Regulation of RNA Polymerase III Transcription by the ID1, ID2, ID3 and E47 Proteins

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Abstract

RNA polymerase III (pol III) is responsible for transcribing a set of genes that are involved in protein synthesis, including transfer (tRNA) and 5S ribosomal RNA (5S rRNA). Pol III transcription levels are increased in many cancers, as increased protein synthesis is required for tumour growth. Furthermore, it has been shown that a number of oncoproteins and tumour suppressor proteins interact directly with the pol III machinery. The work presented in this thesis aimed to investigate whether the inhibitor of differentiation (ID) and E47 proteins regulate pol III transcription, as well as the mechanisms behind these processes.

Members of the ID family of helix-loop-helix (HLH) proteins promote cell proliferation and inhibit differentiation. ID proteins were identified through their ability to bind and antagonise other HLH proteins, such as the E47 transcription factor, but can also interact with non-HLH proteins, including ETS (E twenty-six) domain transcription factors and the retinoblastoma (RB) protein. Elevated levels of ID proteins are seen in many tumour types, including breast and colorectal carcinomas and neuroblastomas. Work in this thesis showed that pol III activity is specifically decreased following RNAi knockdown of ID1, ID2 or ID3 and that, conversely, overexpression of ID2 can induce pol III transcription. Chromatin immunoprecipitation (ChIP) assays demonstrated that ID proteins are present at pol III-transcribed genes *in vivo*, which may be explained by interaction with transcription factor IIIB (TFIIIB). To test this hypothesis, mapping experiments were performed which showed that TFIIIB and ID proteins co-localise near the start of a pol III template. By ChIP assays it was demonstrated that, in an ID2 knockout cell line, pol III and TFIIIB occupancy at pol III-transcribed genes is decreased compared to wild type. By co-immunoprecipitation (co-IP) experiments, it was shown that IDs interact with TFIIIB at physiological ratios. Also, *in vitro* transcription and translation experiments demonstrated that glutathione S-transferase (GST) fusion proteins GST-ID1, GST-ID2 and GST-ID3 interact with TFIIIB. Therefore, ID regulation of pol III activity appears to be through interaction of these proteins with TFIIIB.

Abstract

Members of the E-protein family of basic helix-loop-helix (bHLH) transcription factors are involved in regulation of cell growth and differentiation. E-proteins have been demonstrated to bind to E-boxes and can interact with other members of the HLH proteins as homodimers or heterodimers in order to modulate expression of their target genes. The ubiquitously expressed E47 transcription factor, which is a member of the E-protein family, is negatively regulated by direct interaction with ID proteins, which prevent its binding to DNA. Work in this thesis showed that pol III activity is specifically stimulated following RNAi knockdown of E47 and that overexpression of E47 decreased pol III transcription. It was demonstrated by ChIP assays that endogenous E47 is present at pol III-transcribed genes *in vivo*. Mapping experiments were performed which showed that TFIIIB and E47 co-localise near the start of a pol III template. Co-IP experiments demonstrated an interaction of E47 with TFIIIB. Also, *in vitro* transcription and translation experiments indicated an interaction of GST-E47 fusion protein with TFIIIB. ChIP assays suggested that the ID2 protein is recruited to pol III-transcribed genes in an E47-dependent manner.

In conclusion, work in this thesis identified novel functions concerning the role of ID and E47 proteins in their involvement in the regulation of pol III transcription. These findings have important implications in understanding the molecular basis of cancer development. The ID and E47 proteins therefore join the growing list of oncogene and tumour suppressor gene products that stimulate or repress pol III output.

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List of Abbreviations

°C	degrees Celsius
δ	deletion mutant
Δ	mutant
µCi	microcurie
µg	microgram
µl	microlitre
3T3	3-day transfer, inoculum 3 x 10^5 cells
A	adenine
A_{260}	absorbance at 260nm
AD	activation domain
Ad	adenoviral
Ada	transcriptional adaptor
Alt	alternative
AML	acute myeloid leukaemia
AMP	adenosine monophosphate
ARF	alternative reading frame
Arg	arginine
ARPP P0	acidic ribosomal phosphoprotein P0
ATP	adenosine triphosphate
Bcl-2	B cell lymphoma 2
Bdp1	B double primer 1

bHLH	basic HLH
bp	base pairs
Brf1	TFIIB-related factor 1
Brf2	TFIIB-related factor 2
Bromodomains	brm [brahma]-like domains
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic AMP
Can	canonical
CBP	CREB binding protein
CD4	cluster of differentiation 4
cdc	cell division cycle
CDK	cyclin-dependent kinase
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CHO	Chinese hamster ovarian
CK2	casein kinase II
CMV5	cytomegalovirus 5’
CREB	cyclic AMP response element-binding protein
Ct	threshold cycle
CTD	carboxyl-terminal domain
C-terminal	carboxyl-terminal
DAB	3,3-diaminodbenzidine
dCTP	deoxycytidine triphosphate
DD	degradation domain

DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double-stranded DNA
DSE	distal sequence element
DTT	dithiothreitol
E. coli	*Escherichia coli*
EBER	Epstein-Barr virus-encoded RNA
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra acetic acid
Egr1	early growth response 1
emc	extramachrochaetae
EMSA	electrophoretic mobility shift assays
ERK	extracellular signal-regulated kinase
ETO	eight-twenty-one
ETS	E twenty-six
EWS	Ewing sarcoma
FACS	Fluorescence-Activated Cell Sorting
FBS	foetal bovine serum
FRET	fluorescence resonance energy transfer
g	relative centrifugal force
G	guanine
G418	Geneticin

Gcn5	general control nonderepressible 5
GFP	green fluorescent protein
GOF	gain of function
GST	glutathione-S-transferase
HA	haemagglutinin
HA-Brf1	HA-tagged Brf1
HAT	histone acetyltransferase
HBV	hepatitis B virus
HDAC1	histone deacetylase 1
HDACs	histone deacetylases
Hdm2	human double minute 2
HEK 293	human embryonic kidney cell line 293
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLF	hepatic leukaemia factor
HLH	helix-loop-helix
HPV	human papilloma virus
Hsp	heat shock protein
HTLV-1	human T cell leukaemia virus type I
ICR	internal control region
ID	inhibitor of differentiation
IE	intermediate element
INF-β	Interferon-β
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside

IRES	Internal ribosomal entry site
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
JAB1	Jun activating binding protein 1
kb	kilo base
kDa	kilodaltons
LB	Luria Bertani
Leu	leucine
LOF	loss of function
LS-MEB	low salt microextraction buffer
LYL1	lymphoblastic leukaemia 1
M	molar
MAP	mitogen activated protein
MAX	Myc associated factor X
MBD2	methyl-CpG binding domain protein 2
mCi/mmol	millicuries per millimol
MDM2	mouse double minute 2
MeCP2	methyl CpG binding protein 2
MEFs	mouse embryonic fibroblasts
MEK	MAP kinase kinase
Met	methionine
mg	milligram
MIDA1	mouse ID associated protein 1
miRNA	micro RNA
ml	millilitre

mM	millimolar
MPP11	M-phase phosphoprotein 11
mRNA	messenger RNA
MRP	mitochondrial RNA processing
MTGR 1	myeloid transforming gene-related protein 1
mUbc9	mammalian Ubc9
MycER	Myc-oestrogen receptor fusion protein
MyoD	myogenic determination
N-CoR	nuclear receptor corepressor
NES	nuclear export signals
ng	nanograms
NLS	nuclear localisation signals
nm	nanometers
nt	nucleotide
N-terminal	amino-terminal
Oct-1	octamer-binding protein 1
OD	optical density
PAGE	polyacrylamide gel electrophoresis
Pax	paired box
PBS	phosphate buffered saline
Pbx1	pre-B cell leukaemia homeobox 1
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PEST	proline glutamic acid serine threonine
PI3K	phosphatidylinositol 3-kinase

PKR	Protein kinase R
pmol	picomol
PMSF	phenylmethylsulfonyl fluoride
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III
pol IV	RNA polymerase IV
POU	pituitary-octamer-unc-86
pre-rRNA	precursor rRNA
PSE	proximal sequence element
P-TEFb	positive transcription elongation factor b
PTEN	phosphatase and tensin homologue
PTF	PSE-binding transcription factor
Q-PCR	Quantitative-PCR
rATP	riboadenosine triphosphate
RB	retinoblastoma
RBP-Jκ	recombination signal binding-protein-J-kappa
rCTP	ribocytidine triphosphate
rDNA	ribosomal DNA
rGTP	riboguanosine triphosphate
RIE	Rat intestinal epithelial cells
RNA	ribonucleic acid
RNase	ribonuclease
RPM	revolutions per minute

rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-PCR
S. cerevisiae	*Saccharomyces cerevisiae*
SAGA	Spt-Ada-Gcn5-Acetyltransferase
Saos-2	sarcoma osteogenic
SCL	stem cell leukaemia
SDS	sodium dodecyl sulphate
Sec	selenocysteine
Ser5	serine 5
SINEs	short interspersed nuclear elements
siRNA	small interfering RNA
SL1	selectivity factor 1
SMC	smooth muscle cell
$SNAP_c$	snRNA activator protein complex
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SOC	super optimal broth with catabolite repression
Sp1	stimulating protein 1
Spt	suppressor of Ty
SRP	signal recognition particle
STAT	signal transducers and activators of transcription
SV40	simian virus 40
SWI/SNF	switch/sucrose nonfermentable
T	thymidine

$t_{1/2}$	half-life
TAE	Tris-acetate-EDTA
TAFs	TBP-associated factors
TAL1	T cell acute lymphoblastic leukaemia 1
T-ALL	T cell acute lymphoblastic leukaemia
Taq	*Thermus aquaticus*
TATA	TATA box
TBE	Tris-Borate-EDTA
TBP	TATA box-binding protein
TBS	Tris buffered saline
TCF	ternary complex factor
tDNA	DNA encoding tRNA
TE	Tris-EDTA
Tet-On	Inducible gene expression system in which the rtTA transactivator is active in the presence of doxycycline
TFE3	transcription factor E3
TFIIA	transcription factor IIA
TFIIB	transcription factor IIB
TFIIIA	transcription factor IIIA
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
Thr	threonine
TLR3	Toll-like receptor 3
Tn	termination

TPRs	tetratricopeptide repeats
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	polyethylene glycol tert-octyl-phenyl ether
tRNA	transfer RNA
TRRAP	transactivation/transformation domain associated protein
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr	tyrosine
U	unit
UBF	upstream binding factor
UTP	uridine triphosphate
UV	ultraviolet
V	volt
v/v	volume per volume
VA	viral associated
W	watt
w/v	weight per volume

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Author's Declaration

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

Nikiforos A. Spandidos

Chapter 1

Introduction

1.1 Eukaryotic Transcription

The synthesis of ribonucleic acid (RNA) from deoxyribonucleic acid (DNA) templates is called transcription and is catalysed by enzymes known as RNA polymerases. One strand of DNA is copied by complementary base pairing to one strand of RNA. RNA polymerases start transcription at the initiation site of a gene. This site, which is referred to as the +1 start site, allows an RNA polymerase to move downstream along the gene and synthesise an RNA strand. This process will continue until the RNA polymerase reaches a termination sequence. At this point the RNA transcript is released from the DNA. Interestingly, not all DNA sequences are transcribed into RNA. Non-transcribed regions contain regulatory signals that can direct RNA polymerases to the initiation site of a gene.

Transcription is the initial stage of gene expression and therefore it is subjected to a range of control mechanisms. Post-transcriptional regulation of gene expression can also occur, however the most significant and common level of control is through transcription (White, 2001). Transcriptional regulation is involved in almost all biological processes such as growth and development. This allows the cell to adapt to environmental changes and metabolic requirements. Errors in transcription are associated with a wide range of diseases, such as developmental abnormalities, diabetes and various forms of cancer.

