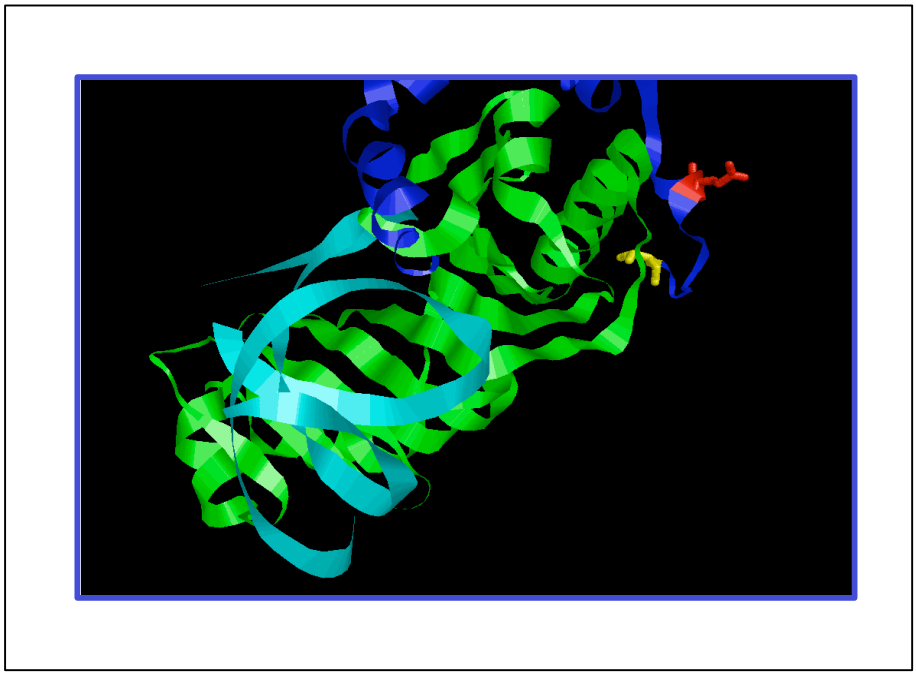


Figure 4.14. Structural model of C terminal portion of yBrf1.
Green represents TBP, dark blue represents Brf1, and pale blue represents DNA. The red residue indicates the yeast equivalent of human S450 and the yellow residue indicates the yeast equivalent of human S426.

Characterisation of mammalian TFIIIB indicated that types I and II promoters used different components of TFIIIB compared to the type III promoters (Schramm et al, 2000). Types I and II promoters were shown to require TBP and the homologue of yeast Brf1 known also as Brf1 (Lobo et al, 1992; White and Jackson, 1992). However, type III promoters use a protein related to the N-terminal but not to the C-terminal region of Brf1, which is referred to as Brf2 (Schramm et al., 2000). Hu et al (2004) showed that Brf2 does not appear to be a target of phosphorylation by CK2 *in vitro*. It is noteworthy that Brf2 not contain the S450 residue and therefore does not contradict the data presented in this chapter.

In summary, through *in vitro* kinase assays and the use of a phospho-specific antibody, this chapter has demonstrated for the first time that CK2 phosphorylates Brf1 at the S450 residue. This modification is highly specific, since CK2 fails to phosphorylate other consensus phosphoacceptor sites within the same region of Brf1.

5 Molecular Mechanism of Activation of Pol III Transcription by CK2.

5.1 Introduction

The data presented in Chapters Three and Four, demonstrate that pol III transcription is activated by CK2-mediated phosphorylation of the Brf1 residue S450. The mechanisms responsible for this increase in pol III transcription are investigated in this chapter.

In order for pol III to bind to the promoters of class III genes, it requires the productive assembly of a pre-initiation complex at the appropriate promoter [reviewed in (Schramm and Hernandez, 2002)]. For the majority of class III genes, recruitment of pol III to the promoter requires two pol III-specific, basal transcription factors, namely TFIIIC and TFIIIB (discussed in Chapter One). Control of transcription initiation and pre-initiation complex assembly, is a major target for the regulation of class III gene expression. In particular, regulation of TFIIIB activity is achieved through protein-protein interactions with various activators or repressors. As discussed in Chapter One, Erk can induce pol III transcription by binding and phosphorylating the Brf1 subunit of TFIIIB (Felton-Edkins et al., 2003a). This stimulates the interaction of TFIIIB with both TFIIIC and pol III, thereby increasing pol III output (Felton-Edkins et al., 2003a). In addition, c-Myc can also target TFIIIB to regulate pol III transcription (Gomez-Roman et al., 2003). c-Myc binds directly to Brf1, and this interaction is responsible for the recruitment of c-Myc to pol III promoters, rather than being recruited through DNA sequences, such as the E-box (Gomez-Roman et al., 2003) and (Kenneth et al., 2007). c-Myc is also able to recruit co-activator complexes such as the histone acetyltransferase (HAT) GCN5 and TRAPP to class III genes, which stimulate acetylation of histone H3 (Kenneth et al., 2007) and ultimately gives rise to an increase in pol III output.

Repressors of pol III transcription such as RB and p53, also target TFIIIB directly when regulating pol III transcription (Crighton et al., 2003; Larminie et al., 1997). Both repressors bind TFIIIB and sequester the protein in an inactive complex when the cell is resting or under stress conditions (Crighton et al., 2003; Larminie et al., 1997; Sutcliffe et al., 2000).

Changes in transcription factor expression may also contribute to the regulation of pol III output. Alterations in transcription factor abundance appear to be an important determinant of pol III transcription rates during mammalian cell differentiation (Alzuherri and White, 1998), viral infection (Felton-Edkins and White, 2002; Larminie et al., 1999; Wang et al., 1995) and tumourigenesis (Winter et al., 2000; Daly et al., 2005).

Studies from Johnston and co-workers showed that CK2 appears to regulate pol III transcription prior to initiation (Johnston et al., 2002). The objectives of this chapter were to examine changes in pol III output and interactions between proteins associated with the pol III machinery, in response to phosphorylation of Brf1 at S450 by CK2. These were performed in order to establish a mechanism for the regulation of pol III transcription during CK2 activation.

5.2 Results

5.2.1 Mutating the CK2 phosphoacceptor site in Brf1, decreases pol III transcription.

The data presented in Chapter Four demonstrated that CK2 can phosphorylate Brf1 on residue S450 both *in vitro* and *in vivo*. This section describes a series of experiments that were performed to examine the effects of this phosphorylation event on pol III transcription. A Brf1 construct was generated by PCR-mediated site directed mutagenesis, in which S450 was mutated to alanine (A) (pBrf1-S450A). Several techniques were used to compare pol III transcription following transient transfection of the wild type Brf1 construct (pBrf1) or this mutant construct (pBrf1-S450A), in either HeLa or CCL39 cells.

In the first instance, primer extension analysis was used to determine pol III output following transfection of the constructs. CCL39 fibroblast cells were transfected with equal amounts of control vector (referred to as empty vector) or vectors encoding either wild type or mutant Brf1. In addition, vectors encoding the adenovirus VA1 RNA (as a pol III reporter) and green fluorescent protein (GFP) (to normalise for transfection efficiency), were also transfected. Western blotting (shown in Figure 5.1A) confirmed that the wild-type and mutant Brf1 constructs were expressed equally in the transfected cells and that mutation had not destabilised Brf1. The primer extension analyses (shown in Figure 5.1B) revealed that transfecting CCL39 cells with wild-type Brf1 stimulated VA1 transcription (upper panel, lane 2) in comparison to the control transfection (upper panel, lane 1). However, mutation of S450 prevented this increase (upper panel, lane 3). Primer extension of GFP RNA showed that transfection efficiency was similar in each case (lower panel). Figure 5.1C shows a bar graph of the fold changes in pol III transcription after normalisation for GFP RNA levels. The wild-type construct stimulated VA1 transcription by 1.8-fold, whereas the S450A mutation decreased VA1 transcription by 2-fold.

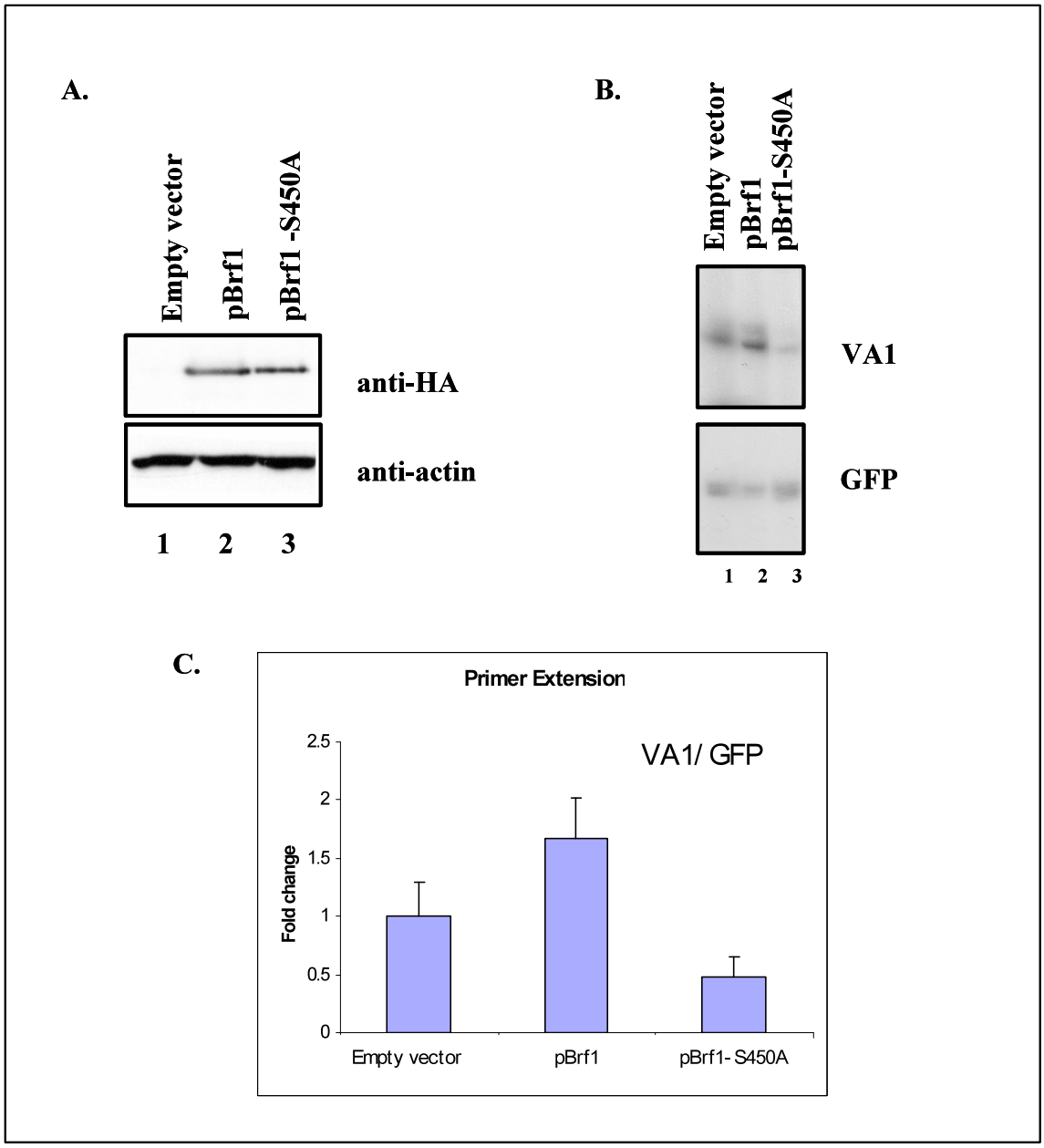


Figure 5.1. Mutating Brf1 S450 decreases pol III transcription.
CCL39 cells were transiently transfected with empty vector, pBrf1 or pBrf1-S450A together with pVA1 and peGFP. A. Cell extracts were resolved by SDS-PAGE and immunoblotted with an anti-HA antibody (top panel) to compare expression levels. Actin (bottom panel) was used as a loading control. B. Total RNA was extracted and VA1 and GFP RNA levels analysed by primer extension. C. VA1 and GFP RNA levels were quantified by densitometry and VA1 expression normalised against GFP expression (Normalisation of 3 independent experiments).

Semi-quantitative RT-PCR analysis was also used to assess the effects of pBrf1-S450A on pol III transcription. HeLa cells were transfected with equal amounts of the control and

Brf1 constructs (Figure 5.2A). Extracted RNA from the transfected cells was then analysed by RT-PCR using primers specific for 5S rRNA, $tRNA^{Tyr}$ and mRNA from the control pol II template, ARPP P0. This RT-PCR analysis (

Figure 5.2B) revealed that there was an increase in the pol III transcripts 5S rRNA and $tRNA^{Tyr}$ when wild-type Brf1 was transfected (upper panel, lane 2), in comparison to the control vector (lane 1). However, expression of the S450A mutant reduced pol III transcription (lane 3). These effects are specific, as the level of ARPP P0 mRNA is unchanged.

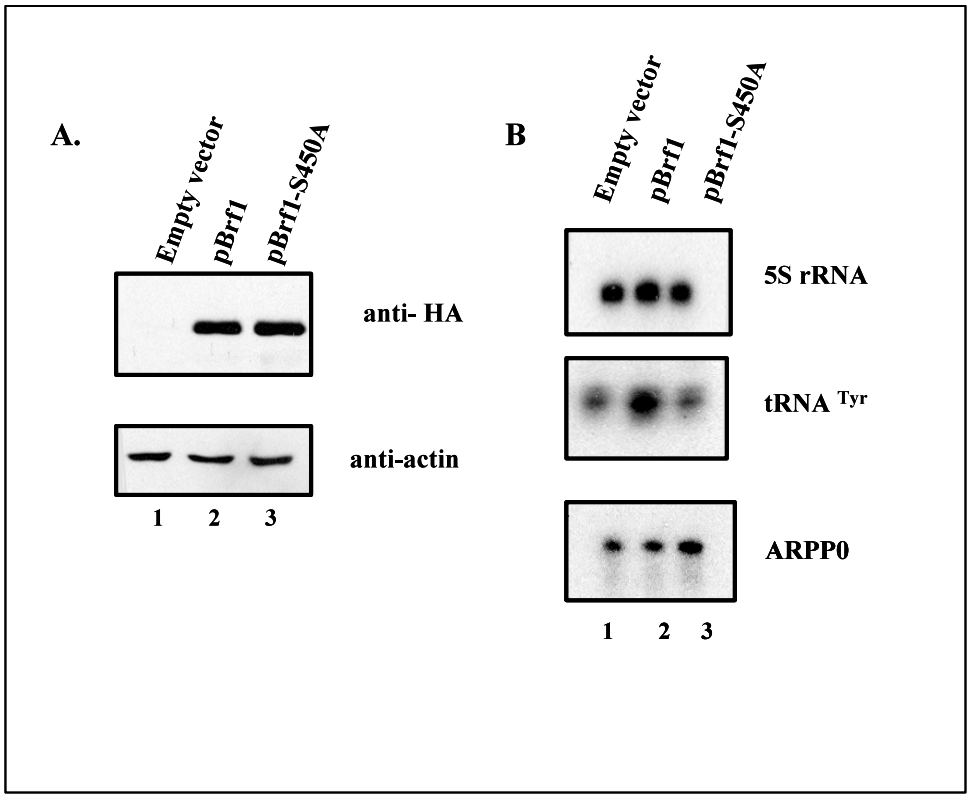


Figure 5.2. S450A decreases pol III transcription in HeLa cells.
HeLa cells were transiently transfected with empty vector, pBrf1 or pBrf1-S450A. A. Cell extracts were resolved by SDS-PAGE and immunoblotted with an anti-HA antibody (top panel) to compare expression levels. Actin levels were also measured to control for equal protein loading (bottom panel). B. Specific primers for the transcripts indicated were used to PCR amplify cDNAs generated from total RNA of the transiently transfected HeLa cells.