Eukaryotes have four nuclear RNA polymerases. In the nucleus of eukaryotic cells three RNA polymerases (pol I, pol II and pol III) transcribe different sets of genes (White, 1998a). Another RNA polymerase, (pol IV), has been discovered and it is expressed from an alternative transcript of the mitochondrial RNA polymerase gene (Kravchenko *et al.*, 2005). Also, a plant-specific polymerase was found to participate in transcriptional silencing (Herr *et al.*, 2005; Wierzbicki *et al.*, 2008). Each RNA polymerase is essential for transcription of a specific set of genes and depends on transcription factors to recognise promoter sequences.

Pol I has 14 subunits and pol II and III have 12 and 17 subunits, respectively. Pol I is devoted to transcribing one set of genes, the ribosomal RNA genes, which are

present in about 400 copies in humans and account for about 35-65% of all nuclear transcription (Moss & Stefanovksy, 2002). Pol II transcribes the protein-encoding messenger RNA genes (mRNA genes), as well as most small nuclear RNA (snRNA) genes (Thomas & Chiang, 2006), and the small nucleolar (sno) and micro (mi) RNA genes and provides 20% of nuclear transcription. Pol III is the largest RNA polymerase with an aggregate mass of 600-700 kDa. Pol III synthesises short untranslated RNA molecules which are usually shorter than 400 base pairs. Many of these have important functions in protein synthesis and cell growth, such as 5S ribosomal RNA (rRNA) and transfer RNA (tRNA). Pol III also synthesises 7SL RNA, an important component of the signal recognition particle, and 7SK RNA, which regulates pol II transcription. Mitochondrial RNA processing (MRP), U6 and H1 RNA molecules are also synthesised by pol III and are required for post-transcriptional processing of rRNA, mRNA and tRNA respectively. Pol III also transcribes short interspersed nuclear elements (SINEs), including Alu genes, of which there are over a million in humans. RNA polymerase IV, which is of mitochondrial origin, is responsible for the transcription of a number of protein-encoding genes (Kravchenko *et al.*, 2005). These polymerases are controlled, so that the co-ordination of gene expression can regulate cell fate (White, 2001).

Pol I and III products are important for protein synthesis and are therefore essential for viability. When cell growth is no longer required, transcription is repressed by the p53, retinoblastoma (RB) and alternative reading frame (ARF) proteins (White, 1998b; Morton *et al.*, 2007). These proteins are targets in cancer where deregulation of pol I and III can occur. A number of tumour and transformed cell types express high levels of pol III products. Pol III-specific transcription factor IIIB (TFIIIB) can be stimulated by transforming agents, such as c-Myc (Gomez-Roman *et al.*, 2003; Kenneth *et al.*, 2007). TFIIIB is bound and activated by oncoproteins such as c-Myc. Normally, c-Myc is involved in cell cycle and cell growth and c-Myc's deregulation is associated with one seventh of cancer deaths in the United States (U.S.) (Dang, 1999).

1.2 RNA polymerases I, II and III in eukaryotic cells

Pol I synthesises only one RNA product, the large ribosomal RNA. A single type of promoter is utilised to synthesise the rRNA molecule, which is processed into 5.8S, 18S and 28S rRNA (Grummt, 2003; Russell & Zomerdijk, 2006). Transcription of rRNA genes occurs in the nucleoli and accounts for 35-65% of all nuclear transcription (Moss & Stefanovksy, 2002). rRNA is exported to the cytoplasm in the form of immature ribosomes where its assembly is finished (Voet & Voet, 1995). High levels of ribosome production are required in order to maintain protein synthesis. Pol I activity is therefore linked with cellular growth, which can become deregulated in cancer. rRNA genes are separated by intergenic spacers where the promoter is present. The core promoter is present about 50 base pairs upstream of the initiation site. Because the efficiency of the promoter is weak it requires the Upstream Binding Factor (UBF), which recruits the selectivity factor 1 (SL1) factor and extends the region of DNA which is covered. Initiation starts when pol I is recruited to the promoter (Paule & White, 2000).

Pol II transcribes the protein encoding-genes (mRNA genes), as well as small nuclear genes (White, 2000). Pol II transcription occurs in the nucleoplasm and accounts for 20% of all nuclear transcription. Pol II transcribes the majority of genes and this is evident in a variety of promoter structures. Promoters have binding sites for transcription factors. The class II gene promoters are located upstream of the initiation site. Factor recognition sites are also located in the transcribed region or downstream of the termination site. Pol II requires six basal transcription factors to start transcription. Initially, TATA binding protein distorts the DNA to create a structure to which the basal transcription factors can bind. Basal transcription is then initiated by pol II.

Pol III transcribes class III genes which encode small RNA molecules, including the 5S rRNA and tRNAs that have important functions in protein synthesis and cell growth (White *et al*., 2001). Pol III transcription accounts for 10-20% of all nuclear transcription (Moss & Stefanovksy, 2002) and pol III could be an important control point in the regulation of growth and proliferation. The location of pol III transcription

has been discovered with the help of electron and confocal microscopy. It was shown that in HeLa cells pol III transcription occurs in about 2000 sites in the nucleoplasm (Pombo *et al.*, 1999). Pol III transcription is regulated by the activities of transcription factors such as TFIIIB and TFIIIC. Also, phosphorylation and dephosphorylation can regulate the activity of transcription factors.

1.3 RNA Polymerase III transcribed genes

RNA polymerase III transcribes genes that encode a number of small RNA molecules that are not translated, and are involved in a number of cellular functions. Table 1.1 lists the functions of these molecules.

Table 1.1: RNA polymerase III gene products and their functions

Pol III products	Known Functions
tRNA	Involved in protein synthesis as a translational adaptor
5S rRNA	Involved in protein synthesis as a component of ribosomes
U6 RNA	Involved in mRNA splicing
H1 RNA	Involved in tRNA processing (Rnase P component)
MRP RNA	Involved in rRNA splicing

7SL RNA	Involved in intracellular protein transport (component of SRP)
7SK RNA	Involved in controlling transcriptional elongation by pol II
SINE transcripts	Possible role in cellular stress responses
VA RNA	Involved in adenovirus translational control
EBER RNA	Involved in Epstein-Barr virus translational control

1.3.1 5S ribosomal RNA

For the synthesis of the ribosome, components from three RNA polymerases are needed. Pol I sythesises the 28S, 18S and 5.8S rRNAs (Doudna & Rath, 2002), pol II transcribes the genes encoding ribosomal proteins and pol III synthesises the 5S rRNA. The four rRNAs are required in equal stoichiometry, one copy of each per ribosome (White, 2001). Similarly to other pol III genes, 5S rRNA is transcribed in the nucleoplasm, however, it is then transferred to the nucleolus where it is incorporated into the large ribosomal subunit (Lafontaine & Tollervey, 2001). 5S rRNA is the smallest rRNA, 121 nucleotides long in humans. The human genome contains 300-400 5S rRNA genes, most of them in clusters of tandem repeats (International Human Genome Consortium, 2004).

1.3.2 Transfer RNAs

tRNAs translate genetic information between the triplet nucleic acid code of mRNA and into a particular amino acid sequence of a protein. Transcribed tRNAs are processed into mature tRNAs between 70-90 nucleotides in length and have a secondary structure as a cloverleaf (Sharp *et al.*, 1984). There is also at least one tRNA per amino acid. In humans there are about 450 tRNA genes which encode 274 different tRNA species (Goodenbour & Pan, 2006). tRNAs are covalently linked to particular amino acids. They contain a three base region, the anticodon, that can base pair to the corresponding base region, codon, on the mRNA nucleotide sequence. This ensures the correct synthesis of the polypeptide chain encoded by the mRNA.

1.3.3 7SL

7SL is a pol III-transcribed gene, which encodes a 300 nucleotide long transcript. It is an essential component of the signal recognition particle (SRP), involved in protein translocation across the endoplasmic reticulum (Walter & Blobel, 1982). The eukaryotic SRP consists of six polypeptides bound to 7SL. 7SL forms the backbone of the SRP and without it the other constituents of the SRP cannot assemble (Walter & Blobel, 1983). 7SL is highly conserved throughout evolution, with the human genome containing four copies (International Human Genome Consortium, 2004).

1.3.4 7SK

7SK is a 330 nucleotide long pol III transcript which is abundant in eukaryotic cells (Mattaj *et al.*, 1993; Murphy *et al.*, 1986). 7SK can function as a negative regulator of the positive transcription elongation factor b (P-TEFb) (Nguyen *et al.*, 2001; Yang *et al.*, 2001). This interaction between 7SK and P-TEFb is crucial for the regulation of transcription of pol II genes (Nguyen *et al.*, 2001; Yang *et al.*, 2001). P-TEFb contains a

carboxyl-terminal domain (CTD) kinase, cyclin-dependent kinase 9 (CDK9), and can promote transcription elongation.

1.3.5 U6 small nuclear RNA

Spliceosomes are large complexes whose role is to remove introns from the pre-mRNAs for the formation of the mature mRNA (Valadkhan, 2005). Spliceosomes consist of five snRNA species, four of which are synthesised by pol II. The smallest, U6, is synthesised by pol III. U6 is the most highly conserved of the spliceosomal RNAs and about 106 nucleotides long (Reddy *et al.*, 1987).

1.3.6 H1 and MRP

H1 is a 369 nucleotide component of ribonuclease (RNase) P, an endoribonuclease that is involved in the processing of the 5'-termini of pre-tRNAs (Bartkiewicz *et al.*, 1989; Lee & Engelke, 1989). The primary sequence of the H1 RNA is not homologous between organisms, however the tertiary structure is highly conserved in order to maintain its function (Morrissey & Tollervey, 1995). MRP is a 265 nucleotide component of an endoribonuclease that has the ability to cleave the mitochondrial transcript to generate an RNA primer for replication of mitochondrial DNA (Chang & Clayton, 1987; Chang & Clayton, 1989). MRP is predominantly found in the nucleolus, where it is involved in the processing of pre-RNA (Clayton, 2001).

1.3.7 Viral genes transcribed by pol III

Several viruses that infect cells can employ pol III to express short transcription units within their genomes. One of the best characterised examples is the adenovirus viral associated I (VAI) and II (VAII) genes which are transcribed by pol III at the late stages of viral infection (Akusjärvi *et al.*, 1980; Söderlund *et al.*, 1976). The VA transcripts stimulate the translation of adenoviral mRNA during the late stages after

infection (Thimmappaya *et al.*, 1982). The Epstein-Barr virus (EBV) contains two genes transcribed by pol III that are expressed in viral infections, the Epstein-Barr virus-encoded RNA 1 (EBER1) and 2 (EBER2) (Rosa *et al.*, 1981). The EBER1 and EBER2 can functionally substitute for the VA genes in adenovirus infection, and therefore they have a similar role in simulating translation (Bhat & Thimmappaya, 1985). EBERs can induce growth in soft agar and can have an oncogenic role (Komano *et al.*, 1999; Ruf *et al.*, 2000).

1.3.8 Short interspersed nuclear elements

Short interspersed nuclear elements are transcribed by pol III and are the majority of pol III templates in mammals. SINEs include the Alu genes found in primates (Jelinek *et al.*, 1980), and the B1 and B2 elements which are found in rodents (Bennett *et al.*, 1984). The B1 genes show a high homology of about 80% to Alu genes and both appear to have evolved from the 7SL gene (Britten, 1994), whereas, the B2 genes are thought to have evolved from tRNA genes (Batzer & Deininger, 2002). Alu genes constitute a significant part of the human genome. There are over a million copies of Alu genes in human cells, providing approximately 10% of the human genome (International Human Genome Consortium, 2001). The B1 and B2 genes in the rat genome are present in approximately 384,000 and 328,000 copies respectively. Although SINEs are abundant, little is known about their function. Many years ago they were regarded as ‘selfish’ DNA (Orgel & Crick, 1980). Later reports have indicated that SINE families are involved in the expression of adjacent genes (Häsler & Strub, 2006). B2 elements are involved in the repression of pol II gene expression after heat shock (Allen *et al.*, 2004; Espinoza *et al.*, 2004), translational regulation (Häsler & Strub, 2006) and control of tumour cell proliferation (Pagano *et al.*, 2007). Therefore, although SINEs initially were regarded as genomic parasites, it appears now that they have important roles.

1.4 Promoters used by RNA polymerase III

Most promoters used by pol III contain crucial elements found within the transcribed region of a gene (White, 1998a). Promoters contain specific elements that direct the recruitment of transcription factors and RNA polymerase (Paule *et al.*, 2000). Three types of pol III promoters exist (White, 1998a) (Figure 1.1). Type I and II are present downstream of the initiation site, whereas type III are present upstream of the site.

1.4.1 Type I promoters

Type I promoters are internal promoters that consist of three elements, which are required for efficient transcription of the gene: the A-block (+50 to +64 bp), the intermediate element (IE) (+67 to +72 bp), and the C-block (+80 to +97), which is known as the internal control region (ICR) (Pieler *et al.*, 1985a; Bogenhagen, 1985). Space between these elements is restricted for effective transcription. Mutations in the A- and C-blocks can reduce transcription (Pieler *et al.*, 1985a). This is in contrast to the flanking regions which display little conservation, showing greater resilience to mutations (Pieler *et al.*, 1985b, Pieler *et al.*, 1987). Type I promoters were initially characterised in *Xenopus laevis* (Bogenhagen *et al.*, 1980) and are unique to 5S rRNA genes (Figure 1.1A).

1.4.2 Type II promoters

Type II promoters contain two important sequence elements: an A-block and a B-block with TFIIIC required for promoter recognition (Figure 1.1B). Most pol III-transcribed genes such as tRNA genes, the adenovirus VA genes and the SINEs contain type II promoters (Bartholomew, 1990). The A-block found in type II promoters is homologous to the type I promoter A-block, and in some species these elements can be interchangeable (Ciliberto *et al.*, 1983). Mutations in the type II elements can lead to

reduced gene expression (Traboni *et al.*, 1984). Typically the A- and B-blocks are separated by 30-40 bp (Baker *et al.*, 1987), however the distance can vary between them and can reach 365 bp, with the blocks still being functional (Fabrizio *et al.*, 1987). The space between the two blocks can increase due to short introns in the coding regions of certain genes (White, 2001).

1.4.3 Type III promoters

Type III promoters differ in that they are external compared to type I and II. They consist of three external elements: a TATA box bound by TFIIIB, which is specific for pol III, a proximal sequence element (PSE) bound by snRNA activator protein complex/PSE-binding transcription factor (SNAPc/PTF) and a distal sequence element (DSE) which is bound by octamer-binding protein 1 (Oct-1) (Schramm & Hernandez, 2002) (Figure 1.1C). Vertebrate U6 (Krol *et al.*, 1987) and 7SK (Murphy *et al.*, 1986), as well as MRP (Topper & Clayton, 1990) and H1 RNA (Baer *et al.*, 1990) genes contain type III promoters. In some cases, genes with type I and II promoters also contain elements upstream of the initiation site that are required for transcription. Surprisingly, yeast U6 snRNA genes have functional A- and B-blocks with homology to type II promoters (Brow & Guthrie, 1990), whereas U6 snRNA genes in humans do not have them, suggesting that the evolution of an extragenic promoter organisation within the pol III system is recent (Paule & White, 2000).

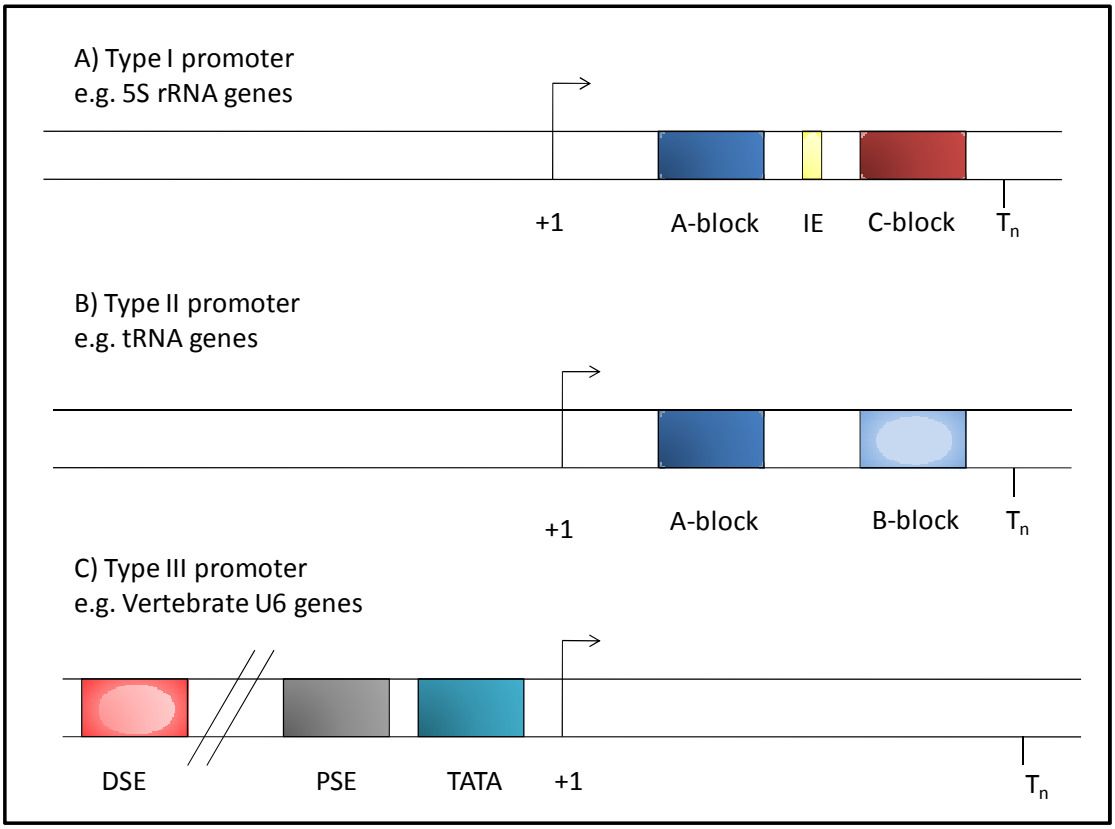

Figure 1.1 Structure of the different types of pol III promoters.

The transcription start site is indicated by +1 and the termination site by T_n (termination). The promoters of the 5S rRNA genes are examples of type I promoters. The promoters of the tRNA genes are examples of type II promoters. The promoters of the U6 snRNA genes are examples of type III promoters. The positions of various promoter elements are displayed: intermediate element (IE), distal sequence element (DSE) and proximal sequence element (PSE). Adapted from White, 2001.

1.5 Transcription Factors used by RNA polymerase III

Transcription by pol III is not specific for a DNA sequence. Pol III requires proteins to position it to the initiation site of a gene. Different promoters require different transcription factors. Type I and II promoters require the assistance of TFIIIB and TFIIIC. Type I promoters also utilise transcription factor IIIA (TFIIIA) (Figures 1.2 and 1.3). Type III promoters can use the SNAPc/PTF, Oct-1, TFIIIC and TFIIIB transcription factors. A way to increase pol III output is by raising the level of transcription factors that it requires.

1.5.1 TFIIIA

Xenopus laevis TFIIIA was the first eukaryotic transcription factor to be purified and cloned (Engelke *et al.*, 1980; Ginsberg *et al.*, 1984). TFIIIA can bind specifically both RNA and DNA and consists of nine zinc fingers that can bind to 5S ribosomal DNA (rDNA) (Miller *et al.*, 1985). TFIIIA can bind to 5S rDNA and then recruit TFIIIC to the promoter. Following the TFIIIC recruitment, TFIIIB binds upstream of the transcription start site which allows recruitment of pol III (Kassavetis *et al.*, 1995; Roberts *et al.*, 1996). TFIIIA is not only a transcription factor, but is also involved in the storage, nuclear export and regulation of the production of 5S rRNA (Guddat *et al.*, 1990; Pieler & Theunissen, 1993).

1.5.2 TFIIIB

The TFIIIB transcription factor can control the production of 5S rRNA and tRNA. TFIIIB is a pol III-specific transcription factor and a determinant of biosynthetic capacity. As a result TFIIIB, is a potential target for tumour suppressors such as p53 and RB, which can inhibit its function (Cairns & White, 1998; White *et al.*, 1996). On the other hand, oncoproteins such as Tax of Human T cell Leukaemia Virus type I (HTLV-

1) can activate TFIIIB (Gottesfeld *et al.*, 1996). Phosphorylation and dephosphorylation can also regulate TFIIIB (Hockman & Schultz, 1996). Deregulation of this factor can be important in tumour progression.

TFIIIB is recruited by TFIIIC, when the latter is assembled on the promoter. TFIIIB consists of TATA box-binding protein (TBP), TFIIB-related factor 1 (Brf1) and B double prime 1 (Bdp1). TBP is a 38 kDa factor which is used by pol I, II and III (Cormack & Struhl, 1992; White *et al.*, 1992a,b). The carboxyl-terminal (C-terminal) domain is widely conserved between species, but the amino-terminal (N-terminal) domain is more species-specific. TBP-Brf1 complexes have been found that are not bound to DNA. One of the characteristics of TBP is that it can bend DNA alone or in a complex with Brf1 (Braun *et al.*, 1992a). TBP can bind to a TATA-box in type III promoters without Brf1 or Bdp1 (Hernandez, 2001). However, Brf1 increases the affinity of TBP for DNA complexes (Librizzi *et al.*, 1998).

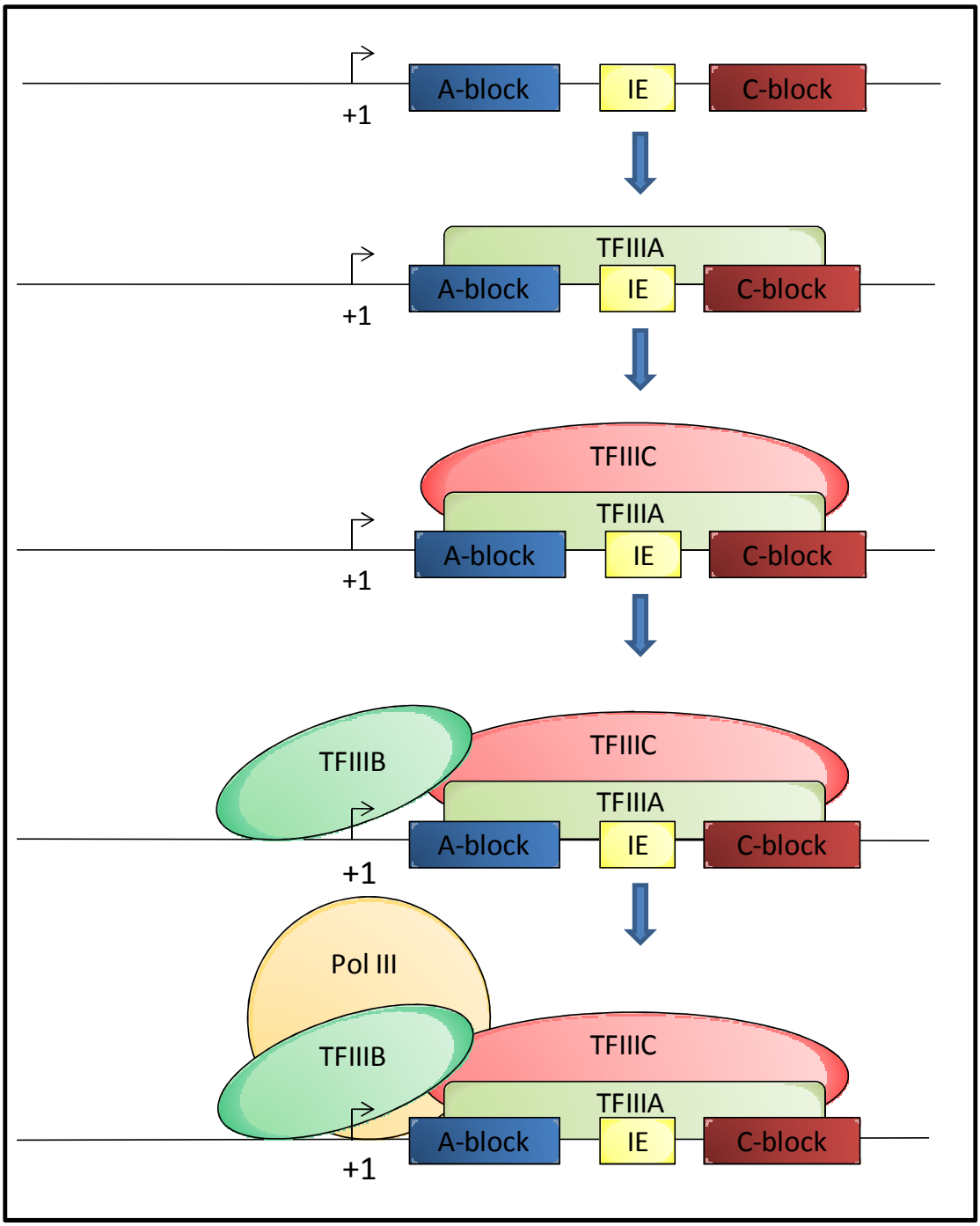

Figure 1.2 Transcription complex assembly on a type I promoter.