The effects of mutating the S450 to aspartate (S450D), which simulates phosphorylation, were also assessed. CCL39 cells were transfected with equal amounts of control and Brf1 constructs. The Western blot shown in Figure 5.3A reveals that all constructs were expressed equally. RT-PCR analysis was used to examine the effects of the Brf1 mutations on pol III transcription (Figure 5.3B). The results reveal that, as with HeLa cells, there was an increase in 5S rRNA and $tRNA^{Tyr}$ when the wild-type Brf1 construct was transfected (lane 2). This increase was reduced when the pBrf1-S450A construct was transfected (lane 3). In contrast, expression of the Brf1-S450D mutant (lane 4) activated tRNA expression even more than wild type Brf1 (lane 2). These effects are specific, as the level of ARPP P0 mRNA was unchanged. The changes in tRNA expression are more striking than the changes in 5S rRNA expression in both HeLa and CCL39 cells. As

discussed in Chapter Three, the primers designed to assay the levels of tRNAs are intron-specific and measure rapidly processed precursors. In contrast, the 5S rRNA primers measure steady-state levels. These differences could explain the discrepancy between 5S rRNA and tRNA induction.

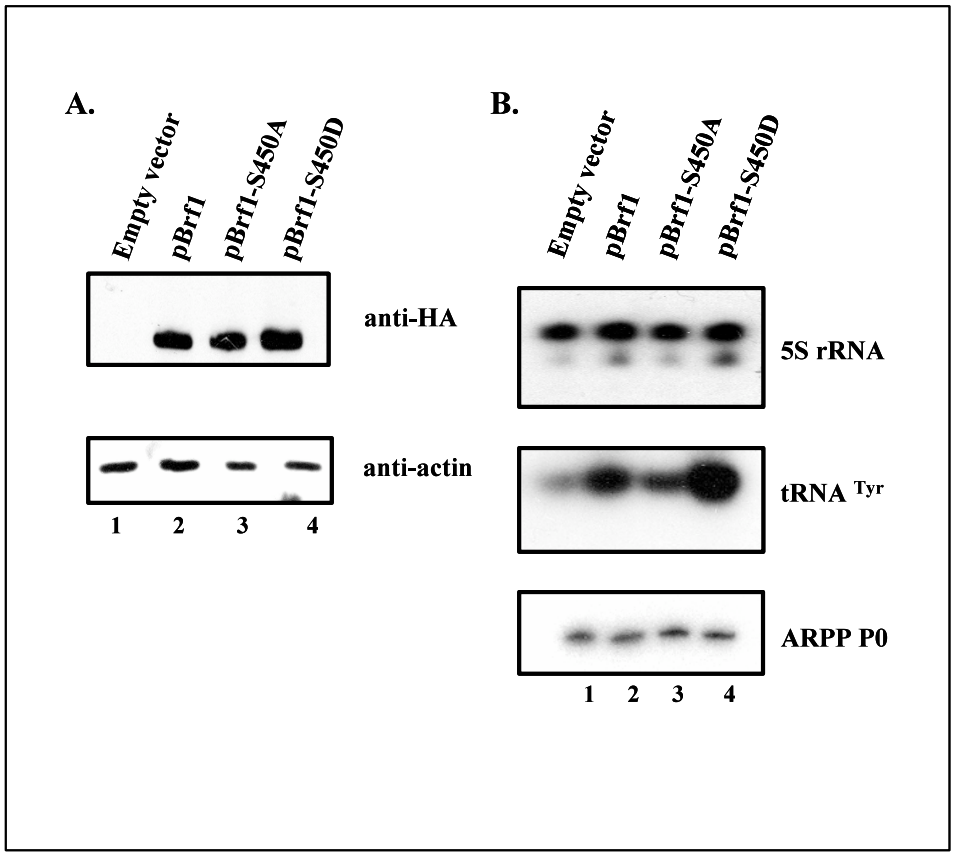


Figure 5.3. Phosphorylation of S450 increases pol III transcription in CCL39 cells. CCL39 cells were transiently transfected with empty vector, pBrf1, pBrf1-S450A or pBrf1-S450D. A. Cell extracts were resolved by SDS-PAGE and immunoblotted with an anti-HA antibody (top panel) to compare expression levels. Actin levels were measured to control for equal protein loading (bottom panel). B. Specific primers for the transcripts indicated were used to PCR amplify cDNAs generated from total RNA of transiently transfected CCL39 cells.

Primer extension and RT-PCR analyses demonstrate that mutation of the Brf1 CK2 phosphoacceptor site S450 to A decreases pol III transcription in HeLa and CCL39 cells. Furthermore, mimicking phosphorylation by mutating S450 to D can increase pol III transcription more than wild-type Brf1. Hence, it can be concluded that phosphorylation of Brf1 at S450 stimulates pol III transcription and that this is not cell type-specific.

5.2.2 CK2 is found at type I and II promoters

To test whether endogenous CK2 is present on pol III-transcribed genes *in vivo*, ChIP assays were performed. Antibodies against CK2β, CK2 holoenzyme, c-Myc and TFIIA

were used to precipitate their respective proteins that had been cross-linked with chromatin in asynchronously growing HeLa cells.

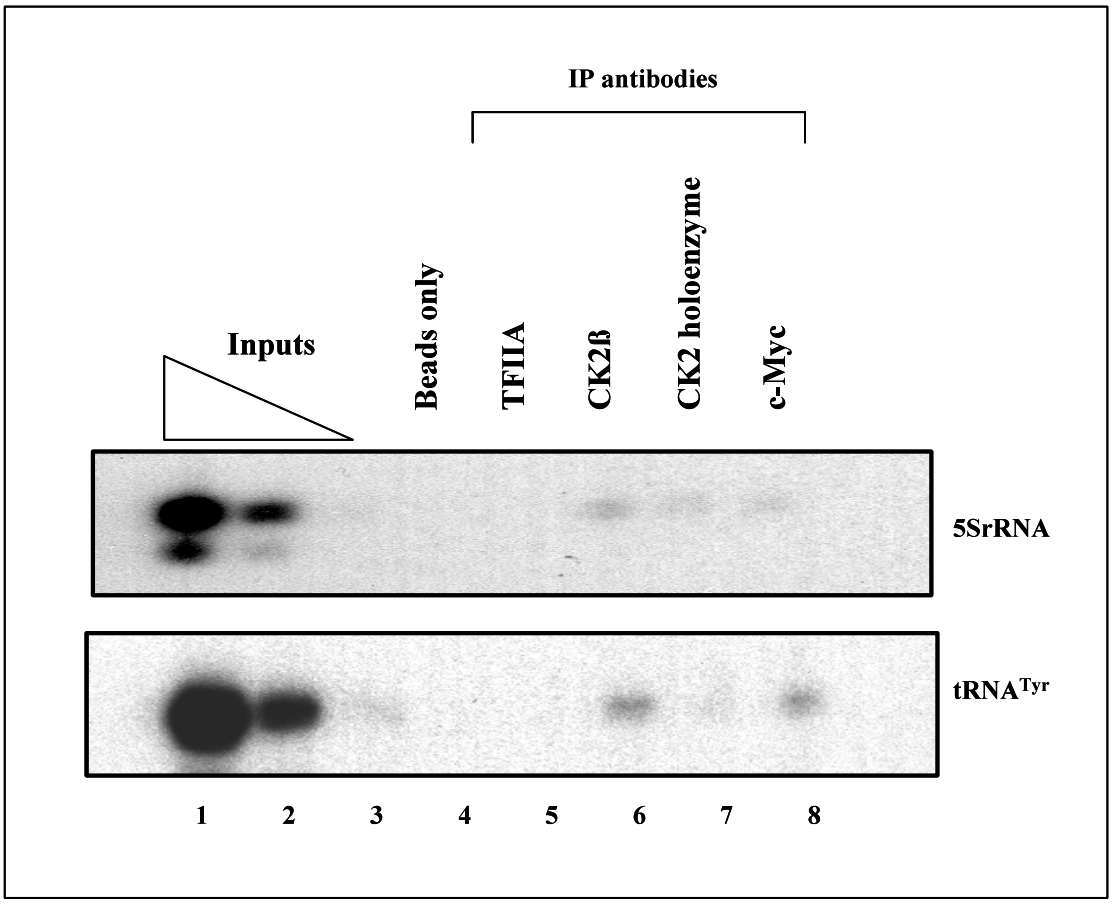


Figure 5.4. CK2 associates with pol III templates *in vivo*.
Chromatin immunoprecipitation of HeLa cells were performed with antibodies against CK2β, CK2 holoenzyme and c-Myc, as indicated. As negative controls, TFIIA and beads only were used. Association of each factor with 5S rRNA (top panel) and tRNA^{Tyr} (bottom panel) genes was quantified by PCR analysis using gene-specific primers. Input genomic DNA (10%, 2% and 0.4% of that used in the ChIPs) was analysed in the same PCR reactions.

Endogenous c-Myc has been previously detected at pol III-transcribed genes (Gomez-Roman et al., 2003) and the anti-c-Myc antibody was used as a positive control in ChIP assays (lane 8). Figure 5.4 shows that in addition to c-Myc, CK2 is found at 5S rRNA (type I promoter, upper panel) and tRNA^{Tyr} (type II promoter, lower panel) genes. The TFIIA antibody and beads were used as negative controls and, as expected, do not precipitate the 5S rRNA and tRNA^{Tyr} genes. The presence of the CK2β subunit (lane 4) at these promoters is more striking than the presence of the CK2 holoenzyme (lane 5). This could reflect differences in the affinities of these antibodies.

5.2.3 CK2 does not affect c-Myc binding to TFIIIB.

c-Myc has been shown to interact with Brf1. Therefore, the next experiment was to determine whether phosphorylation of Brf1 by CK2 could be a pre-requisite for c-Myc to bind Brf1. HeLa cell extracts were pre-treated with the specific CK2 inhibitor DMAT, and the interaction between Brf1 and c-Myc was investigated.

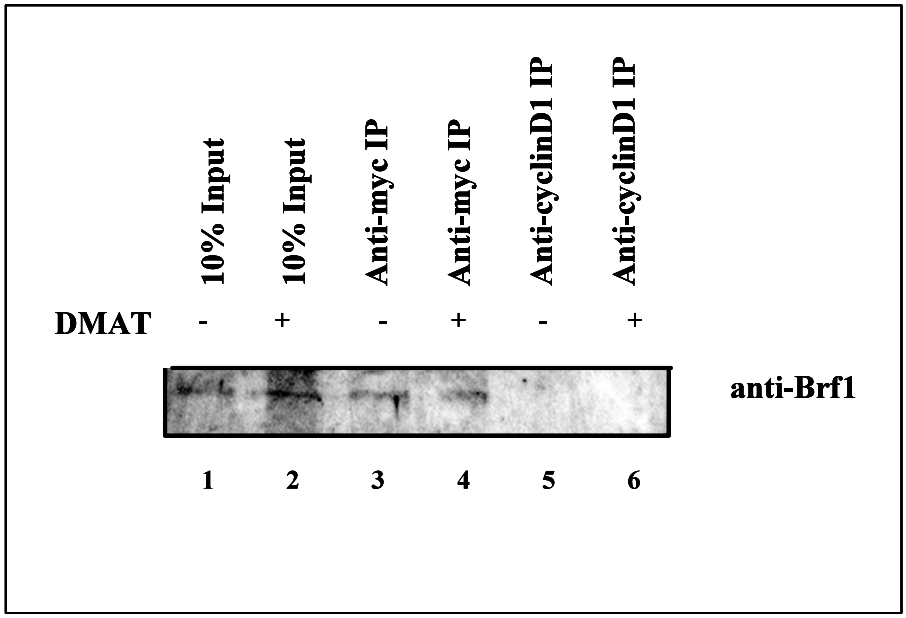


Figure 5.5. c-Myc binding to Brf1 is not affected by DMAT treatment
HeLa extract was treated with either DMSO alone (lanes 1, 3 and 5) or with 10 µM DMAT (lanes 2, 4 and 6). The extracts were then immunoprecipitated using antibodies against either c-Myc or cyclin D1. The precipitates were immunoblotted for Brf1 (using the 128-4 antibody).

Co-immunoprecipitation experiments (shown in

Figure 5.5) reveal that the association of endogenous c-Myc with Brf1 was not affected by the presence of the selective CK2 inhibitor and that Brf1 did not bind to the negative control cyclin D1. The data suggest that the binding of c-Myc to Brf1 and the phosphorylation of Brf1 by CK2 are two events which can occur independently of each other.

5.2.4 Phosphorylation by CK2 increases pol III and TFIIIB recruitment to type 1 and 2 promoters

Enhanced promoter occupancy of polymerase at target genes often results in increased transcriptional output. ChIP analyses were carried out to determine whether the binding of pol III and its transcription factors (TFIIIB and TFIIIC) to type I and II promoters was subject to regulation by CK2 phosphorylation. Formaldehyde cross-linked chromatin was prepared from asynchronously growing A31/3T3 cells either treated with DMAT or with DMSO only for 4 hours. Antibodies against a pol III subunit (RPC 155), TFIIIC subunit (TFIIIC110) and TFIIIB subunit (Brf1) were used to determine their occupancy. TFIIA was used as a negative control, as it is not present on pol III- transcribed genes.

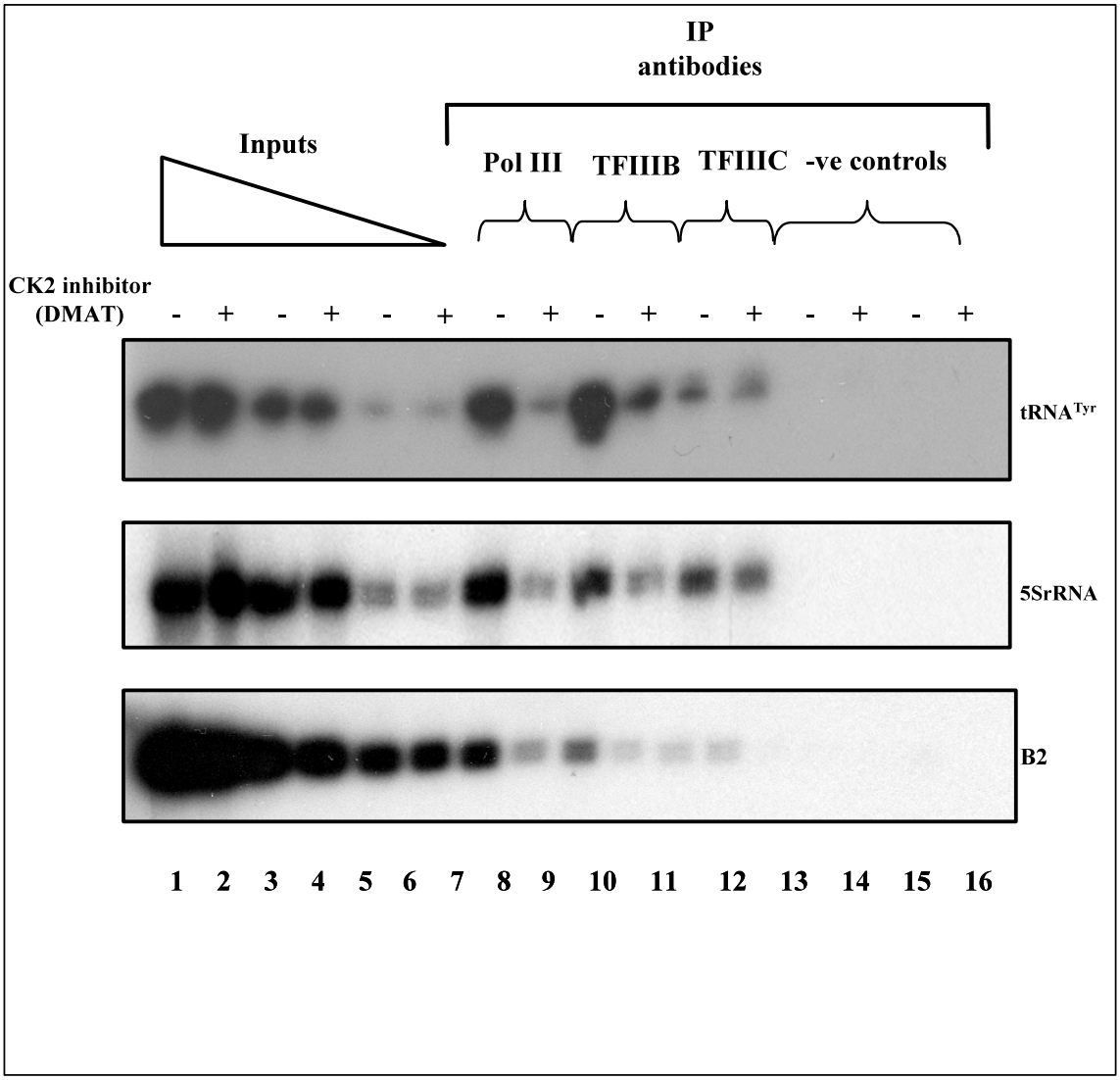


Figure 5.6. DMAT reduces TFIIIB and pol III, but not TFIIIC, binding to class III gene promoters *in vivo*.
A31/3T3 fibroblast cells were treated with either 50 µM DMAT or DMSO for 4 hours. ChIPs were performed with antibodies against Brf1, TFIIIC110, and the largest pol III subunit (RPC155), as indicated. As negative controls, ChIPs were carried out using a TFIIA antibody and beads alone. Association of each factor with 5S rRNA, B2, $tRNA^{Leu}$ and $tRNA^{Tyr}$ promoters, in DMAT-treated and DMSO-treated cells, was quantified by PCR with gene-specific primers. Input genomic DNA (10%, 2% and 0.4% of that used in the ChIPs) was analysed in the same PCR reactions.

PCR analysis showed that gene occupancy of pol III (Figure 5.6, lanes 7 & 8) and Brf1 (Figure 5.6, lanes 9 & 10), on all promoter types, was diminished after the addition of DMAT. In contrast, the levels of TFIIIC on each promoter type were similar in both DMAT-treated and DMSO – treated cells (Figure 5.6, lanes 11 & 12).

The polymerase is recruited to class III genes by protein-protein interactions with TFIIIB. To examine whether TFIIIB recruitment is affected following phosphorylation of Brf1-S450 by CK2, ChIP analyses were performed using HeLa cells which had been transfected with either the pBrf1 or the pBrf1-S450A expression vectors. TFIIIB recruitment was assessed using an antibody against HA. As a negative control, an anti-TFIIA antibody was also used.

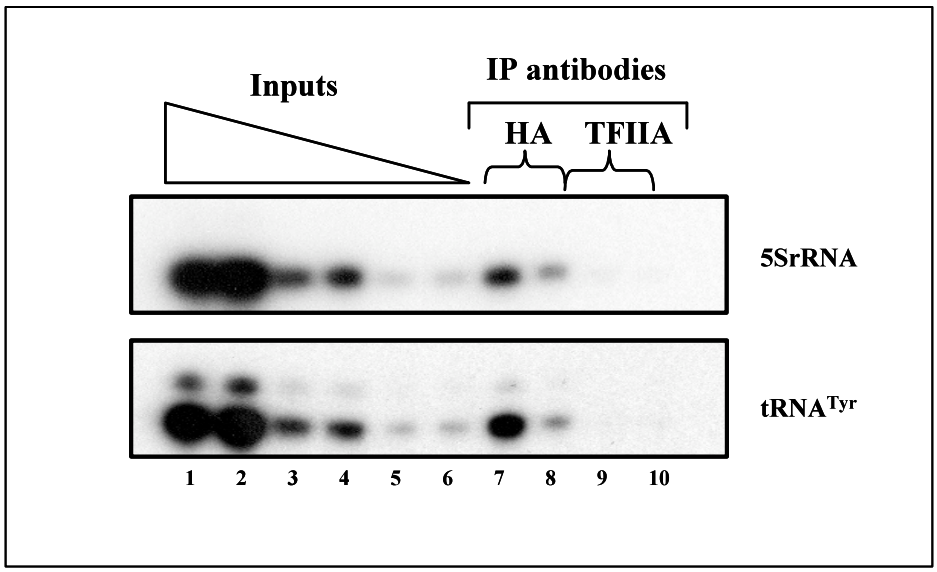


Figure 5.7. Mutation of S450A decreases association of TFIIIB with pol III genes *in vivo*.
HeLa cells were transiently transfected with either pBrf1 (lanes 1, 3, 5, 7 and 9), or pBrf1S450A (lanes 2, 4, 6, 8 and 10). ChIP assays were performed with antibodies against HA and TFIIA (negative control), as indicated. Association of HA-tagged Brf1 or Brf1-S450A with 5S rRNA and $tRNA^{Tyr}$ promoters, in transfected cells, was quantified by PCR with gene-specific primers. Input genomic DNA (10%, 5% and 0.4% of that used in the ChIPs) was analysed in the same PCR reactions.

Figure 5.7 reveals that the mutation of Brf1 residue S450 to A caused a marked decrease in TFIIIB binding to 5S rRNA and tRNA genes *in vivo*.

The increased association of TFIIIB with promoters following phosphorylation by CK2 is likely to account for the enhancement of polymerase binding to type I and II promoters. This enrichment could explain the enhanced class III gene expression that occurs following Brf1 phosphorylation by CK2, as demonstrated in Chapter Three.

The increase in TFIIIB occupancy at pol III templates in response to phosphorylation by CK2 might be a direct result of changes in abundance of TFIIIB. Therefore, Western blot

analysis was performed using extracts treated with or without 50 μM DMAT to measure the levels of this transcription factor.

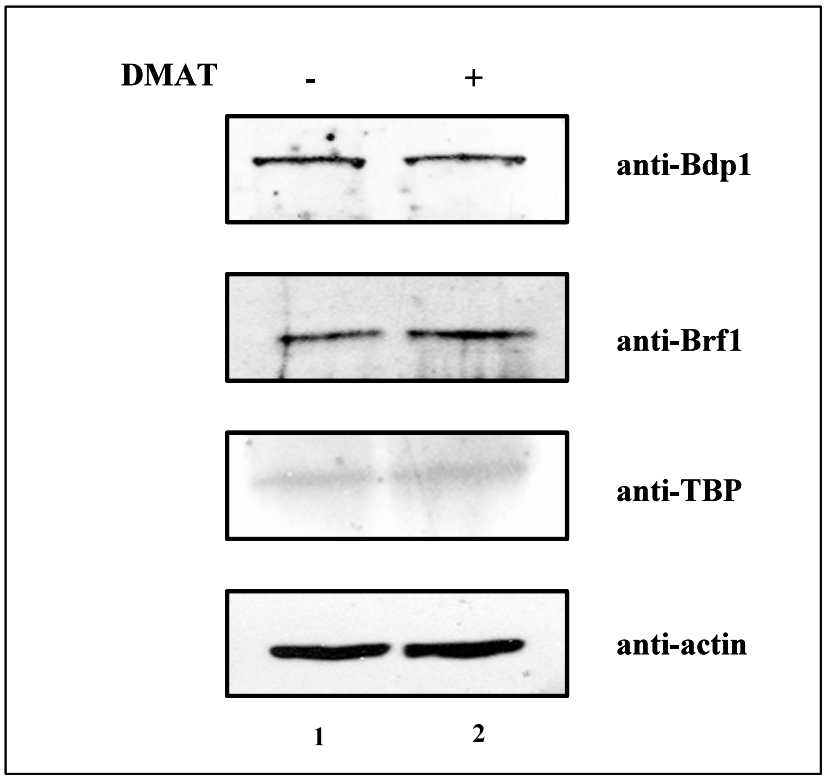


Figure 5.8. DMAT does not affect the levels of the TFIIIB subunits.
HeLa extracts were treated with either DMSO alone or 50 μM DMAT and subjected to Western blotting with antibodies against Bdp1 (upper panel), Brf1 (second panel), TBP (third panel) and actin (bottom panel) as a loading control.

No change in the abundance of TFIIIB subunits Brf1, Bdp1 or TBP was detected after the addition of DMAT, as shown in Figure 5.8 above.

5.2.5 Phosphorylation of TFIIIB increases interaction with TFIIIC.

The results so far demonstrate that the CK2-mediated phosphorylation of Brf1 at residue 450 results in an enrichment of TFIIIB and pol III at class III genes (shown in Figure 5.6 and Figure 5.7). Furthermore, CK2 phosphorylation of TFIIIB did not appear to affect its stability (Figure 5.8). Phosphorylation of transcription factors can modulate their activity by triggering conformational change and generating binding sites for interaction partners. The next series of experiments was performed to investigate the interactions of Brf1 with components of the pol III machinery. In the first instance, a GST pull-down assay was performed using GST fusion proteins of the wild-type H2 region of Brf1 (GST-H2), and

the H2 region bearing a mutation at S450 (GST-H2S40A). The binding of these fusion proteins to the TFIIIC subunit TFIIIC110 and the TFIIIB subunit TBP were then compared as shown in Figure 5.9 below.

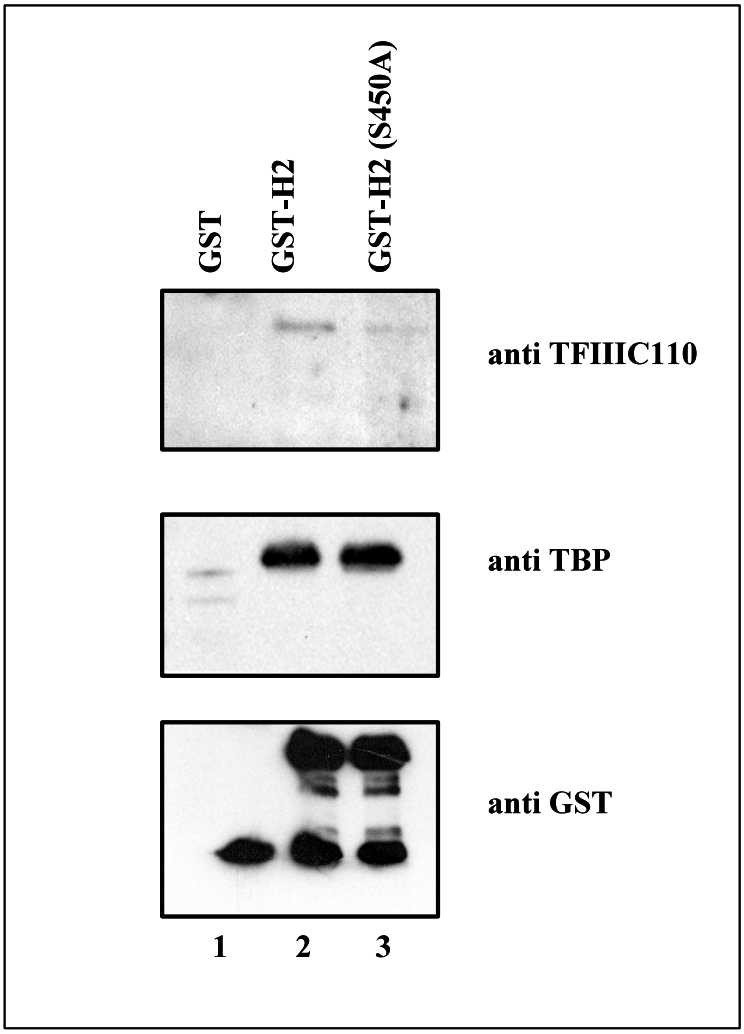


Figure 5.9. S450A specifically reduces TFIIIC binding but not TBP binding to Brf1.
HeLa nuclear extracts were subjected to pull-down assays with the indicated fusion proteins or GST alone and tested for TFIIIC binding (top panel) and TBP binding (middle panel). Proteins bound to the GST beads were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. The bottom panel shows the relative amounts of recombinant fusion proteins used in each instance.

The GST pull-down assay showed that the interaction of Brf1 and TFIIIC is compromised when S450 is mutated to A, suggesting that phosphorylation of this residue is important for the association of TFIIIC to Brf1. In addition, the association of TBP and Brf1 does not seem to be affected by this mutation, indicating that the mutated protein is folded properly and is stable.