The transcription factor TFIIIA binds to the internal promoter. TFIIIC is then recruited, followed sequentially by TFIIIB and pol III. Once pol III is recruited, transcription is initiated at the transcriptional start site +1.

Type III promoters in mammalian cells use an alternative TFIIIB complex. In this case the TFIIIB complex consists of TBP, Bdp1 and a Brf family factor known as TFIIB-related factor 2 (Brf2) (Schramm *et al.*, 2000). In order to distinguish the two different complexes, the complexes consisting of TBP, Bdp1 and Brf2 are referred to as TFIIIBα, and the complexes consisting of TBP, Bdp1 and Brf1 referred to as TFIIIBβ (Schramm *et al.*, 2000). The C-terminal domain of TBP recruits Brf2 to the TATA box of the U6 gene (Cabart & Murphy, 2001). If Brf2 is depleted, this impairs the transcription of U6 genes, and even if recombinant Brf2 is added transcription cannot be reconstituted (Teichmann *et al.*, 2000). This is an indication that possible Brf2-associated polypeptides are required for transcription.

TFIIIB has also been shown to be a target of various tumour suppressors, such as RB and p53. Overexpression of RB can inhibit pol III transcription (White *et al.*, 1996; Chu *et al.*, 1997; Larminie *et al.*, 1997; Hirsch *et al.*, 2000). This occurs because RB binds to TFIIIB, which can block the interaction with TFIIIC and pol III (Larminie *et al.*, 1997; Sutcliffe *et al.*, 2000). RB knockout mice show increased levels of pol III transcription and high levels of tRNA and 5S rRNA synthesis when compared with wild type cells (White *et al.*, 1996; Scott *et al.*, 2001). Therefore, inactivation of RB will derepress TFIIIB and will increase pol III transcription. It is thought that RB must be compromised for cancer development. This can occur by mutations in the RB gene. In most cases these mutations are present in the large pocket of RB (Harbour, 1998). This domain is important for RB to bind to TFIIIB and repress pol III activity (White *et al.*, 1996; Chu *et al.*, 1997; Larminie *et al.*, 1997). Also many DNA tumour viruses encode oncoproteins that neutralise RB by binding to its pocket (Vousden, 1995). These are the E7 product of human papilloma virus (HPV), the large T antigen of simian virus 40 (SV40) and the E1A product of adenovirus, all of which can release TFIIIB from repression and activate pol III transcription (White *et al.*, 1996; Larminie *et al.*, 1999; Sutcliffe *et al.*, 1999). One of the most common mechanisms by which RB loses its function in tumours is by deregulated hyperphosphorylation by cyclin-D and E-dependent kinases (Hunter & Pines, 1994; Sherr, 2001). These kinases have the ability to stimulate pol III transcription because phosphorylated RB is not able to bind to

TFIIIB (Scott *et al.*, 2001). Therefore it has been shown that derepression of TFIIIB can result from mechanisms that control RB function.

TFIIIB is also repressed by p53 (Cairns & White, 1998; Crighton *et al.*, 2003). When bound by p53, TFIIIB cannot associate with TFIIIC or become recruited to pol III templates (Crighton *et al.*, 2003). As a result, p53 activation will cause a decrease in TFIIIB activity and pol III transcription. It has been shown that in p53 knockout mice, synthesis of 5S rRNA and tRNA increases (Cairns & White, 1998; Crighton *et al.*, 2003). Oncoproteins such as human double minute 2 (HDM2) and HPV E6 can target p53 for degradation and derepress a pol III reporter (Stein *et al.*, 2002). Mutations in p53 are found in half of human cancers and 75% of these contain substitutions in the central core domain (Hollstein *et al.*, 1994). Therefore, these (Cairns & White, 1998) experimental data indicate that loss of p53 will contribute to derepression of TFIIIB in a large number of cancers.

TFIIIB is also a target for a number of oncoproteins that can stimulate its activity. c-Myc can interact with TFIIIB in humans and in mice and have a stimulatory effect on pol III transcription (Gomez-Roman *et al.*, 2003). Chromatin immunoprecipitation (ChIP) experiments have shown that endogenous c-Myc is present at pol III-tranascribed genes in transformed and untransformed cells (Felton-Edkins *et al.*, 2003a; Gomez-Roman *et al.*, 2003). The oncogenic kinase casein kinase II (CK2) can bind and phosphorylate TFIIIB in yeast and mammals (Hockman & Schultz, 1996). It can cause lymphomas in mice and is hyperactive in various human cancers (Notterman *et al.*, 2001). Another oncogenic kinase which has been shown to stimulate the assembly of pol III transcription complexes is the extracellular signal-regulated kinase (ERK) (Felton-Edkins *et al.*, 2003b). This occurs due to the binding of ERK to the Brf1 subunit of TFIIIB, which becomes phosphorylated (Felton-Edkins *et al.*, 2003b). One of the components of TFIIIB is TBP and TBP expression was shown to be raised by hepatitis B virus (HBV) and Ras activation. Also, high levels of TBP are sometimes found in colon cancer (Johnson *et al.*, 2003). Nuclear pol I, II and III use TBP, so increased levels will likely have an effect on a number of genes (Hernandez, 1993). One of the subunits of TFIIIB, Bdp1 has been shown to be expressed in high levels in cell lines transformed by polyomavirus (Felton-Edkins & White, 2002). The

third subunit of TFIIIB, Brf1 was found to have elevated expression in cervical carcinomas (Daly *et al.*, 2005).

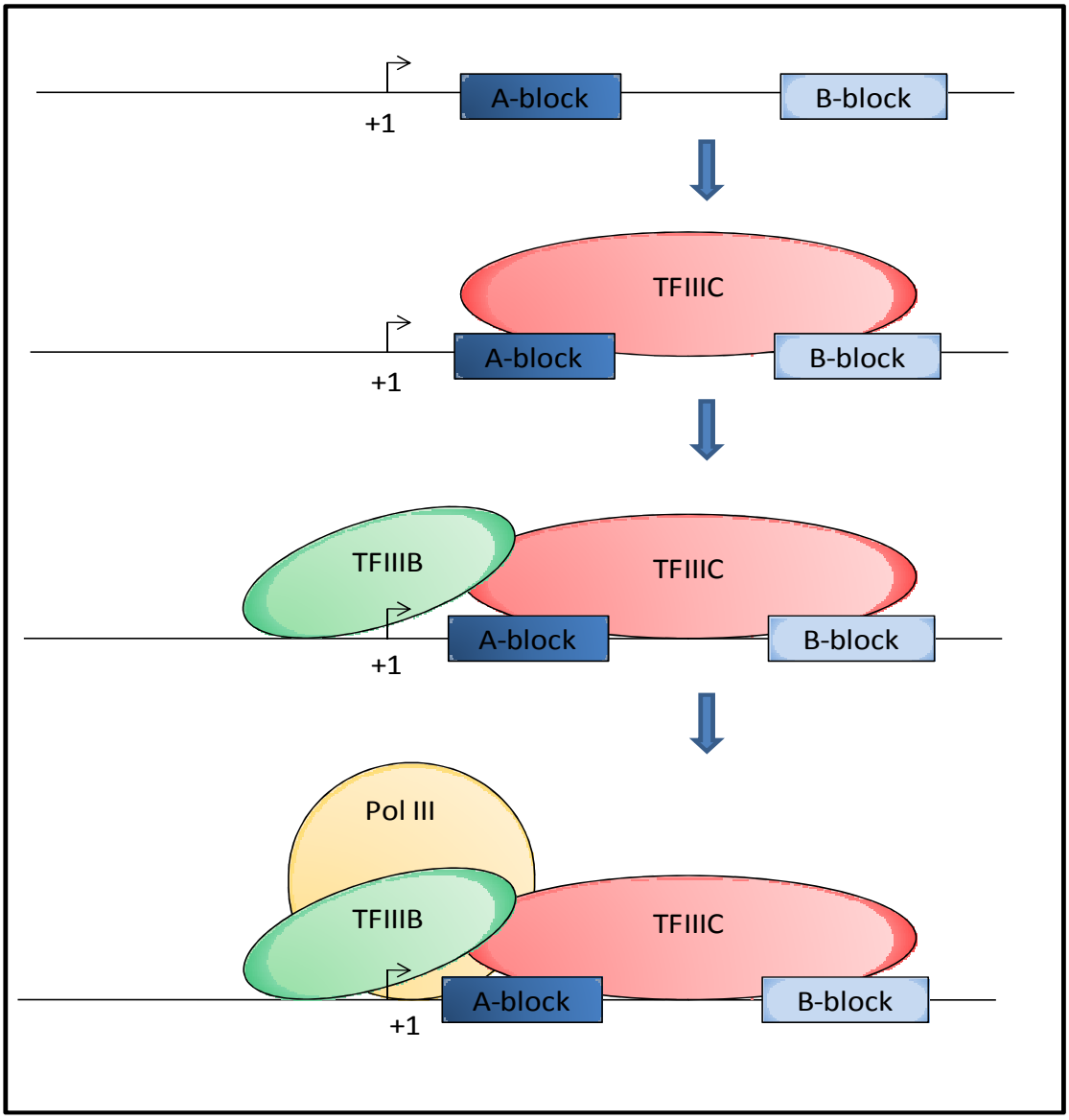

Figure 1.3 Transcription complex assembly on a type II promoter.
Transcription factor TFIIIC binds to the A- and B-block on the promoter. Once TFIIIC is bound to DNA it recruits TFIIIB by protein-protein interactions. TFIIIB subsequently recruits pol III and transcription is initiated. The transcription start site is indicated by +1.

1.5.3 TFIIIC

TFIIIC in *Saccharomyces cerevisiae (S. cerevisiae)* consists of six subunits, TFC1, TFC3, TFC4, TFC6, TFC7 and TFC8, with a cumulative mass of 520 kDa (Manaud *et al.*, 1998; Deprez *et al.*, 1999; Geiduschek & Kassavetis, 2001). These six subunits form two domains: the τ_A which consists of TFC1, TFC4 and TFC7 that recognises the A-block, and the τ_B which consists of TFC3, TFC6 and TFC8, that recognises the B-block (Huang & Maraia, 2001). TFC8 allows TFIIIC to contact both blocks. When the space between the blocks of DNA is long, TFIIIC can loop out the DNA in order to bind the two blocks simultaneously (Schultz et al., 1989). Human TFIIIC consists of six polypeptides: TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90, TFIIIC63 and TFIIIC35 (Kovelman & Roeder, 1982; Dumay-Odelot *et al.*, 2007). All the subunits are required for pol III transcription of class III genes consisting of type I and II promoters.

The TFIIIC220 subunit binds to the B-block (L'Etoile *et al.*, 1994). The C-terminal domain interacts with TFIIIC102 and TFIIIC63, whereas the N-terminal domain interacts with TFIIIC110 (Shen *et al.*, 1996). TFIIIC110 is the second largest subunit of TFIIIC and was isolated using a complementary DNA (cDNA) library from a human myeloid cell line (Sinn *et al.*, 1995). A blast search has revealed sequence homology between human TFIIIC110 and TFC6 from *S. cerevisiae* (Huang *et al.*, 2000). The TFIIIC110 gene is located on chromosome 2. The TFIIIC102 gene is located on chromosome 9 and has the highest homology of all the human subunits with the yeast counterpart TFC4 subunit (Hsieh *et al.*, 1999a). The N-terminal domain consists of acidic regions, a helix-loop-helix (HLH) motif and contains 11 tetratricopeptide repeats (TPRs) (Hsieh *et al.*, 1999a). Through its TPRs, TFIIIC102 associates with TFIIIC63 and TFIIIC90 and can recruit TFIIIB to the promoter by its interaction with both Brf1 and Bdp1. The yeast counterpart TFC4 interacts with Brf1, and this interaction is mediated by the first 168 residues of TFC4. Another subunit, TFIIIC90, was cloned with the use of a cDNA library (Hsieh *et al.*, 1999b). The TFIIIC90 subunit can specifically acetylate the K14 on histone H3. Pol III interaction with TFIIIC90 has also been shown (Hsieh *et al.*, 1999b). It acts as a linker between the 220 and 110 subunits that are

located on the B-block and the 63 and 102 subunits that are present on the A-block. The TFIIIC90 gene is located on chromosome 9. The gene of the TFIIIC subunit TFIIIC63 is also located on chromosome 9. TFIIIC63 interacts with TFIIIC102, TFIIIC90 and the pol III subunit C62 (Hsieh *et al.*, 1999a). The TFIIIC63 protein contains a central helix-turn-helix domain and a C-terminal acidic region.

In order to increase pol III output, the levels of some of its transcription factors can be raised. It has been shown that adenoviral infection can raise the levels of TFIIIC (Hoeffler & Roeder, 1985). Five of the subunits of TFIIIC are overexpressed at both the mRNA and protein levels in 3T3 fibroblast cells transformed by polyomavirus (Felton-Edkins & White, 2002). Elevated levels of TFIIIC were found in each of nine human ovarian cancers compared to control tissue (Winter *et al.*, 2000).

1.5.4 SNAPc/PTF and Oct-1

U6 and 7SK genes, which contain type III promoters, are expressed by transcriptional complexes containing TFIIIBα but not TFIIIC (Figure 1.4). The PTF, also called SNAPc, is a factor of five subunits which interacts with the PSE (Schramm *et al.*, 2000; Teichmann *et al.*, 2000). The initiation complex is assembled with the binding of the PSE by SNAPc (Henry *et al.*, 1996; Wong *et al.*, 1998). The SNAP43 and SNAP19 interact with the N-terminal domain whereas the SNAP45 associates with the C-proximal region of SNAP190 (Mittal *et al.*, 1999). Oct-1 binds the upstream DSE to make contact with SNAPc and stimulates promoter occupancy by protein-protein interactions (Mittal *et al.*, 1996; Murphy *et al.*, 1992). Oct-1 contains activation domains and a POU domain. The POU domain contains a bipartite DNA binding domain which consists of two joined helix-turn-helix structures, the N-proximal POU specific and the C-proximal POU homeodomain (Herr & Cleary, 1995). SNAPc can also regulate the binding of TBP to DNA. Oct-1 is not essential for basal transcription (Hu *et al.*, 2003). Assembly of the TFIIIB/$SNAP_c$ complex at the type III promoters recruits pol III.

1.6 RNA Polymerase III

Pol III is the largest and most complex of the eukaryotic nuclear RNA polymerases. Pol III consists of 17 subunits in yeast and human, all of which are essential in yeast, and has an aggregate mass of 600-700 kDa (Geiduschek & Kassavetis, 2001; Schramm & Hernandez, 2002; White, 2001). Pol III shares common subunits with pol I and pol II. Of the 17 subunits, ten are unique to pol III, five are common to pol I, II and III and two are common to pol I and pol III (Schramm & Hernandez, 2002; White, 2001). The 17 subunits are essential for cell viability and function (Chédin *et al.*, 1998; Ferri *et al.*, 2000). The 17 subunits range from 10 to 160 kDa. The ten subunits which are unique to pol III are the following: C11, C17, C25, C31, C34, C37, C53, C82, C128 and C160. The five which are shared by the three polymerases are the following: ABC 10α, ABC 10β, ABC 14.5, ABC23, ABC27. The two common subunits in pol I and pol III are AC19 and AC40 (Bréant et al., 1983; Buhler et al., 1980; Chédin et al., 1998). The two largest polypeptides of pol III are homologous to the two largest subunits of pol I and pol II. The conserved subunits together with the shared subunits are thought to form the RNA polymerase catalytic core (White, 2001). The unique subunits are likely to perform other roles such as interaction with transcription factors (Geiduschek & Kassavetis, 2001). Therefore, it is no surprise that the three RNA polymerases carry out a similar role, transcribing a DNA template to produce a complementary RNA strand.

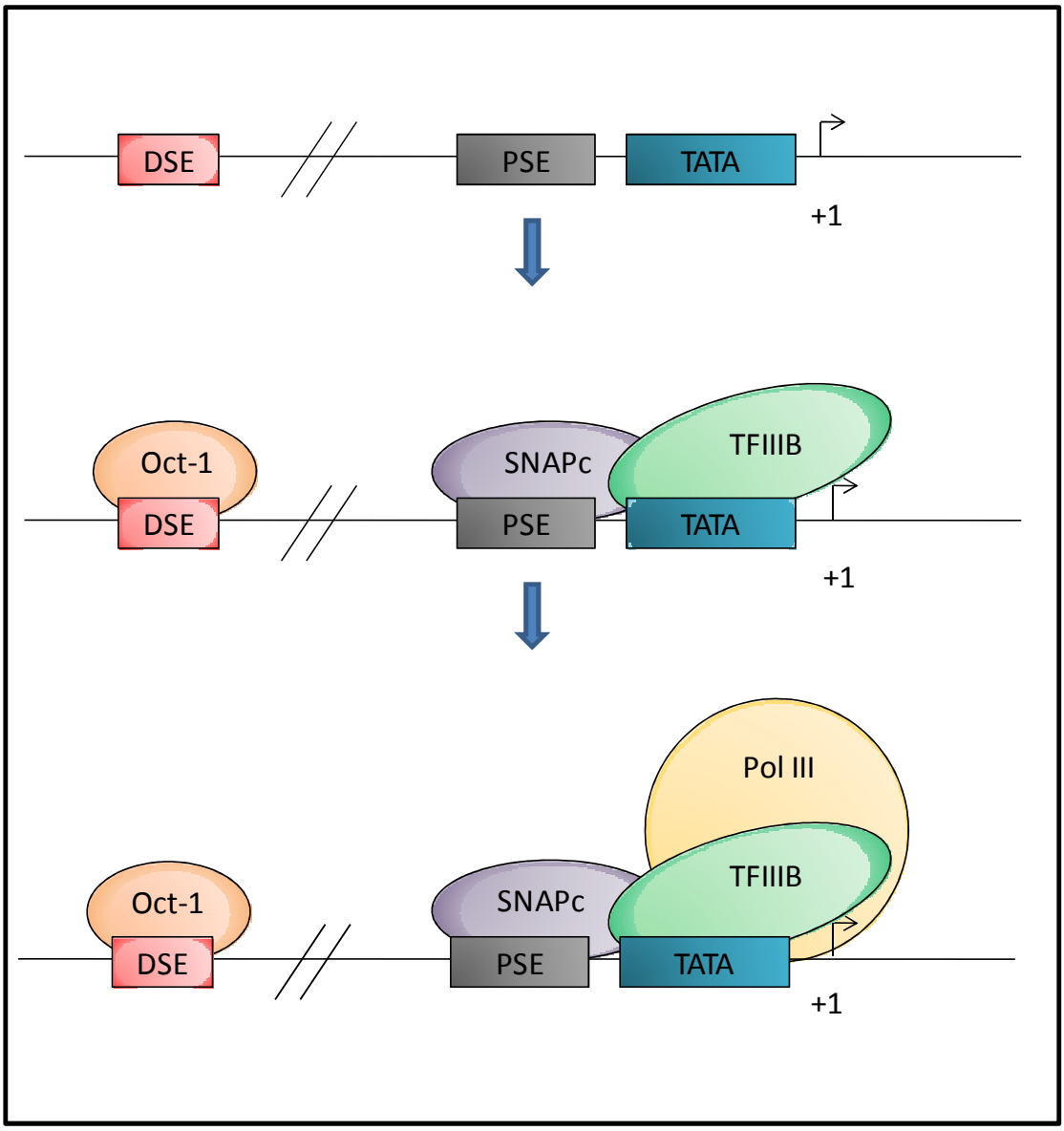

Figure 1.4 Transcription complex assembly on a type III promoter.

TFIIIB and SNAPc cooperatively bind to the TATA box and PSE respectively on type III promoters. Binding of Oct-1 to the upstream DSE enhances SNAPc/TFIIIB recruitment. Pol III binds and transcription is initiated following the SNAPc/TFIIIB recruitment. The transcription start site is indicated by +1.

1.7 Transcription by RNA polymerase III

1.7.1 Transcript initiation

Pol III recruitment to TATA-less promoters is well characterised in yeast, where several steps are involved. Initially, TFIIIC binds to the promoter. The locations of TFIIIC subunits have been found by photocrosslinking to chemical probes (Bartolomew, 1990). Binding to tDNA (DNA encoding tRNA) is ensured by TFC3 and TFC6 interacting with the B-block (Arrebola *et al.,* 1998). TFC1 and TFC7 bind to the A-block at opposite sites of the DNA helix (Bartolomew, 1990). TFC4 binds DNA upstream of the initiation site, which is occupied by TFIIIB, and downstream between the A- and B-blocks. Finally, TFC8 is not thought to bind DNA.

When TFIIIC binds to tDNA it recruits TFIIIB. Initially, Brf is recruited to DNA, by interacting with TFC4 (Moir *et al.,* 2000). Brf recruits TBP which is bound to it. This stabilises the TFIIIC-DNA complex by enhancing the cross-linking of TFC4 to DNA. When the Brf/TBP-TFIIIC-DNA complex has been formed, Bdp1 enters the complex. TFC4 and Brf1 recruit Bdp1 (Dumay-Odelot *et al.,* 2002; Ishinguro *et al.,* 2002). TFIIIC is essential in determining the position that TFIIIB occupies at the tDNA. TBP can scan a region to choose an optimal site, where it occupies a region about 40 bp upstream of the transcription start site (Braun *et al.,* 1992b). Bdp1 and Brf1 are present at opposite sides of the TBP-DNA core. Brf binds the TBP-DNA complex 15 bp downstream of the TATA-like sequence and 1 bp upstream of it through its C-terminal region (Colbert *et al.,* 1998). The N-proximal segment of Bdp1 cross-links to the upstream end of the TFIIIB-DNA complex 8 bp upstream of the TATA-like sequence (Shah *et al.,* 1999).

Pol III is recruited to the initiation site when the TFIIIC-TFIIIB-tDNA complex is formed. However, TFIIIB alone is capable of recruiting pol III. This was evident from the stability of the TFIIIB-DNA complex in yeast, where high concentrations of salt or heparin cannot dissociate the complex. On the contrary, TFIIIA and TFIIIC are easily removed (Kassavetis *et al.,* 1990). Following these treatments, pol III can still be recruited to class III genes by TFIIIB (Kassavetis *et al.,* 1990). Pol III is recruited

through Brf-C34 interactions, however Brf-C17 and TBP-C34 interactions can also occur (Wang & Roeder, 1997; Ferri *et al.*, 2000). At the tDNA, C34 binds upstream at -17 bp and -3/-2 bp of the initiation site of the non-transcribed strand, whereas C53 makes contact near the downstream end (Persinger & Bartholomew, 1996; Tate *et al.*, 1998). When pol III binds to DNA, the double stranded DNA melts and a bubble forms that extends from -11 bp and propagates downstream of the initiation site. The double stranded DNA is separated spontaneously and, unlike pol II, without ATP hydrolysis (Kassavetis *et al.*, 1992). Brf unpairs DNA around the initiation site, whereas Bdp1 is involved in unpairing the upstream sequences (Kassavetis *et al.*, 2001).