As discussed in Chapter One, TFIIIB is brought to the promoter of class III genes by protein-protein interactions with TFIIIC. Once TFIIIB is bound to the promoter, it recruits pol III and places it over the start site. To investigate whether phosphorylation of Brf1 at S450 by CK2 could influence these interactions *in vivo*, co-immunoprecipitations were carried out. CCL39 cells were transfected with empty vector, pBrf1, pBrf1-S450A or pBrf1-S450D. The cell lysates were then used in co-immunoprecipitation assays. In the first instance, the interactions between TFIIIC and wild-type or mutant Brf1 were examined, as shown in Figure 5.10 below.

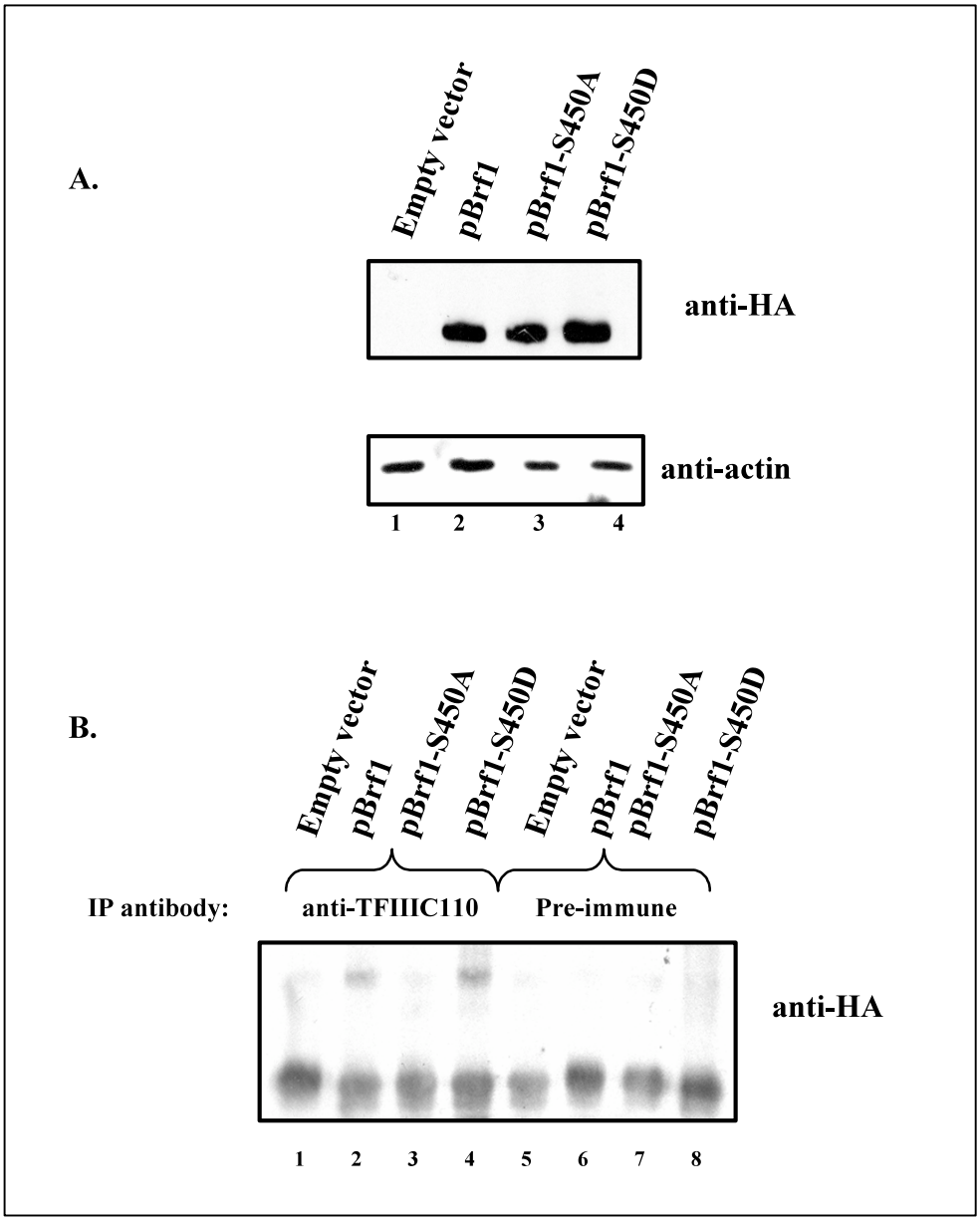


Figure 5.10. S450A compromises binding of TFIIIB to endogenous TFIIIC.
A. Extracts from CCL39 cells transfected with empty vector, pBrf1, pBrf1-S450A and pBrf1-S450D, were subjected to Western blotting using an anti-HA antibody to verify expression levels and an anti-actin antibody to control for equal protein loading. B. Subsequently the cell lysates were immunoprecipitated with a pre-immune serum (lanes 5-8) and anti-TFIIIC antibody (subunit 110, lanes 1-4). The samples were resolved by SDS-PAGE and subjected to Western blotting using an antibody against the HA tag on the transfected wild-type Brf1 or mutant Brf1.

The data reveal that when an anti-TFIIIC antibody was used to immunoprecipitate proteins from the transfected cells, only a small amount of TFIIIC was found to bind to Brf1-S450A (lane 3) in comparison to the wild-type Brf1 (lane 2). However, the binding of Brf1-

S450D (lane 4) and TFIIIC was increased in comparison to wild-type Brf1 (lane 2). No interaction was observed between Brf1 and TFIIIC, when the empty control vector was used, or when the negative pre-immune serum was used (lanes 5-8).

Next, the interactions between pol III and wild-type or mutant Brf1 (S450A) were investigated. As before, CCL39 cells were transfected with empty vector, pBrf1 or pBrf1-S450A and the cell lysates were used in co-immunoprecipitation assays.

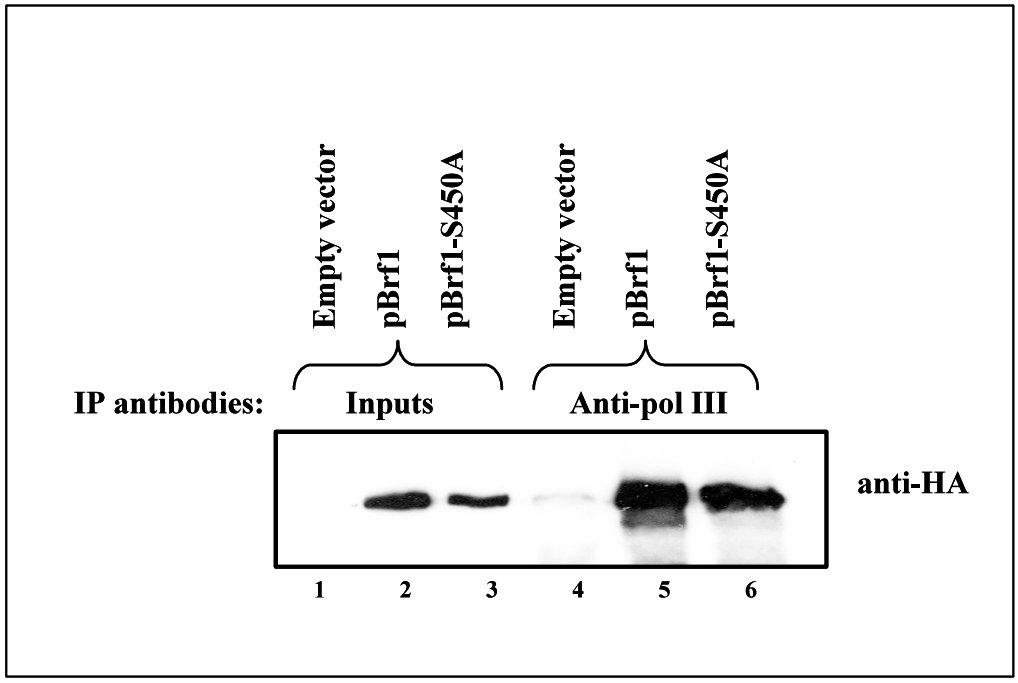


Figure 5.11. Mutation of S450 to A does not compromise TFIIIB binding to pol III. Extracts from transfected cells were immunoprecipitated with an anti-pol III antibody (lanes 4-6). The samples were resolved by SDS-PAGE and subjected to Western blotting using an antibody against the HA tag on the transfected wild-type or mutant Brf1.

The results indicate that binding of pol III to Brf1 is unaffected by the mutation of S450 to A.

The combined data from the GST pull-down and co-immunoprecipitation assays suggest that the association of TFIIIC and TFIIIB is increased specifically when CK2 phosphorylates Brf1.

5.3 Discussion

This chapter explores the influence of CK2 on molecular events involved in controlling transcription initiation complex assembly on class III genes with type I and II promoters, and provides an insight into how CK2 phosphorylation of Brf1 may be involved in the activation of pol III transcription. Primer extension and RT-PCR assays revealed that mutation of the CK2 phosphoacceptor site found in the H2 region of Brf1 to A reduces pol III output in human and rodent cells. Furthermore, mutation of this same residue to aspartate activates pol III transcription. The data suggest that the CK2-mediated phosphorylation of this Brf1 residue can contribute to the positive role of CK2 on pol III transcription. This is consistent with the data presented in Chapter Three and previous studies which suggest that CK2 is a positive regulator of pol III transcription in yeast (Ghavidel and Schultz, 1997; Hockman and Schultz, 1996) and mammals (Johnston et al., 2002; Hu et al., 2004).

The role of CK2 as a regulator of pol III transcription is further supported by the detection of endogenous CK2 on chromosomal 5S rRNA and tRNA genes (Figure 5.4). ChIP analyses by the Hernandez and Reinberg laboratories also found that CK2 can associate with promoter elements. Specifically, Hu and co-workers demonstrated that CK2 binds to the U6 promoter (type III, pol III promoter) (Hu et al., 2004), and Lewis and co-workers have shown association of CK2 with the downstream promoter element (DPE) of a number of pol II-transcribed genes (Lewis et al, 2005). Thus, association with regulatory promoter regions may be a common means through which CK2 can influence gene expression.

c-Myc is known to regulate pol III transcription by binding to TFIIIB (Gomez-Roman et al., 2003). In particular, Gomez-Roman and co-workers demonstrated that c-Myc binds to the Brf1 subunit of TFIIIB (Gomez-Roman et al., 2003). In order to assess whether CK2 phosphorylation of Brf1 could stimulate this binding, the effects of pre-incubating HeLa extracts with DMAT on c-Myc and Brf1 interactions were examined.

Figure 5.5, shows that c-Myc binding to Brf1 is not affected by CK2 inhibition. Consistent with this finding, unpublished data (N. Kenneth and R.J.White) identified the N-terminus of Brf1 as the site of c-Myc binding. Since CK2 was found to phosphorylate the C-

terminal region of Brf1, this could explain why the interactions between c-Myc and Brf1 were not affected by blocking CK2-mediated phosphorylation.

Order-of-addition studies by Johnston and co-workers suggested that CK2 acts prior to transcription initiation (Johnston et al., 2002). ChIP analyses were performed to establish whether CK2 could regulate pol III transcription during pre-initiation complex assembly. Addition of the CK2 inhibitor DMAT to fibroblast cells decreased TFIIIB and pol III recruitment, but not TFIIIC recruitment. Therefore, the combined data suggest that CK2 phosphorylation results in an increase in TFIIIB recruitment, which in turn is likely to account for increased pol III binding and enhanced pol III transcriptional output Regulation of TFIIIB recruitment to class III genes is commonly used to control pol III transcription. For example, reduction in TFIIIB promoter occupancy has also been observed when cells were treated with Erk inhibitors (Felton-Edkins et al., 2003a), following p53 induction (Crighton et al., 2003) and during mitosis (Fairley et al., 2003).

The abundance of pol III transcription factors is elevated in a number of transformed cell types. For example, TFIIIB has been shown to be elevated in fibroblasts transformed by SV40 (Fairley et al., 2003; Larminie et al., 1999) and Py (Felton-Edkins and White, 2002). However, the results in this chapter demonstrate that this does not occur following CK2 activation, as inhibition of CK2 activity with DMAT does not change the levels of the individual TFIIIB subunits.

Previous studies in *S.cerevisiae* have shown that the binding of Brf1 to TFIIIC represents the rate-limiting step in the assembly of the pol III pre-initiation complex (Moir et al, 2004). GST-pull down and co-immunoprecipitation assays revealed that S450A mutation compromises the binding of endogenous TFIIIC and Brf1 *in vitro* and in fibroblasts. This effect is very selective, since the binding of pol III and TBP to Brf1 is not compromised when S450 is mutated to A. The interactions between TFIIIC and Brf1 have been studied in S.*cerevisiae* and it has been shown that Brf1 can interact with the tetratricopeptide repeat (TPR)-containing TFIIIC subunit, TFC4 subunit. TPR motifs play a general role in protein-protein interactions and complex assembly (D'Andrea, 2003). Mutations in the region which contains the TPR motif of TFC4 can result in the activation of pol III transcription *in vitro* and *in vivo* (Moir et al, 2002) and (Moir et al, 1997). The mutations cause a conformational change in Tfc4 that can increase its affinity to Brf1, and hence the

recruitment of pol III (Moir et al, 1997). In addition, a recent study by Liao and co-workers has shown that the H region II of Brf1 is important for binding to TFC4 (Liao et al, 2006). The region of TFC4 that contains the TPR motif is conserved in the human homologue of this subunit (TFIIIC102) and the H2 region of Brf1 is also conserved through evolution, it is therefore likely that the interactions between TFIIIC and Brf1 would be a target of regulation by CK2 in mammalian cells, consistent with the findings shown in this chapter.