1.7.2 Transcript elongation, termination and reinitiation

As pol III moves into the gene, the bubble of melted DNA moves with it (Kassavetis *et al.*, 1992). TFIIIC and TFIIIA are present in regions that will be transcribed by pol III, but this does not affect transcription by the enzyme. This leads to an important question on how pol III can transcribe through the DNA when factors such as TFIIIC and TFIIIA are stably bound to it, covering the entire gene without being removed after several rounds of transcription (Bogenhagen *et al.*, 1982; Jahn *et al.*, 1987). A possibility is that pol III transiently displaces a particular factor during transcription, but the factor remains associated with another factor, and because of protein-protein interactions remains bound to DNA sites (White, 2001). The TFIIIC obstacle delays pol III by 0.2 seconds at a site upstream of the B-block without affecting total transcription rates. If, however, pol III transcribes in the antisense direction, TFIIIC will pause for 9 seconds before continuing through the B-block (Bardeleben *et al.*, 1994). tRNA elongation in yeast at 20°C occurs at a rate of about 20 nt/second (Matsuzaki *et al.*, 1994).

Whereas pol I and II require accessory factors to terminate transcription, pol III can recognise termination sites accurately without help from other factors. While pol III is sufficient for termination, additional factors may be involved in human cells. Pol III transcription usually terminates at simple clusters of four or more thymidine (T) residues (Bogenhagen & Brown, 1981). The region surrounding the T cluster can affect the

termination signal recognition by pol III (Braglia *et al.*, 2005). Pol III does not dissociate from the template after the synthesis of the first transcript, allowing multiple rounds of transcription (Jahn *et al.*, 1987). TFIIIB and TFIIIC are involved in polymerase recapture and re-initiation of transcription (Ferrari & Dieci, 2008; Ferrari *et al.*, 2004). Because of the bending of DNA by pol III transcription factors, re-attachment of pol III to the transcription unit is more rapid. During multiple rounds of tRNA gene transcription, synthesis occurs every 35 seconds, whereas initiation of the first transcript takes about 5 minutes (Dieci & Sentenac, 1996).

1.8 The cell cycle

In order for a cell to proliferate it has to increase its mass as well as its DNA content. The cell cycle is a period between two mitotic divisions. Cell growth is a continuous process, whereas DNA replication occurs at a specific point. The cell cycle is comprised of four phases. During the M phase or mitosis, a cell divides into two cells. In the G1 phase, which follows the mitotic phase, RNA and proteins continue to be synthesised. During the S phase, the DNA is replicated. In the G2 phase, the cell continues to grow until the next mitosis begins. Each cell cycle phase is controlled by proteins expressed periodically and this regulation occurs at the transcriptional level (Lewin, 2000).

1.9 Cell growth and RNA polymerase III

Normal cell proliferation requires an equilibrium between cell growth and the cell cycle. Cells grow by increasing their levels of protein and macromolecules (Baxter & Stanners, 1978). Since 80-90% of the dry mass of a cell is protein, the growth rate is proportional to the rate of accumulation of protein (Zetterberg & Killander, 1965). Even a 50% decrease in protein synthesis will cause cells to exit the cell cycle and quiesce (Brooks, 1977). Therefore, an increase in protein synthesis is essential for cell growth.

Ribosomes and tRNAs are part of the translational machinery and their availability is important for the rate of translation. Because 5S rRNA and tRNA are pol III products, increased pol III transcription is essential for cell growth. In yeast it has been demonstrated that reduced levels of the initiator tRNA affects cell growth and prolongs cell doubling time (Francis & Rajbhandary, 1990). Recently, it was shown that increased levels of $tRNA_i^{Met}$ synthesis can stimulate cell proliferation and oncogenic transformation (Marshall et al., 2008). Pol I and pol III are required to maintain high rates of transcription to sustain ribosome synthesis and therefore protein production (Clarke *et al.*, 1996). The discovery of the requirement for high levels of pol I and pol III transcription came from studies that restricted rRNA and tRNA synthesis. Halving the concentration of $tRNA_i^{Met}$ can cause a threefold increase in cell mass doubling time, as was demonstrated in *S. cerevisiae* (Francis & Rajbhandary, 1990).

1.10 Regulation of pol III transcription

Pol III transcription is linked to cell growth (increase in cell mass), which is important for cell proliferation and leads to an increase in cell number. Pol III is responsible for the transcription of tRNA and 5S rRNA, which are required for protein synthesis and growth. The level of pol III transcription varies according to the requirements of the cell. When the cells are stimulated by serum it has been shown that pol III can be activated (Johnston *et al.*, 1974; Mauck & Green, 1974). To increase pol III output, the levels of the transcription factors that are limiting pol III transcription can be raised (White, 2001). In the following sections, a number of factors that regulate pol III transcription will be discussed.

1.10.1 Overexpression of pol III transcription factors

Overexpression of the basal transcription factors TFIIIB or TFIIIC can occur in transformed cells. The three subunits of TFIIIB have been found at elevated levels in transformed cell types (Felton-Edkins & White, 2002; Larminie *et al.*, 1999). TBP can

be induced following infection with hepatitis B virus (Wang *et al.*, 1997). TBP is also expressed at high levels in colon carcinomas (Johnson *et al.*, 2003). The Brf1 subunit has been found at elevated levels in cervical carcinomas (Daly *et al.*, 2005). TFIIIC overexpression can result following transformation by simian virus 40, polyomavirus, adenovirus infection and Epstein-Barr virus (White *et al.*, 1990; Felton-Edkins & White, 2002; Hoeffler *et al.*, 1988; Felton-Edkins *et al.*, 2006). Also, a study comparing human ovarian carcinomas relative to healthy adjacent tissue, revealed elevated levels of TFIIIC and pol III transcripts in the ovarian tumours (Winter *et al.*, 2000).

1.10.2 Repressors of pol III transcription

1.10.2.1 The retinoblastoma protein

The retinoblastoma protein is a 105 kDa nuclear phosphoprotein that is ubiquitously expressed in normal mammalian cells (Knudsen & Knudsen, 2006). Overexpression of RB can inhibit pol III transcription (White *et al.*, 1996; Chu *et al.*, 1997; Larminie *et al.*, 1997; Hirsch *et al.*, 2000). RB knockout mice show increased levels of pol III transcription and high levels of tRNA and 5S rRNA synthesis when compared with wild type cells (White *et al.*, 1996; Scott *et al.*, 2001). RB is involved in controlling the cell cycle if growth factors are limiting. The RB protein regulates the cell cycle by preventing the passage of cells from G1 into S phase. This occurs due to RB's ability to bind and repress several transcription factors, such as TFIIIB and E2F. When TFIIIB is bound to RB it is unable to interact with Pol III and TFIIIC and remains inactive (Sutcliffe *et al.*, 2000). RB binds and represses TFIIIB only in the underphosphorylated form which occurs during the G0 and early G1 phase (Scott *et al.*, 2001). Increased tRNA synthesis occurs at the G1/S transition, which corresponds with hyperphosphorylation of RB by cyclin-dependent kinases.

1.10.2.2 p53

p53 is a tumour suppressor protein that inhibits pol III transcription. The p53 protein is lost or mutated in more than 50% of human cancers and 75% of these mutations comprise substitutions in the central core domain (Hollstein *et al.*, 1991; Hollstein *et al.*, 1994; Vousden, 2000). p53 is induced by hypoxia and oncogenic stimuli which can lead to cell death or cell cycle arrest (Vousden & Lu, 2002). p53 interacts with TFIIIB and sequesters it away from the promoter, inhibiting pol III transcription (Crighton *et al.*, 2003). This interaction occurs through TBP. The binding of p53 to TFIIIB blocks the interaction of TFIIIB with TFIIIC (Crighton *et al.*, 2003). Also, high levels of tRNA and 5S rRNA are present in fibroblasts from p53-knockout mice (Cairns & White, 1998). Fibroblasts from patients with Li-Fraumeni syndrome, that inherit a mutated p53 allele, also exhibit high levels of pol III transcription (Stein *et al.*, 2002). p53 can be neutralised by the HPV E6 or HDM2, which leads to elevated levels of pol III transcription (Stein *et al.*, 2002). Therefore, experimental data indicate that loss or mutation of p53 will contribute to derepression of TFIIIB in a large number of cancers.

1.10.2.3 Maf1

Maf1 has been shown to negatively regulate pol III transcription (Goodfellow *et al.*, 2008; Johnson *et al.*, 2007; Upadhya *et al.*, 2002). Pol III transcription is repressed by a variety of signalling pathways that are activated in response to nutrient deprivation, DNA damage and secretory defects (Upadhya *et al.*, 2002; Desai *et al.*, 2005). Repression of pol III transcription requires Maf1 to incorporate the different signals and regulate the polymerase recruitment (Desai *et al.*, 2005; Willis & Moir, 2007). Maf1 homologues are found in mammals and plants (Pluta *et al.*, 2001). Also, human Maf1 directly binds tRNA promoters and interacts with TFIIIB and pol III (Johnson *et al.*, 2007). Maf1 can be phosphorylated or dephosphorylated under various conditions (Oficjalska-Pham *et al.*, 2006). Maf1 is dephosphorylated under repressing conditions and accumulates in the nucleus, where it represses pol III transcription.

1.10.3 Activators of pol III transcription

1.10.3.1 Myc

Myc plays an important role as a regulator of tumourigenesis in human cancers (Schmidt, 1999). Inhibiting Myc significantly affects tumour growth and proliferation (Ponzielli *et al.*, 2005; Stewart *et al.*, 2001; Stewart *et al.*, 2002). c-myc N-Myc and L-Myc show oncogenic activity in cancers (Marcu *et al.*, 1992; Nau *et al.*, 1985). Under physiological conditions, these three oncoproteins are expressed in foetal development. c-myc is an immediate-early response gene whose expression is important for G1/S progression (Heikkila *et al.*, 1987).

The c-myc oncogene encodes the c-Myc transcription factor and was first identified as the cellular homologue of the viral oncogene v-myc of the avian myelocytomatosis retrovirus (Vennstrom *et al.*, 1982). Elevated levels or deregulation of c-Myc have been detected in a wide range of cancers and is often associated with aggressive and poorly differentiated tumours. These cancers include, breast, colon, cervical, small cell lung carcinomas, osteosarcomas, glioblastoma, melanoma and myeloid leukaemias (Dang, 1999; Nesbit *et al.*, 1999). c-Myc not only functions as a regulator of gene transcription of RNA pol II-transcribed genes, but is involved in the regulation of RNA pol I and III-transcribed genes. Chromatin immunoprecipitation assays have shown that c-Myc is present at chromosomal 5S rRNA, tRNA and B2 genes in untransformed fibroblast cells, ovarian epithelial cells and in the HeLa transformed cervical cell line (Felton-Edkins *et al.*, 2003a; Gomez-Roman *et al.*, 2003). This recruitment occurs due to protein-protein interactions of TFIIIB with the N-terminal transactivation domain of c-Myc, which leads to direct activation of transcription. Experiments using RNA interference to knockdown c-myc in HeLa cells and c-myc knockout rat fibroblasts showed that the expression of the 5S rRNA, tRNA and B2 genes was compromised (Felton-Edkins *et al.*, 2003a; Gomez-Roman *et al.*, 2003). These data suggest that c-myc contributes significantly to the level of pol III transcription in mammalian cells. Experimental data show that Myc and Myc associated factor X (Max) bind at non canonical E-box sequences located within the ribosomal DNA. This is

followed by the recruitment of transactivation/transformation domain associated protein (TRRAP), enhanced histone acetylation, recruitment of pol I and activation of rDNA transcription (Grandori *et al.*, 2005).

1.10.3.2 CK2

CK2 is a ubiquitous active Ser/Thr protein kinase with a high number of substrates that is implicated in cellular growth and proliferation (Meggio & Pinna, 2003). CK2 has been found to activate pol III transcription by binding to TFIIIB (Johnston et al., 2002). CK2 binds directly to and phosphorylates Brf1, which causes recruitment of TFIIIB to TFIIIC. Overexpression of CK2 is associated with human cancers, including breast (Landesman-Bollag *et al.*, 2001), head and neck (Faust *et al.*, 1996) and prostate (Yenice *et al.*, 1994). CK2 appears to have oncogenic activity in transgenic mice (Seldin & Leder, 1995).

1.10.3.3 ERK

Several kinases have been demonstrated to have a direct role in regulating pol III transcription. They respond to mitogenic stimuli through a signalling pathway and one example is the mitogen activated protein (MAP) kinase cascade. Induction of this pathway causes activation of ERK. ERK responds through a signalling cascade that involves the Ras-Raf-Mek pathway (Downward, 2003). Ras mutations are found in about 20% of human cancers (Downward, 2003). Because of the mutations in Ras and Raf and other abnormalities, the ERK kinases are hyperactive in 30% of cancers (Downward, 2003). ERK can also interact with and phosphorylate the Brf1 subunit of TFIIIB. This results in stimulating transcription of tRNA and 5S rRNA genes (Felton-Edkins *et al.*, 2003b; Goodfellow & White, 2007).

1.11 The inhibitor of differentiation (ID) family of helix-loop-helix transcription factors

1.11.1 The ID family

The ID family of helix-loop-helix proteins function as positive regulators of cell growth and as negative regulators of differentiation. The human prototype of a subfamily of these proteins was discovered nearly 20 years ago (Benezra *et al.*, 1990). It was named ID1, which relates to its functional properties as both an inhibitor of DNA binding and an inhibitor of differentiation. Mammalian genes that encode for other ID proteins were discovered by homology cloning (ID2, ID4) (Sun *et al.*, 1991; Biggs *et al.*, 1992; Riechmann *et al.*, 1994) or as mitogen-responsive early response genes (ID3) (Christy *et al.*, 1991; Deed *et al.*, 1993). Homologues of ID genes have been identified in *Drosophila* (Ellis *et al.*, 1990; Garrell & Modohell, 1990), *Xenopus* (Wilson & Mohun, 1995) and zebrafish (Sawai & Campos-Ortega, 1997). The four members of the ID family (ID1, ID2, ID3 and ID4) in humans are all present on different chromosomes (20q1, 2p25, 1p36 and 6p22-21 respectively) (Mathew *et al.*, 1995; Deed *et al.*, 1994; Pagliuca *et al.*, 1995). The HLH family of transcription factors consists of >200 members (Norton, 2000). Basic HLH transcription factors can bind DNA as homodimers or heterodimers. The ID proteins lack a basic DNA binding domain and function as dominant-negative regulators of basic HLH proteins by forming inactive ID-bHLH (basic HLH) heterodimers. ID family proteins interact with the ubiquitously expressed bHLH E-protein transcription factors that include HEB, E2-2, and the E2A gene products, E47 and E12, which regulate differentiation linked genes (Massari & Murre, 2000) (Figure 1.5). ID proteins interact with other proteins such as the RB protein (Iavarone *et al.*, 1994), the ETS (E twenty-six) proteins and the Pax (paired box) DNA binding proteins (Iavarone & Lasorella, 2004; Roberts *et al.*, 2001; Yates *et al.*, 1999). In this way, the cell can regulate proliferation and differentiation by altering the activity of one gene.

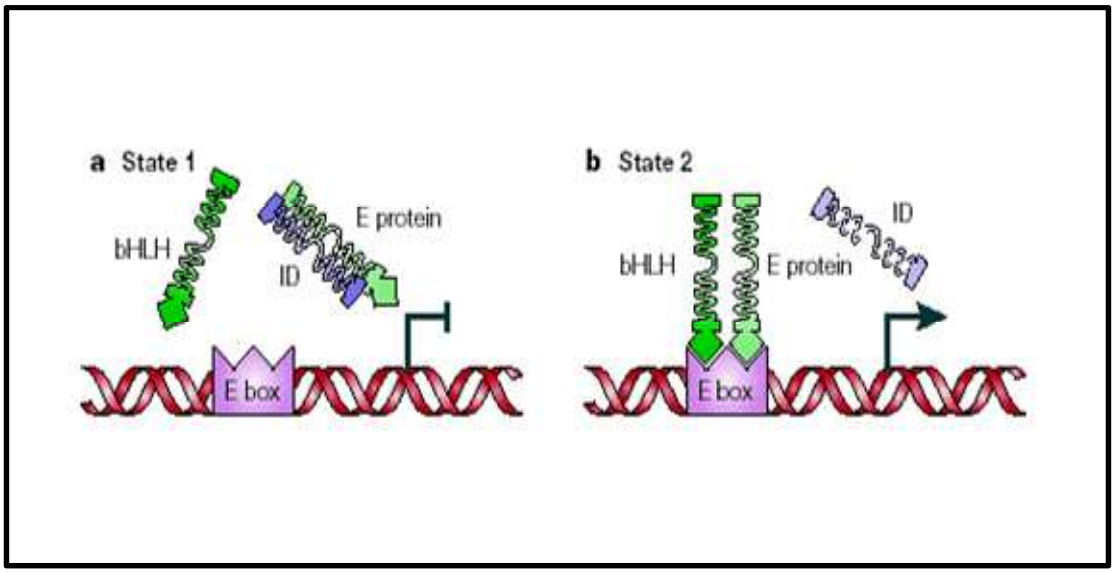

Figure 1.5 Cell fate determination by ID and E-proteins.
(a) Cells are sustained in state 1 by the ID proteins, which lack the basic DNA binding region. In state 1, E-proteins are sequestered by ID proteins resulting in inhibition of E-box-dependent gene expression. This results in block of differentiation and stimulation of proliferation. (b) In state 2, the E-protein transcription factors activate transcription by binding to promoter E-boxes as E-protein–E-protein homodimers or as E-protein–tissue-specific bHLH factor heterodimers. Formation of ID protein–E-protein dimers prevents E-proteins from forming DNA-binding transcriptionally active complexes. This results in induction of differentiation and inhibition of proliferation (state 2) (taken from Perk *et al.*, 2005).

1.11.2 The structure of ID genes and proteins

All ID proteins range from 13 kDa to 20 kDa in size (Norton *et al.*, 1998). The HLH domain for the ID proteins is highly conserved, however the rest of the sequence is very divergent, except for four small domains. The HLH region consists of two amphipathic α helices, which are 15-20 residues long, and are separated by an intervening loop. The HLH domain confers homo- or hetero-dimerisation, which is

required for DNA binding and transcriptional regulation. The HLH monomers form a four-α-helix structure which is stabilised by a combination of polar and electrostatic interactions at the interface of the monomer (Wibley *et al.*, 1996). Heterodimerisation appears to depend on the variable loop region and ID-bHLH dimers may be stabilised by forming a multiple oligomeric state (Pesce & Benezra, 1993; Fairman *et al.*, 1993). ID family genes have a similar genomic organisation of exon-intron boundaries in their coding regions, which might suggest that they have evolved from a familial ancestral ID gene (Deed *et al.*, 1994).

1.11.3 Functions of ID transcription factors

One of the best characterised interactions of ID proteins are with the E-protein transcription factors. ID proteins inhibit cell differentiation by binding and sequestering bHLH transcription factors that are responsible for regulating differentiation linked genes (Ruzinova & Benezra, 2003). Slight changes in the equilibrium of heterodimeric interactions between ID and bHLH proteins cause remarkable changes in cell fate determination (Barndt & Zhuang, 1999; Jan & Jan, 1993). Even though all ID proteins bind to E-proteins, they have different preferences for E-proteins (Langlands *et al.*, 1997; Deed *et al.*, 1998). In some cases, ID proteins can act as transcriptional activators. Fused ID proteins to a heterologous Gal4-DNA binding domain can cause activation of Gal4-dependent transcription (Bounpheng *et al.*, 1999a). Although this occurs through sequestering bHLH proteins, which are involved in transactivation, the ID-bHLH heterodimers cannot bind DNA making this system unlikely to be physiological. Nevertheless, ID proteins could have a role as part of DNA-bound transcriptional complexes.

1.11.4 Role of ID proteins in cancer

In human tumours, elevated levels of IDs have been reported in breast cancer (Lin *et al.*, 2000), prostate cancer (Ouyang *et al.*, 2002a), ovarian cancer (Schindl *et al.*, 2003), thyroid cancer (Kebebew *et al.*, 2000) and colon cancer (Wilson *et al.*, 2001). Overexpression of ID proteins is associated with loss of differentiation and enhanced malignancy (Lasorella *et al.*, 2001; Perk *et al.*, 2005). Also, ID proteins have been shown to be involved in tumour progression (Fong *et al.*, 2004). In breast cancer, the overexpression of ID1 is associated with infiltrating, more aggressive tumours, which suggests a relationship between the expression of ID1 and disease progression (Lin *et al.*, 2000). ID overexpression has been shown to exhibit oncogenic properties (Benezra *et al.*, 2005). When overexpressed with B cell lymphoma 2 (Bcl-2) they can immortalise primary fibroblasts (Norton & Atherton, 1998) and ID1 can immortalise primary keratinocytes (Alani *et al.*, 1999). In mice, overexpression of ID1 or ID2 in thymocytes results in T cell lymphoma (Kim *et al.*, 1999; Morrow *et al.*, 1999). ID proteins can be regulated by a number of oncoproteins such as Ras, Myc and the EWS (Ewing sarcoma) - ETS fusion protein, found in Ewing's sarcoma. The Ras signalling pathway leads to the activation of early growth response 1 (Egr1), which causes induction in the levels of ID1 (Tournay & Benezra, 1996) and ID3 (Bain *et al.*, 2001). ETS1 can become inhibited by ID proteins, which in turn inhibit $p16^{INK4a}$ expression and allow phosphorylation of RB (Ohtani *et al.*, 2001). Myc oncoproteins can directly bind to the ID2 promoter/enhancer and activate expression of ID2 (Lasorella *et al.*, 2000). Increased levels of ID2 are required by Myc to overcome RB-mediated cell cycle arrest. ID1 and ID3 are also downstream targets of Myc. Elevation of ID1 is essential for Myc to drive breast cancer cells to enter S phase (Swarbrick *et al.*, 2005). Myc also binds to the E-box in the ID3 promoter/enhancer similar to ID2. In Ewing's sarcoma, the EWS-ETS fusion protein activates ID2 by binding to the promoter directly (Nishimori *et al.*, 2002; Fukuma *et al.*, 2003). This was examined by retroviral transduction of EWS-ETS that lead to transcriptional activation of ID2.

1.11.5 ID proteins as a therapeutic target

ID proteins are overexpressed in tumour cells and are involved in proliferation, invasiveness, metastasis and angiogenesis (Lasorella *et al.*, 2000; Perk *et al.*, 2005; Ruzinova & Benezra, 2003). Therefore, ID proteins are now regarded as interesting targets for anti-cancer drugs against tumour progression. ID proteins are expressed at low levels in adult tissue but are reactivated in cancer. Target therapy that has low toxicity and that is able to aim at the tumour and its support network would be very attractive. However, transcription factors are difficult to inhibit as they are present in the cell's nucleus and act through protein-protein or protein-DNA interactions. Small molecules have been used to target protein-protein interactions (Berg, 2003). The Myc-Max interaction has been inhibited as well as the interaction between β-catenin and cAMP response element-binding protein (CREB) (Berg, 2003; Emami *et al.*, 2004). In the case of ID proteins, a developed antitumour agent has been shown to downregulate ID1 in tumour cells *in vivo* (Henke *et al.*, 2008). An antisense oligonucleotide fused to a peptide was used, which homed to tumour neovessels, and when delivered caused a decrease in the ID1 protein levels. Delivery of the drug lead to inhibition of primary tumour growth and metastasis, effects which are similarly seen in ID1 knockout mice. When combined with the heat shock protein 90 (Hsp90) inhibitor 17-(allylamino)-17-demethoxygeldanamycin, this resulted in complete growth suppression of breast tumours (Henke *et al.*, 2008).