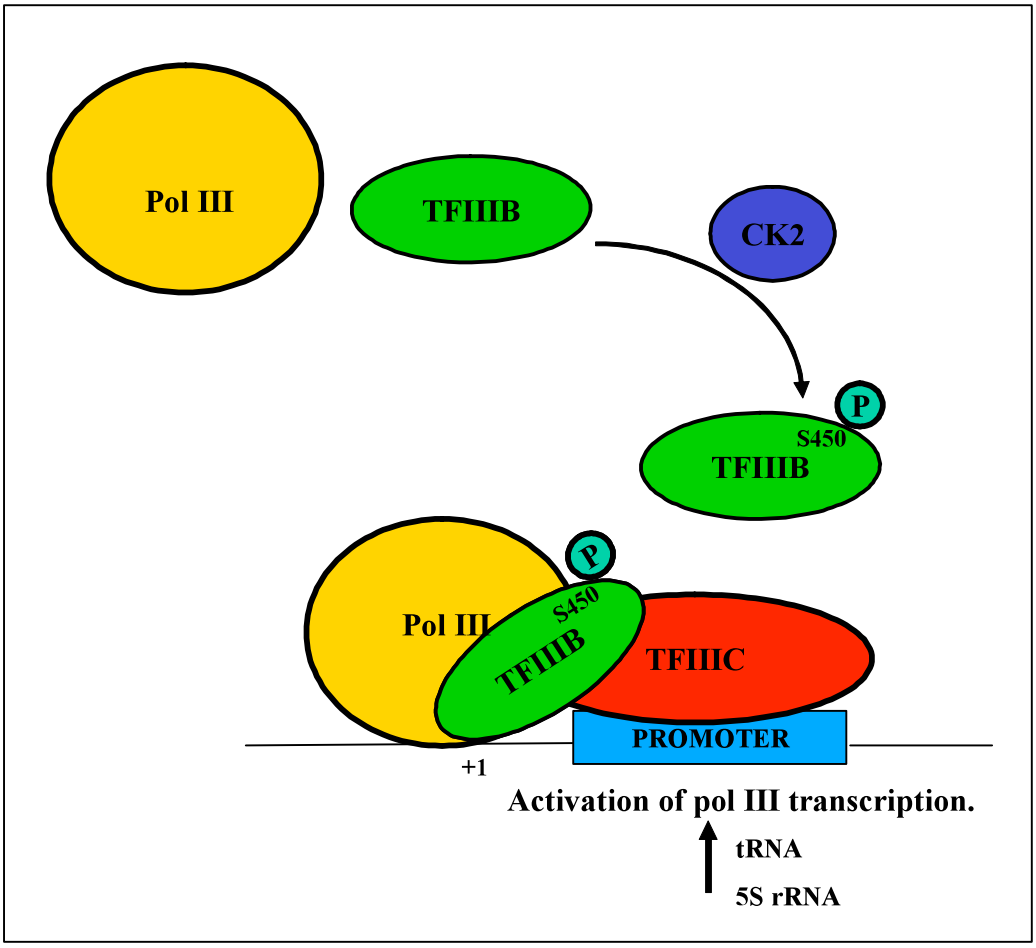


Figure 5.12. Model of regulation of pol III transcription by CK2.
Phosphorylation of Brf1 in the C-terminal domain can result in an increased association with DNA-bound TFIIIC. This can result in an increase in pol III recruitment to the promoters of class III genes and hence a rise in pol III output.

The critical role that CK2 plays in general transcription has been recently emphasised by studies showing that this protein kinase is present on the promoters of pol I, II and III-transcribed genes (Panova et al, 2006; Lewis et al, 2005; Hu et al., 2004). This study reinforces the concept that this protein kinase is an important regulator of nuclear transcriptional machineries. With respect to pol III transcription, the experiments

described in this chapter suggest that CK2 is recruited to pol III promoters and phosphorylates the Brf1 residue S450. Inhibition of CK2-mediated phosphorylation of Brf1 leads to a decrease in TFIIIB occupancy to class III genes, leading to a reduction in pol III transcription.

The data in this chapter support the role of CK2 as an activator of pol III transcription. However, studies from Hu and co-workers have also suggested that CK2 can phosphorylate the Bdp1 subunit of TFIIIB and contribute to the repression of pol III transcription during mitosis (Hu et al., 2004). The next chapter will examine the phosphorylation status of Brf1 during virus-induced cell transformation and mitosis.

6 The regulation of Brf1 by phosphorylation

6.1 Introduction

Chapters Three, Four and Five have demonstrated that CK2 can phosphorylate the Brf1 residue S450, and that this phosphorylation event can activate pol III transcription. The objectives of this chapter were to investigate whether phosphorylation of Brf1 S450 can change during processes in which fluctuations in pol III activity have been observed, for example during virus induced-transformation and mitosis.

6.1.1

6.1.2 Regulation of pol III transcription in response to viruses of the Polyomaviridae family.

Viruses can induce cellular transformation and represent an important risk-factor for cancer development in humans. About 15% of human cancers are thought to arise from viral infection (Rosenthal, 2001). Two classes of viruses can give rise to tumour formation, the DNA tumour viruses and RNA tumour viruses. RNA tumour viruses consist of the *Retroviridae* family, and DNA tumour viruses consist of six different families namely *Adenoviridae*, *Hepadnaviridae*, *Herpesviridae*, *Polyomaviridae*, *Papillomaviridae* and *Poxviridae* [reviewed in (Knipe D.M., 2007)].

The *Polyomaviridae* family can cause cellular transformation by integrating their DNA into the host genome, and allow a high level of expression of viral oncogenic proteins (White, 2004a). Py (Stewart et al., 1958) and SV40 (Sweet and Hilleman, 1960) were the first polyomaviruses to be discovered and are the two best studied. Py is able to induce a variety of different tumours when inoculated into newborn mice (Stewart et al., 1958), and several groups have reported that SV40 can cause tumours in rodents as well as induce transformation of primary cultures of human cells (Eddy et al., 1961; Eddy et al., 1962; Shein and Enders, 1962; Rabson and Kirschstein, 1962; Rabson et al., 1962).

Py and SV40 are very closely related in genome structure and DNA sequence, but differ slightly with respect to the oncoproteins they encode (White, 2004a). Both viruses encode a multifunctional nuclear phosphoprotein termed large T antigen that is directly involved in the initiation of viral DNA replication (DeLucia et al., 1983; Jones and Tjian, 1984). Furthermore, large T antigen has been shown to be sufficient to transform a variety of primary rodent cells (Bikel et al., 1987; Zhu et al., 1992). The transforming properties of large T antigen rely on its ability to bind and perturb the tumour suppressor proteins p53 (Lane and Crawford, 1979) and RB (DeCaprio et al., 1988). This results in aberrant stimulation of the cell cycle and is the driving force for oncogenic transformation. In addition, both viruses encode a low molecular weight protein termed small t antigen that contains sequence in common with the amino terminus of large T antigen and its own unique C-terminal sequence. Unlike SV40, Py encodes a distinct third early protein, termed middle T antigen (Ito, 1979). Middle T antigen also shares the N-terminus of large T antigen and small t antigen (Schaffhausen et al, 1982). Middle T antigen is an integral membrane protein primarily associated with the plasma membrane (Ito, 1979; Schaffhausen et al, 1982).

Both SV40 and Py can elevate pol III transcription (Camacho et al., 1990; Carey et al., 1986; Scott et al., 1983). In fact, studies with SV40-transformed murine fibroblasts were the first to suggest that pol III transcription changes in response to cell transformation (Carey et al., 1986; Scott et al., 1983). Comparison of different SV40-transformed cell lines indicates that rates of pol III transcription are higher in those that most efficiently induce tumours, compared to the less tumourigenic cell lines (Camacho et al., 1990; Scott et al., 1983). In addition, experiments using temperature sensitive mutants of the SV40 oncoprotein large T antigen (Yang et al., 2001), and expression vectors carrying single viral oncogenes (Loeken et al., 1988; Damania et al., 1998) have demonstrated that pol III transcription is increased following transformation.

To increase pol III output, these two viruses employ several distinct mechanisms which involve the basal transcription factors TFIIIB and TFIIIC. Indeed, one of the mechanisms by which the *Polyomaviridae* viruses have been shown to increase pol III transcription is by promoting the release of TFIIIB from repression by RB (Felton-Edkins and White, 2002; Larminie et al., 1999), due to the ability of large T antigen to bind and neutralise RB (DeCaprio et al., 1988). As a consequence, the proportion of endogenous TFIIIB

associated with RB is substantially diminished in SV40-and Py-transformed cells (Felton-Edkins and White, 2002; Larminie et al., 1999)

Cells transformed by these viruses overexpress the Bdp1 subunit of TFIIIB at both the protein and mRNA levels (Felton-Edkins and White, 2002). Despite the overexpression of Bdp1, the abundance of the other TFIIIB components is unperturbed following SV40 and Py transformation. In contrast, all five subunits of TFIIIC are elevated in fibroblasts transformed by Py and SV40 (Larminie et al., 1999; Felton-Edkins and White, 2002). Increased levels of these subunits contribute to the enhancement of pol III transcription.

Both viruses employ the same mechanisms to activate pol III transcription. Py, however, differs from SV40 in encoding a highly oncogenic middle T antigen. Middle T antigen localises outside the nucleus, activates several signal transduction pathways and can serve as a potent activator of a pol III reporter in transfected cells (Felton-Edkins and White, 2002).

Data presented in this chapter will reveal whether the phosphorylation of Brf1 S450 changes when mouse fibroblasts have been transformed by either SV40 or Py.

6.1.3 Mitotic regulation of pol III transcription.

Nuclear gene expression is repressed when cells enter mitosis (Prescott and Bender, 1962). Mitotic repression of transcription can occur via several mechanisms. At the onset of mitosis, chromosome condensation occurs. This process could, in principle, limit the accessibility of the DNA template to the transcriptional machinery and result in repression of pol III transcription (Johnson and Holland, 1965). In addition, reversible inhibitory phosphorylation of the polymerase and/or the basal transcription factors could result in their displacement from the mitotic chromosomes, and thus also account for the repression of pol III transcription (Martinez-Balbas et al., 1995; Hershkovitz and Riggs, 1995; Segil et al., 1996)

Mitotic repression of pol III transcription has been studied in both Xenopus egg and HeLa extracts [reviewed in (Gottesfeld and Forbes, 1997)]. Hartl and colleagues (1993) established that mitotic repression of transcription could be reproduced *in vitro* by the

addition of purified cyclin B to Xenopus egg extracts (Hartl et al., 1993). The activity of pol III observed in extracts obtained during interphase was dramatically decreased when extracts were converted to mitosis in this way (Hartl et al., 1993). The authors also demonstrated that the repression did not require normal chromatin condensation (Hartl et al., 1993), but instead revealed that phosphorylation of a component of the transcription machinery by a protein kinase, either cdc2 kinase and/or a kinase activated by it, caused the mitotic repression of pol III transcription (Hartl et al., 1993). In addition, Gottesfeld and co-workers showed that repression of Xenopus pol III transcription by cdc2 could be reversed by addition of highly purified TFIIIB, whereas TFIIIC had no effect, and revealed that Xenopus egg mitotic extract showed a greatly diminished TFIIIB activity (Gottesfeld et al., 1994). The authors concluded that TFIIIB appeared to be the target for inhibitory phosphosphorylation during mitosis in Xenopus (Gottesfeld et al., 1994).

Xenopus eggs only undergo S and M phases, whereas somatic cells undergo a more complex cell cycle that involves sequential passage through G_1, S, G_2 and M phases. White and colleagues (1995) used HeLa cells that were synchronised at mitosis to investigate the molecular basis of transcriptional repression in a human system. Their findings demonstrate that mitotic HeLa cells repress pol III transcription in a similar manner to Xenopus oocytes and found that transcriptional activity could be restored in the mitotic cells with the addition of TFIIIB derived from interphase cells (White et al., 1995). This suggests that TFIIIB is targeted for mitotic phosphorylation in human cells.

In both the frog and human systems, TBP becomes hyperphosphorylated during mitosis. However, mitotic repression of transcription appeared to be due to a specific loss of TAF activity, since expression could not be restored by the addition of recombinant TBP to mitotic extracts (White et al., 1995; Gottesfeld et al., 1994). In contrast, affinity-purified TFIIIB TAFs reversed repression efficiently and could restore transcription in mitotic extracts (White et al., 1995; Gottesfeld et al., 1994). Using Xenopus eggs, it was shown that the mitotic kinase cdc2/cyclin B phosphorylates a 92kDa TAF (possibly the Xenopus homologue of Brf1) (Gottesfeld et al., 1994). Fairley and co-workers found that Brf1 is hyperphosphorylated when HeLa cells are arrested in metaphase (Fairley et al., 2003). However, the authors found that this repression was caused by a kinase other than cdc2-cyclin B in humans.

Plk1is a protein kinase that is active at late G_2 and during M phases of the cell cycle, and has several roles in mitosis. Unpublished data (J. Fairley and R.J.White) show that Plk1 can inhibit the expression of pol III-transcribed genes during mitosis and that Plk1 can bind and phosphorylate Brf1. Hyperphosphorylation of Brf1 by a mitotic kinase such as Plk1 can weaken the interaction between Brf1 and Bdp1, resulting in the release of Bdp1 from types I and II promoters (Fairley et al., 2003). The productive assembly of the pre-initiation complex is prevented, and pol III transcription is repressed during mitosis.

Hu and colleagues found that Bdp1 is also hyperphosphorylated during mitosis (Hu et al., 2004). In addition, they confirmed that Bdp1 is released from promoters, preventing the productive recruitment of pol III (Hu et al., 2004). The authors suggested that CK2 is the kinase involved in phosphorylating Bdp1 during mitosis.

Upon exit from mitosis, the hyperphosphorylation of TFIIIB is reversed rapidly. Despite this, TFIIIB activity remains low in early G_1 and then increases only gradually as cells move through G_1 into S phase. Maximal expression is reached during S and G_2 phases (White et al., 1995). In G_1, pol III transcriptional activity is mainly regulated by RB. Figure 6.1, below, illustrates the regulation of TFIIIB throughout the cell cycle.

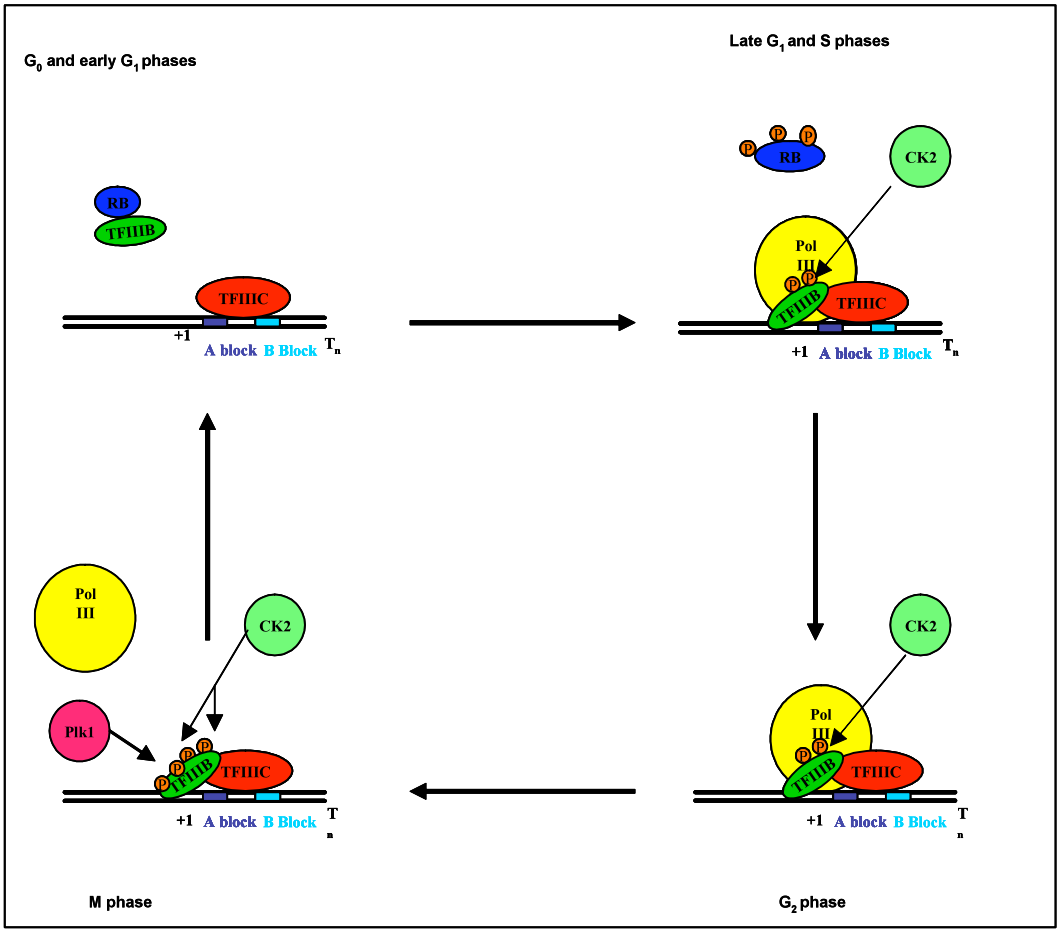


Figure 6.1 Schematic model of the cell cycle regulation of TFIIIB in mammalian cells.
During G_0 and early G_1, underphosphorylated RB binds and represses TFIIIB, but this interaction decreases as cells approach S phase, since cyclin D and E-dependent kinases phosphorylate RB and prevent its binding to TFIIIB. CK2 phosphorylates TFIIIB during interphase and activates pol III transcription. During mitosis, TFIIIB is hyperphosphorylated by several kinases including Plk1 and CK2. This results in the repression of pol III transcription.

TFIIIB is an important target for the regulation of pol III transcription during both mitosis and interphase of actively proliferating HeLa cells. This chapter investigates whether the phosphorylation of Brf1 S450 could contribute to the decrease in pol III transcription during mitosis.

6.2 Results

6.2.1 Phosphorylation of Brf1 in transformed cells

Previous studies have shown that DNA tumour viruses employ several mechanisms to increase pol III transcription (as described in Section 6.1 above). The next set of experiments was performed to determine whether the phosphorylation of S450 varies in cells transformed by SV40 or Py, compared to untransformed control cells.

Cell extracts were obtained from cells transformed by SV40 (SV3T3Cl38) and from the parental Balb/c 3T3 A31 cells. The cell lysates were resolved by SDS-PAGE and were subjected to Western blotting with anti-phosphoS450Brf1, anti-total Brf1 and anti-actin antibodies (Figure 6.2A). The extracts were also subjected to a CK2 kinase activity assay (Figure 6.2B).

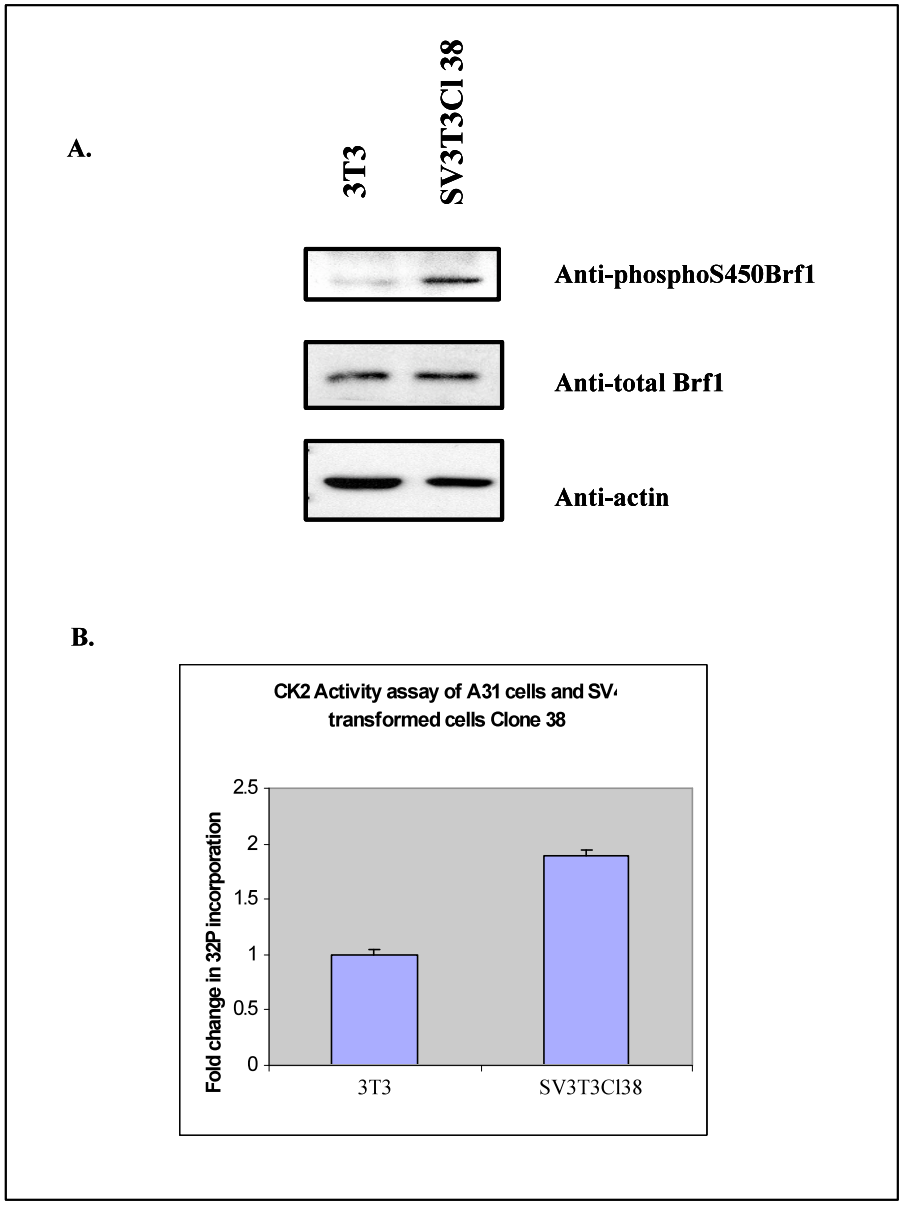


Figure 6.2. The Brf1 residue S450 is phosphorylated following transformation by SV40.
A. Cell lysates (50µg) from Balb/c 3T3 A31 (lane 1) or SV3T3Cl38 cells (lane 2), were resolved by SDS-PAGE and subjected to immunoblotting with antibodies against phosphoS450Brf1 (upper panel), total Brf1 (middle panel) and actin (bottom panel). B. CK2 activity assay of extracts from Balb/c 3T3 A31 and SV3T3 Cl38 cells. The activity of the parental control cell extracts was normalised to 1, and the activity of the transformed cell extracts is represented as a fold change of the control (the error bars indicate the standard deviation from the mean, where n=3).

The results demonstrate that there is an increase in phosphorylation of Brf1 S450 following SV40 infection. This coincides with elevated CK2 activity in the SV3T3Cl38 cell extracts.

In addition, the phosphorylation of the Brf1 S450 was also examined in cells transformed by Py (Py3T3). Extracts derived from the murine cell line Py 3T3 and the parental A31 cells (an immortalised Balb/c 3T3 line) were resolved by SDS-PAGE and subjected to Western blot analysis with antibodies against phosphoS450Brf1, total Brf1 and actin (shown in

Figure 6.3, below).

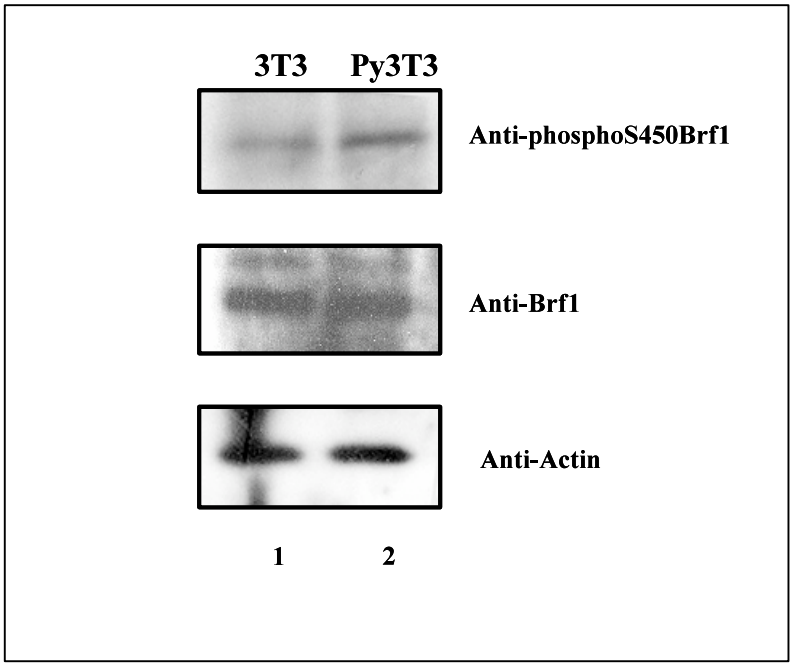


Figure 6.3 Brf1 S450 is phosphorylated following transformation by Py.
Cell lysates (50µg) from Balb/c 3T3 (lane 1) and Py3T3 (lane 2) cells were resolved by SDS-PAGE and subjected to immunoblotting with anti-phosphoS450Brf1 (upper panel), anti-total Brf1 (middle panel) and anti-actin (lower panel) antibodies.

The results indicate that, as with SV40 transformed cell extracts, there is an increase in phosphorylation of Brf1 S450 in the Py-transformed cell extracts compared to the parental Balb/c 3T3 A31 cell extracts.

In conclusion, the combined data from Figure 6.2 and 6.3, indicate that there is an increase in phosphorylation of Brf1 S450 following transformation of A31 cells by SV40 and Py.

6.2.2 Phosphorylation of S450 in mitosis

Pol III transcription is repressed and Brf1 is hyperphosphorylated during mitosis (Fairley et al., 2003). To determine whether the phosphorylation of Brf1 S450 is changed during mitosis, cell lysates from cells that had been arrested in either S or M phase were analysed by Western blotting with anti-phosphoS450Brf1 antibody, anti-total Brf1 and anti-actin antibodies (Figure 6.4A). In addition, the activity of CK2 in these two phases was also assessed by a CK2 activity assay (Figure 6.4B).

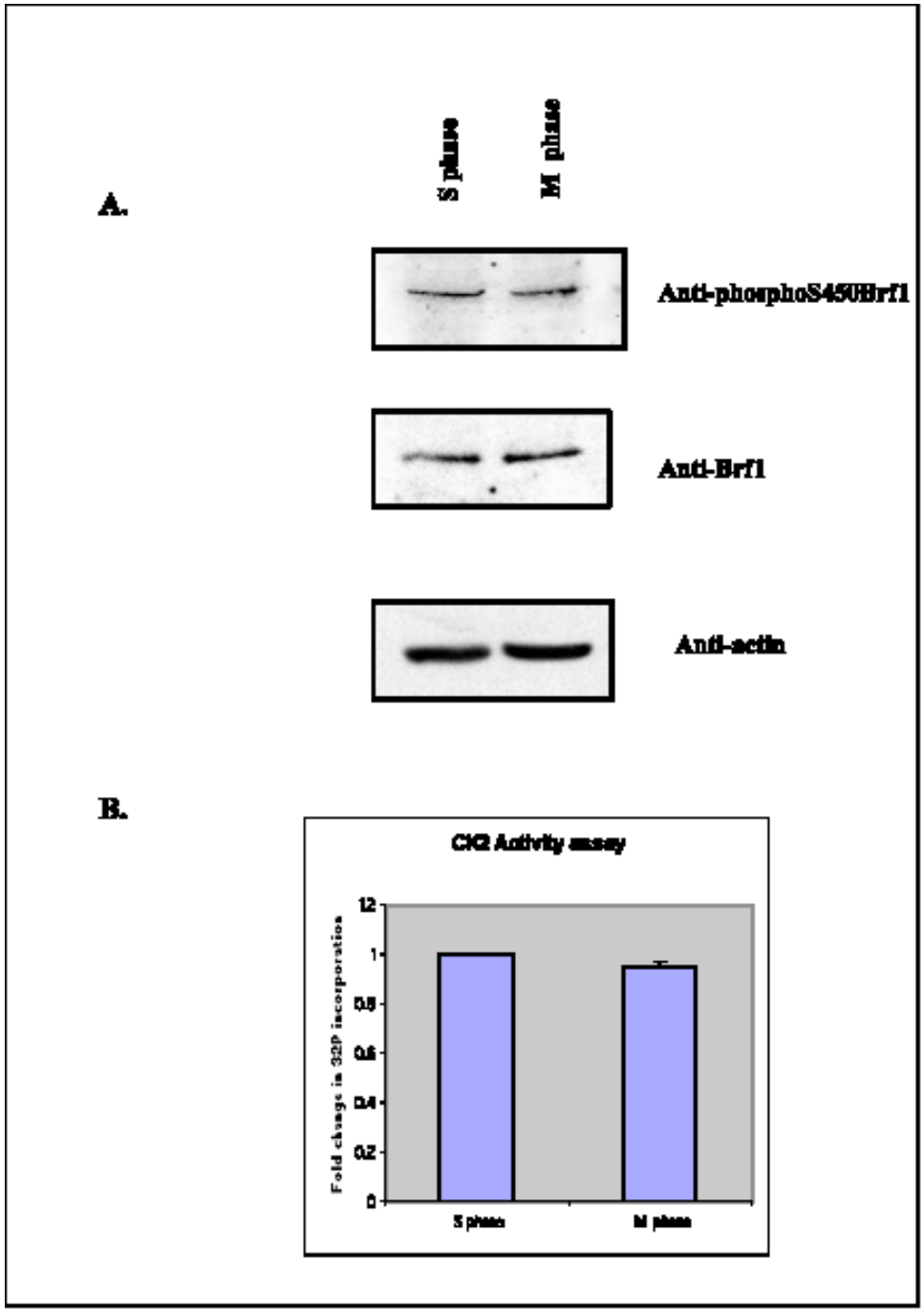


Figure 6.4 Phosphorylation of Brf1 S450 does not change during mitosis.
A. Cell lysates (50 µg) from HeLa cells arrested in S phase (lane 1) or arrested in M phase (lane 2), were resolved by SDS-PAGE and subjected to immunoblotting with antibodies against phosphoS450Brf1 (upper panel), total Brf1 (middle panel) and actin (bottom panel). B. The activity of the S phase extracts was normalised to 1 and the activity of the mitotic extracts is shown as a fold difference of the S phase extracts (the error bars indicate the standard deviation from the mean, n=3).

Figure 6.4A reveals that the phosphorylation of Brf1 S450 remains the same in S and M phases. In addition, no change in the activity of CK2 was observed (Figure6.4B).

The repression of pol III transcription is caused by inhibitory phosphorylation of TFIIIB during mitosis. Fairley and co-workers (2003) found that Brf1 is phosphorylated during mitosis, resulting in the weakening of the interactions between Brf1 and Bdp1. Unpublished data by Fairley and co-workers suggest that Plk1 can phosphorylate Brf1 on T270 during mitosis. Co-immunoprecipitation analysis was performed to determine whether mutating T270 to non-phosphorylatable alanine (T270A) or to the phosphorylation mimic aspartate (T270D) would affect binding of TFIIIB to TFIIIC or pol III.

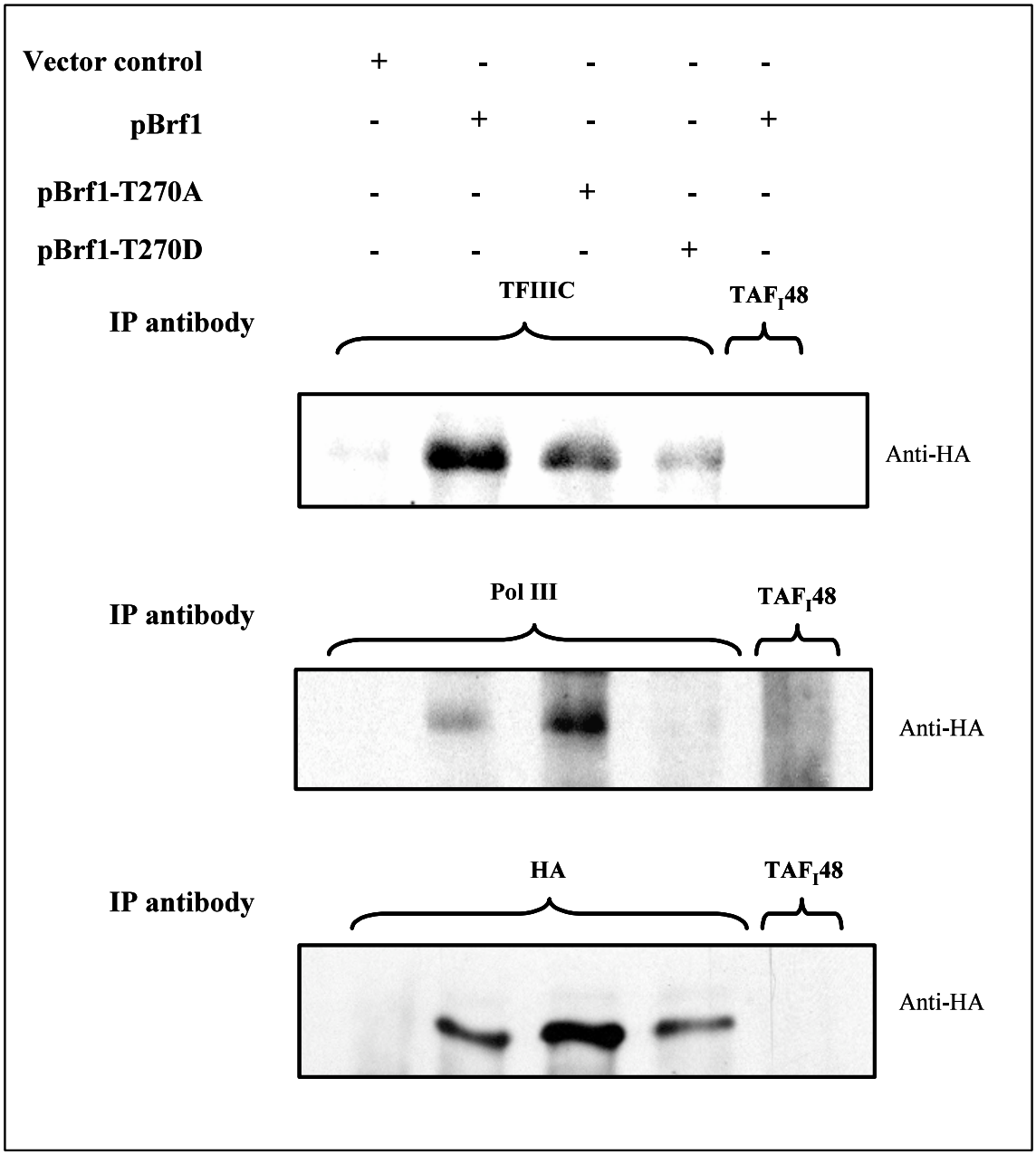


Figure 6.5 Mutation of T270 to aspartate decreases interactions between Brf1 and pol III.
Extracts from HeLa cells transfected with empty vector, pBrf1, pBrf1-T270A and pBrf1-T270D were subjected to immunoprecipitation using anti-HA, anti-TFIIIC, anti-pol III or anti-TAF$_I$48 (negative control) antibodies. The samples were resolved by SDS-PAGE and subjected to Western blotting using anti-HA antibody.

The co-immunoprecipitation analysis revealed that binding of Brf1 to TFIIIC is not greatly affected by mutation of T270 to either A or D (Figure 6.5). However, the binding of Brf1 to pol III does appear to decrease when T270 is mutated to D, but increased slightly when the residue is mutated to A.

Discussion

Several DNA tumour viruses have been shown to induce expression of pol III products including adenovirus, hepatitis B virus, SV40, Py and human papilloma virus (Camacho et al., 1990; Carey et al., 1986; Felton-Edkins and White, 2002; Larminie et al., 1999a; Scott et al., 1983). This up-regulation of pol III transcription in transformed cell lines occurs mainly through mechanisms which target TFIIIB. This chapter focused on elucidating the effects that members of the *Polyomaviridae* family, SV40 and Py, have on the phosphorylation of Brf1 S450. Figures 6.2 and 6.3 reveal that the phosphorylation of Brf1 S450 increases following transformation by both SV40 and Py. As demonstrated in Chapter Five, the phosphorylation of Brf1 at this residue can increase pol III output. The increase in phosphorylation of Brf1 S450 could contribute to the ability of DNA tumour viruses to enhance pol III transcription, which is consistent with previous studies that have shown that these viruses can elevate the expression of pol III transcripts by several shared mechanisms (Felton-Edkins and White, 2002; Larminie et al., 1999). Figure 6.2 demonstrates that there is an increase in CK2 activity following transformation by SV40. CK2 has been shown to phosphorylate and regulate large T antigen (Grasser et al., 1988). Indeed, phosphorylation of the large T antigen by CK2 has been shown to increase its nuclear import (Grasser et al., 1988). The increase of large T antigen in the nucleus could promote the release of more TFIIIB from RB repression and activate pol III transcription.

The onset of mitosis is accompanied by an increase in the level of protein phosphorylation. Indeed, Fairley and co-workers (2003) reported that Brf1 is hyperphosphorylated during mitosis. Figure 6.4 examined whether phosphorylation of Brf1 S450 changes throughout the cell cycle. The data reveal that S450 phosphorylation does not vary between mitotic or interphase cells. In addition, CK2 activity remained unchanged when cells are in mitosis. This is consistent with findings from Bosc and co-workers who examined the expression of CK2α, CK2α' and CK2β, as well as CK2 activity, in cells during different phases of the cell cycle and found no major fluctuations in the expression and activity of CK2 (Bosc et al., 1999).

Mitosis is associated with a decrease in transcription, and TFIIIB was found to be the target of inhibitory phosphorylation. Brf1 residue T270 was found to be specifically phosphorylated during mitosis and mutation of T270 to aspartic acid resulted in the

repression of pol III transcription (J.Fairley and R.J.White, unpublished data). The data suggest that T270 is a mitotic site of phosphorylation by Plk1 and contributes to the mitotic repression of pol III transcription. Fairley and co-workers showed that upon mitotic Brf1 hyperphosphorylation, the interactions between Bdp1 and Brf1-TBP are affected, resulting in the release of Bdp1 from the promoter (Fairley et al., 2003). The resulting Brf1-TBP complex is not sufficient to recruit pol III to the promoters. Indeed, ChIP assays revealed a reduction of polymerase at the promoters of pol III-transcribed genes, but found no change in the occupancy of TFIIIC and Brf1 (Fairley et al., 2003). Figure 6.5 examined whether mutation of T270 to A or to aspartic acid would affect the interactions of Brf1 with pol III and TFIIIC. The data reveal that Brf1 binding to pol III was disrupted when this T residue was mutated to aspartic acid, which is consistent with an inhibitory role for the phosphorylation of the residue. In contrast, the Brf1 interactions with TFIIIC were unaffected when the residue was mutated to either A or aspartic acid. These results are consistent with the model of mitotic pol III transcriptional repression suggested by Fairley and colleagues (Fairley et al., 2003)

Transcription in mitotic extracts was restored by cellular overexpression of not only Brf1 but also Bdp1 (Fairley et al., 2003). Indeed, Hu and colleagues (2004) showed that Bdp1 is also hyperphosphorylated during mitosis. The authors suggested that CK2 phosphorylation of Bdp1 is directly responsible for the mitotic transcription shut-down. It is uncertain how CK2, which is constitutively active and is a positive regulator of pol III transcription during interphase, can induce Bdp1 phosphorylation specifically at mitosis giving rise to a repression of pol III transcription (Hu et al., 2004). CK2 has been shown to specifically phosphorylate DNA topoisomerase IIα during mitosis, Escargueil and colleagues (2007) found that phosphorylation of this protein is suppressed during interphase due to competing dephosphorylation by protein phosphatase 2A. Protein phosphatase 2A is excluded from the nucleus during early mitosis which then allows CK2 to phosphorylate topoisomerase IIα (Escargueil et al, 2007) Therefore; it is possible that a similar mechanism could regulate the phosphorylation of Bdp1 whereby an inhibitory site of phosphorylation found on Bdp1 could be unavailable for phosphorylation by CK2 during interphase. Despite no change in CK2 activity, Bdp1 could be phosphorylated specifically during M phase due to the exit of protein phosphatase 2A from the nucleus. This mechanism could contribute to the mitotic repression of pol III transcription.

Reversible phosphorylation is a prevalent mechanism by which the activity of eukaryotic transcription factors is regulated rapidly. Indeed, this chapter has shown that the multisite phosphorylation of TFIIIB is an important regulatory step used to control pol III output in response to changes in cellular environment.

7 Final Discussion

7.1 Activation of pol I and III transcription by CK2.

Pol I and pol III are together responsible for generating the four rRNAs (28S, 18S, 5.8S and 5S), which are required in equimolar amounts for ribosome assembly. Therefore, synthesis of rRNA by these two polymerases is often regulated co-ordinately. Indeed, CK2 has been found to stimulate transcription by pols I and III in both mammals and yeast (Ghavidel and Schultz, 1997; Ghavidel and Schultz, 2001; Hu et al., 2003; Johnston et al., 2002; Lin et al., 2006; Panova et al, 2006). Consistent with these findings, Chapter Three, demonstrated by several techniques, such as *in vitro* transcription assays, Northern blotting and RT-PCR (shown in Figures 3.1 to 3.5), that CK2 increases pol III transcription *in vitro* and *in vivo*. Pol III output is significantly reduced upon inhibition of endogenous CK2 with specific chemical inhibitors such as TBB and DMAT, in comparison to vehicle treated cells or cell extracts. To confirm that the effects of these inhibitors on pol III activity were mediated by the blockage of CK2, rather than of other kinases, an alternative method of decreasing endogenous CK2 activity, such as RNA interference (RNAi) could have been used. RNAi against CK2, is a non-pharmacological and selective method of decreasing CK2 catalytic activity, and has been successfully carried out in other areas of CK2 research. For example, Di Maira (2005) and colleagues were studying the effects of CK2 phosphorylation on Akt/PKB, they were able to reduce the level of expression and activity of the CK2 catalytic subunit by about 60%, using this approach. In addition to the assays measuring pol III output following CK2 inhibition, ChIP assays show that CK2 is associated with class III genes with types I and II promoters (Figure 5.4) and type III promoters (Hu et al, 2004). Collectively, these data support the role of CK2 as a regulator of pol III transcription.

7.2 Brf1 S450 as a target for CK2 phosphorylation

The transcription of a class III gene is dependent on pol III recruitment to the appropriate promoter. For the majority of genes transcribed by pol III, this is determined by the transcription factors TFIIIC and TFIIIB, which bind specific promoter sequences and directly recruit the polymerase, respectively [reviewed (Schramm and Hernandez, 2002)].

Transcription factors are frequently targeted by numerous control strategies according to changes in the cellular environment. In particular, TFIIIB, has been well documented as a key regulator of pol III transcription in eukaryotic cells (summarised in Figure 7.1 below) (Felton-Edkins et al., 2003a; Crighton et al., 2003; Johnston et al., 2002; Gomez-Roman et al., 2003; Larminie et al., 1997).

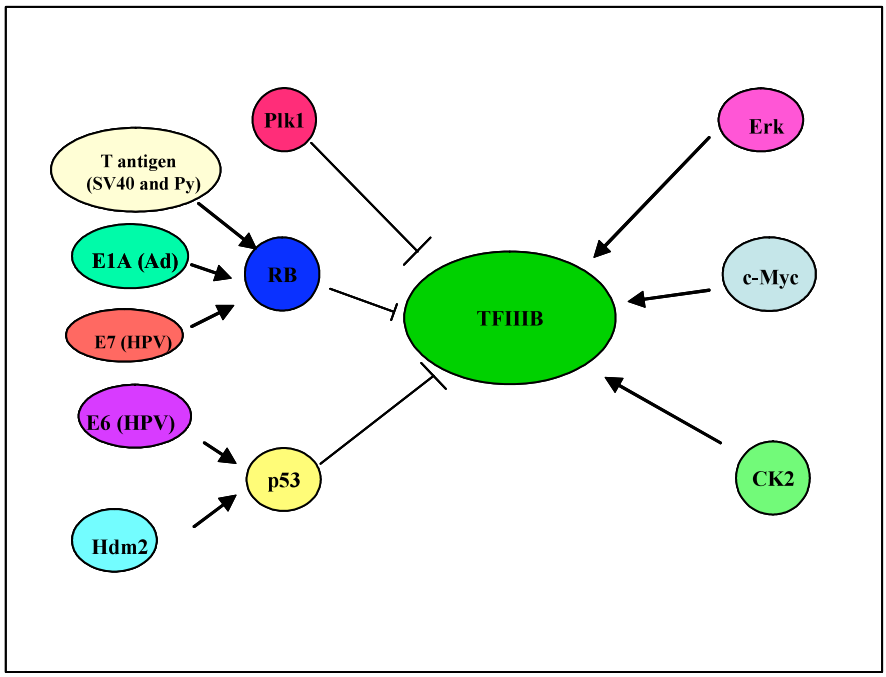


Figure 7.1 TFIIIB is highly regulated by several activators and repressors. The growth repressors RB and p53 can repress TFIIIB by direct interactions. Their function can be blocked by several viral proteins including large T antigen from SV40 and Py, the adenovirus E1A, the HPV E6 and E7 and the cellular oncoprotein Hdm2. The mitotic kinase Plk1 can also repress pol III transcription by phosphorylating TFIIIB directly. In contrast, a variety of oncoproteins have been shown to stimulate TFIIIB directly, including Erk, c-Myc and CK2. Figure was adapted from (White, 2004c).

Chapters Three and Four confirmed that the Brf1 subunit of TFIIIB is a substrate for phosphorylation by the protein kinase CK2 (Figures 3.6, 4.4 to 4.7 and 4.12), in agreement with previous observations (Johnston et al., 2002). Furthermore, the present study has advanced the current knowledge by showing that the Brf1 residue S450 is a major target for phosphorylation by CK2. Analysis of available structural data for a portion of the C-terminal domain of Brf1 (Figure 4.14), S450 is exposed and readily available for phosphorylation, thus further strengthening the conclusion that this residue is indeed phosphorylated by CK2.

S450 of human Brf1 is equivalent to the residue E463 in yeast. Both of these residues lie within the H2 region of Brf1, which is well conserved from yeast to humans (Larminie, 1998). Despite the divergence between yeast E463 and human S450, both phospho-S and E are acidic. This indicates that Brf1 function may require acidity at this particular region but may also reflect a difference in the regulation of transcription between lower and higher eukaryotes.

The remainder of this thesis focused on the H2 region of Brf1, and particularly on the phosphorylation of residue S450. However, Figures 4.4 and 4.5 indicate that the H2 region could also be a target for phosphorylation by CK2. To further examine potential sites within Brf1, several attempts were made to express the H3 region and core domain of Brf1 as GST fusion proteins. However, these attempts were unsuccessful and, therefore, these regions were not tested in the phosphorylation assays. Thus, the possibility that other Brf1 residues in addition to S450 are targets of CK2 phosphorylation cannot be ruled out and should be examined further.

The preferred method for identifying phosphorylation sites would have been through the use of mass spectrometry. Indeed, several months of this project were dedicated to trying to locate the phosphorylation sites within Brf1 by matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry. However, the results obtained were inconclusive due to several experimental issues, including problems during phosphopeptide enrichment of the sample by high performance liquid chromatography (HPLC), low counts of ^{32}P incorporation in Brf1, as well as technical problems such as mass spectrometry instrument failure. Due to time constraints, the remainder of the project was focused on characterising the S450 site which was identified using *in vitro* kinase assays and confirmed *in vivo* with a phosphospecific antibody. Future studies using an improved protocol and better mass spectrometry facility would be of great interest and should be pursued.

7.3 CK2 and regulation of pol III transcription during cell transformation.

A wide variety of transformed cell lines and tumours have abnormally high levels of pol III (Carey et al., 1986; Daly et al., 2005; Scott et al., 1983; Winter et al., 2000) and CK2 activity (Ahmed et al., 2000; Dessauge et al., 2005; Tawfic et al., 2001). These include cell lines transformed by DNA tumour viruses of the *Polyomaviridae* family (Felton-Edkins and White, 2002; Larminie et al., 1999b; Scott et al., 1983). The levels of phosphorylation of Brf1 S450 were higher in the SV40- and Py- transformed fibroblasts compared to the untransformed parental control cells (Figure 6.2 and

Figure 6.3). This increase in phosphorylation could contribute to the mechanisms employed by these viruses to achieve the high pol III output, as previously observed in these cell lines (summarised in Figure 7.2, below).

Virus-induced transformation in cell culture has played a major role in elucidating the molecular mechanisms of oncogenesis. This thesis has only investigated the phosphorylation of Brf1 S450 following transformation by Py and SV40. Other types of DNA and RNA tumour viruses can induce pol III transcription and it would be interesting to assess whether they share similar deregulatory mechanisms.

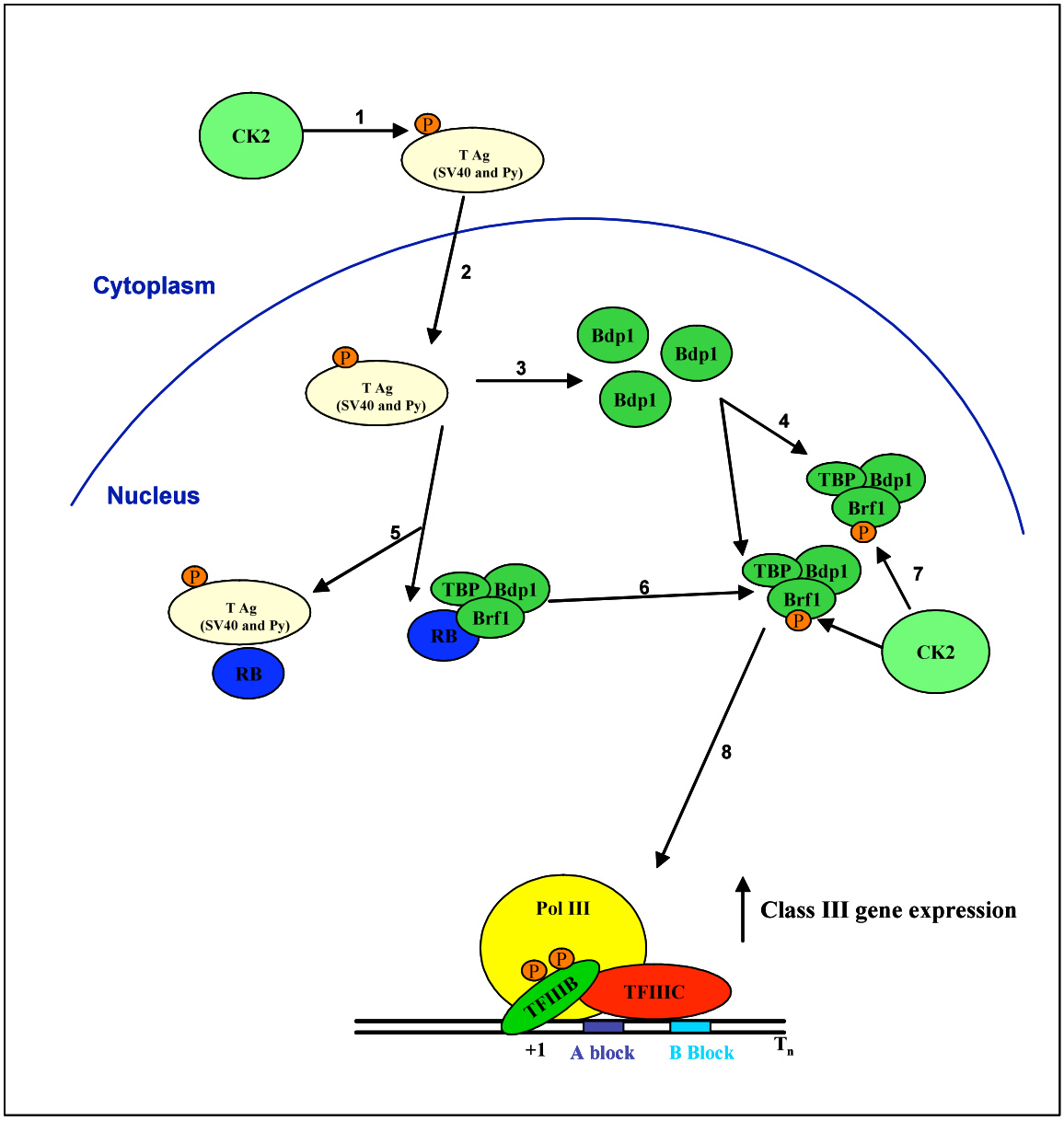


Figure 7.2 Regulation of pol III transcription during cell transformation by *Polyomaviridae* viruses.
CK2 phosphorylates the large T antigen and enhances its nuclear import (1 & 2). Following transformation, the cells overexpress Bdp1, resulting in an increase in the number of TFIIIB complexes (3 & 4). The large T antigen can bind and neutralise RB, releasing TFIIIB from RB inhibition (5 & 6). The free TFIIIB complexes are available for phosphorylation by CK2 and to recruit pol III to the promoters of class III genes(7) and. transcription of class III genes is enhanced (8) (Felton-Edkins and White, 2002; Larminie et al., 1999; Scott et al., 1983).

Neoplastic change is also associated with changes in the intracellular localisation of CK2, so that a higher nuclear localisation is observed in tumour cells compared to normal cells (Tawfic et al., 2001). Pol III products are overexpressed in tumour samples (Daly et al., 2005; Winter et al., 2000). Therefore, it would be interesting to investigate whether the

nuclear shuttling of CK2 during neoplastic changes contributes to an increase in S450 phosphorylation, and thus pol III output.

7.4 Regulation of Brf1 during mitosis

The entry into mitosis is accompanied by an increase in the level of protein phosphorylation and a decrease in nuclear gene transcription (Prescott and Bender, 1962). Several studies have described the direct involvement of inhibitory phosphorylation of TFIIIB to be one of the likely mechanisms which organisms use to repress pol III transcription during M phase (White et al, 1995). Fairley and colleagues (2003) found that cdc2 is unlikely to account for the mitosis-specific phosphorylation of Brf1 in mammalian cells. Instead, Hu and colleagues (2004) suggested that CK2 is responsible for phosphorylating Bdp1 and leading to a decrease in pol III output during mitosis. Figure 6.4 investigated whether the phosphorylation of Brf1 S450 varies when cell lysates from M phase were compared to cell lysates in S phase, and revealed no change in the phosphorylation status of that residue. Plk1 is activated during mitosis and unpublished data (J.Fairley and R.J.White) show that this mitotic kinase phosphorylates the Brf1 residue T270.

Figure 6.5 showed when Brf1 T270 was mutated to aspartate (which mimics phosphorylation) interactions of Brf1 with pol III were diminished, whereas the interactions between Brf1 and TFIIIC interactions were not greatly affected. These findings support the role of T270 as an inhibitory mitotic phosphorylation site. When S450 is phosphorylated it activates pol III transcription (Figures 5.1 to 5.3); despite this, it remained phosphorylated during mitosis (Figure 6.4A). The increase of level and activity of Plk1 can shift the balance towards inhibitory phosphorylation, allowing transcription to be repressed.

Hu and colleagues proposed that CK2 phosphorylation of pol III itself is required for activating for pol III transcription at a type III promoter (Hu et al., 2003). Further work from this group suggested 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). They reported that CK2 treatment of pol III 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). A minimal system consisting of recombinant and purified factors was used by Hu and colleagues and their findings remain to be shown *in vivo*. It remains unclear how CK2 which is constitutively active throughout the cell cycle (Figure 6.4B; Bosc et al, 1999), could have opposite effects on pol III transcription depending on phase. Another way to induce mitotic phosphorylation is by reduction in the level or activity of the protein phosphatase during mitosis. Escargueil and Larsen (2007) provide evidence of an alternative mechanism to attain specific phosphorylation by CK2 during mitosis. The authors found that topoisomerase II is co-localised with both CK2 and PP2A during interphase and argue that PP2A is able to dephosphorylate the CK2 site during interphase. During early mitosis, PP2A is translocated from the nucleus to the cytoplasm, giving CK2 the possibility to phosphorylate the phosphoacceptor site. This model could be a possible explanation for the observations made by Hu and co-workers (2004) who found that Bdp1 can be phosphorylated by CK2 specifically during mitosis.

7.5 Mechanism of regulation of pol III transcription by CK2

ChIP assays were used to monitor pre-initiation complex assembly on class III genes in response to CK2 phosphorylation. This revealed a specific increase in TFIIIB promoter occupancy on tRNA and 5S rRNA genes, while the association of TFIIIC was essentially unaffected (as shown in Figures 5.6 and 5.8). Therefore, it was concluded that polymerase binding, and hence transcription initiation, are regulated at the level of TFIIIB recruitment. Phosphorylation can modulate the activity of transcription factors at various levels of regulation, such as facilitating protein-protein interactions (reviewed in Holmberg et al, 2002). Indeed, through the use of phospho-mimicking/ablation mutants of Brf1 S450, it was established that the phosphorylation of Brf1 S450 by CK2 can enhance interactions with TFIIIC, and thereby stimulate pre-initiation complex assembly. This results in an increase in class III gene expression.

The regulation of TFIIIB by phosphorylation is an important regulatory step used in many instances in which the growth potential of a cell has to be altered. Experiments from

Michael Schultz's laboratory have shown that CK2 normally associates through its β subunit with the TBP subunit of TFIIIB, phosphorylates TBP and activates pol III transcription (Ghavidel et al., 1999; Ghavidel and Schultz, 1997; Hockman and Schultz, 1996b). Indeed, blockage of CK2 causes the synthesis of tRNA and 5S rRNA to decrease by 80-90%. They also found that transcriptional repression induced by DNA damage is achieved by the down regulation of TBP-associated CK2, which occurs through the release of the catalytic subunits from the complex (Ghavidel et al, 2001). A role for CK2 in mediating pol III transcription repression following DNA damage signals remains to be investigated in mammalian cells.

7.6 Conclusions

Transcription is essential to every living organism and it impinges upon all biological processes, such as growth, development and the ability to respond to environmental circumstances. Defects in transcription are known to characterise the majority of diseases. This thesis has provided evidence in support of the direct role of CK2 in pol III transcription. Induction of Brf1 was found to increase cell proliferation and cause oncogenic transformation. (Marshall et al, 2008) and phosphorylation of Brf1 by CK2 could be significant during cell transformation. The work presented contributes to the current understanding of the mechanisms underlying CK2 phosphorylation of Brf1. Appreciating the molecular basis of CK2 activation of pol III transcription could have important implications for the design of treatments aimed at the prevention of cancer.

8 References

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