1.12 E2A basic helix-loop-helix transcription factors

The basic HLH proteins are categorised in different classes depending on functional and biochemical properties. E-proteins belong to class I HLH proteins and are transcription factors that have been demonstrated to bind to E-boxes (Lazorchak *et al.*, 2005; Sun, 2004). E-proteins are widely expressed and can interact with other members of the HLH proteins as homodimers or heterodimers in order to modulate expression of their target genes. The E-protein family consists of four members in mammals, the E47, E12, E2-2 and HEB. The E2A genes encode two transcription factors, E47 and E12, which were discovered by expression screening of a lymphoid library using a DNA probe that corresponded to an E-box (Murre *et al.*, 1989; Sun & Baltimore, 1991). E-proteins are regarded to play a crucial role in regulating cell growth and differentiation (Slattery *et al.*, 2007).

The E2A gene consists of four coding exons and one non-coding exon (Hata & Mizuguchi, 2004). The DNA-binding and dimer assembly domains of E47 and E12 are present on the C-terminal regions. The transcriptional activity is located on two domains, AD1 and AD2, which have been mapped to the N-terminal domain (Aronheim *et al.*, 1993; Henthorn *et al.*, 1990). More information has emerged on the transactivation properties of E-proteins. The p300 activator has been shown to interact with E47 when E47 is bound to DNA (Eckner *et al.*, 1996). Since p300 has acetyltransferase activity, it is possible that E-proteins recruit enzymes that play a role in chromatin modification (Bannister & Kouzarides, 1996; Ogryzko *et al.*, 1996; Eckner *et al.*, 1996). MyoD and p300 interact at the bHLH region of MyoD and do not require the transactivation domains. This was unexpected, since the bHLH domain does not have intrinsic transactivation potential. Also, it has been shown that a conserved motif, LDFS, found in the AD1 domain binds to a histone acetyltransferase (HAT) complex named SAGA. Some of the subunits of SAGA are the HAT general control nonderepressible 5 (Gcn5), the transcriptional adaptor (Ada) and suppressor of Ty (Spt) proteins (Grant *et al.*, 1998a; Ogryzko *et al.*, 1996). Therefore, it is possible that E2A could recruit a nuclear HAT such as p300 or a SAGA complex such as Gcn5 to target genes and affect

the acetylation of nucleosomal histones (Ogryzko *et al.*, 1996). E2A proteins can be negatively regulated by the ubiquitin-proteasomal pathway (Huggins *et al.*, 1999). CK2 has been shown to phosphorylate two sites within the residues N-terminal to the bHLH domain (Sloan *et al.*, 1996). The phosphorylation of these residues resulted in inhibiting the DNA binding ability of E47 homodimers (Sloan *et al.*, 1996).

Several studies have indicated that E-proteins play a role in regulating cell growth. E47 is capable of growth suppression in NIH3T3 cells. When E47 was overexpressed in colony forming assays this suppressed the growth of NIH3T3 cells (Peverali *et al.*, 1994). E-proteins have been implicated in various forms of cancer. Mice that are E47 deficient develop T cell lymphoma (Yan *et al.*, 1997; Bain *et al.*, 1997). Cells from patients with T lymphoblastic leukaemia have chromosomal translocations involving the bHLH T cell acute lymphoblastic leukaemia 1 (TAL1) gene (Park *et al.*, 1999). The E-protein transcriptional activity is inhibited by TAL1 that causes leukaemia in mice (O'Neil *et al.*, 2004). Therefore, it is proposed that E-proteins act as tumour suppressors in human T lineage cells (Bain *et al.*, 1997). In childhood pro-B and pre-B leukaemias, E-proteins are thought to be involved. Chromosomal translocation events in both types of leukaemias replace the E2A bHLH region with a leucine zipper within the hepatic leukaemia factor (HLF) gene or homeodomain pre-B cell leukaemia homeobox 1 (Pbx1) domain (Inaba *et al.*, 1992; Aspland *et al.*, 2001). The regulation of E47 by Ras signalling through activation of ID genes raises the question whether E47 has a role in various developmental systems as a tumour suppressor (Perk *et al.*, 2005). ID proteins are overexpressed in several cancers and it is possible that the E47 protein is a target of Ras-mediated transformation. Since E47 proteins regulate cell growth, suppressing their activity may be crucial for the development and progression of several malignancies.

1.13 Aims of PhD

Pol III transcription can be regulated by various proteins and conditions. Conditions such as cell cycle progression, growth, differentiation and transformation can be regulated by several proteins such as transcription factors. The transcription factors ID1, ID2 and ID3 are important due to their oncogenic potential. The E47 transcription factors are regarded as suppressors of cell growth. Elucidating the function of these proteins in pol I/III transcription would be novel, since no studies have been reported on whether these proteins regulate pol I and/or pol III, and of great importance in the field. The work in this thesis attempts to uncover the role of these novel proteins in the regulation of pol III transcription.

One of the first goals was to determine if ID1, ID2, ID3 and E47 transcription factors can activate or repress pol III transcription. Another goal was to determine the precise interaction of these transcription factors with the pol III transcription machinery. This will give us an insight into the mechanism underlying the regulation of pol III transcription. Finally, this thesis will investigate the synergistic role of ID1, ID2, ID3 and E47 transcription factors on the regulation of pol III transcription. By understanding of the role of these proteins in cancer, tools to explore these proteins as therapeutic targets can be provided.

Chapter 2

Materials and Methods

2.1 Cell culture

A class II hood was used for all cell culture work, using aseptic techniques and sterile equipment and reagents.

2.1.1 Cell lines and maintenance

HeLa, HEK 293, MEF ID2$^{+/+}$ and MEF ID2$^{-/-}$ cells were maintained in DMEM (Dulbecco's Modified Eagle Medium, Cambrex) supplemented with 10% foetal bovine serum (FBS, Sigma), 2 mM L-Glutamine (Sigma), 100 U/ml penicillin (Sigma) and 100 U/ml streptomycin (Sigma). ID1 U2OS and ID3 U2OS stable cell lines were maintained in the same way as the above cell lines, with the addition of 300 µg/ml G418 (Promega) in the culture medium. Stably transfected HeLa TET-ON cells were maintained in 10% FBS (tetracycline free), 100 U/ml penicillin, 100 U/ml streptomycin, 100 µg/ml G418 and 100 µg/ml hygromycin. Expression of HA-Brf1 (haemagglutinin tagged Brf1) was induced for 48 hours before harvesting by the addition of 1 µg/ml doxycycline.

Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and passaged when sub-confluent, approximately every 2-3 days. In order to passage the cells, the medium was aspirated from the flask and 2 ml of buffered trypsin-EDTA [0.05% (w/v) trypsin, 0.02% (w/v) EDTA, Sigma] were added to the cells in a 75cm^2 flask and aspirated immediately. A further 2 ml were added and left for approximately 3 minutes at 37°C. Following the addition of trypsin, fresh medium was immediately added to the dissociated cells which were then centrifuged at 1,000xg for 5 minutes. The medium was removed and the pelleted cells were resuspended in fresh medium and transferred to new flasks.

2.1.2 Adenoviral infection of cells

Cell culture using adenoviruses was performed in a category II hood, using aseptic techniques and sterile equipment and reagents. HeLa and HEK 293 cells were plated 1 day prior to adenoviral (Ad) infection. The cells were infected with an adenovirus expressing ID2 (Ad-ID2), E47 (Ad-E47) or with the parental virus adenovirus-cytomegalovirus 5' internal ribosomal entry site green fluorescent protein (Ad-CMV5-IRES-GFP) (Ad-Vector) at a multiplicity of infection of 100. The infection was allowed to proceed for 2 hours prior to termination by the addition of excess culture medium to the plates.

2.1.3 Cryo-storage of cells

Cryo-freezing was used for the storage of all cell lines. Cells were trypsinised as described and, after pelleting by centrifugation at 1,000xg for 5 minutes, were resuspended in a solution of 80% DMEM, 10% FBS and 10% dimethyl sulphoxide (DMSO, Sigma). From a sub-confluent 75 cm^2 flask, 1.5 ml of cells were aliquoted into 2 ml cryo-tubes and frozen in two steps. Initially, the cells were placed at -80°C overnight before being transferred to liquid nitrogen for long-term storage. The cells were recovered by placing the cryo-tubes in a water bath at 37°C until thawed. Cells were mixed with fresh media and pelleted by centrifugation. The supernatant was aspirated to ensure removal of DMSO and the cells were resuspended in maintenance media.

2.2 Transformation of competent *Escherichia coli* (*E. coli*) cells

For plasmid propagation, *E. coli* XL-1 blue supercompetent cells (Stratagene) were transformed with plasmid DNA. The supercompetent cells, which are highly

temperature sensitive, were stored at -80°C and thawed on ice, to avoid loss of their transformation ability. 50 µl of competent cells were incubated with 20 ng of plasmid DNA on ice for 30 minutes. The cells were heat-shocked at 42°C for 30 seconds and quickly transferred on ice for a further 2 minutes. The cells were incubated at 37°C for 1 hour on an orbital shaker (250 rpm) following the addition of 500 µl of SOC medium (LB Broth, 0.05% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$). LB-agar plates (LB Broth, 2% agar, 100 µg/ml ampicillin) were then plated with 150 µl of the transformation mixture and incubated at 37°C overnight to allow growth and colony formation.

2.3 Preparation of plasmid DNA

A single isolated bacterial colony was selected from the LB-agar plates and was used to inoculate 5 ml of LB medium containing 100 µg/ml ampicillin as the selection antibiotic. The culture was incubated for 6 hours at 37°C in an orbital shaker (250 rpm). 1 ml was transferred to a larger culture containing 500 ml of LB-broth and was incubated overnight at 37°C in an orbital shaker (250 rpm). The cells were harvested by centrifugation at 4500 rpm for 30 minutes at 4°C. Plasmid DNA was isolated by Molecular Technology Services using a Qiagen 9600 robot. The concentration of plasmid DNA was measured spectrophotometrically using the BioPhotometer instrument (Eppendorf) and a quartz cuvette, according to the formula 1 A_{260} = 50 µg/µl dsDNA. Plasmids were stored at -20°C. For a description of the plasmids see table 2.1.

Table 2.1: Description of plasmids

Plasmid	Description	Origin
PGex2T	Encoding GST, used as a control	Gift from Bob Eisenman (Gomez-Roman *et al.*, 2003)
PGex2T-ID1	Encoding full length ID1 protein	Gift from Antonio Iavarone (Lasorella *et al.*, 1996)
PGex2T-ID2	Encoding full length ID2 protein	Gift from Antonio Iavarone (Lasorella *et al.*, 1996)
PGex2T-ID3	Encoding full length ID3 protein	Gift from Antonio Iavarone (Lasorella *et al.*, 1996)
PGex2T-ID2-HLH	Encoding helix-loop-helix domain of ID2 protein	Gift from Antonio Iavarone (Lasorella *et al.*, 1996)
PGex2T-ID2-δHLH	Encoding ID2 protein lacking the helix-loop-helix domain	Gift from Antonio Iavarone (Lasorella *et al.*, 1996)
PGex2T-E47	Encoding full length E47 protein	Gift from Antonio Iavarone (Lasorella *et al.*, 1996)

2.4 Restriction endonuclease digestion

EcoRI and BamHI (Promega) restriction endonucleases were used to digest plasmid DNA. Six plasmids based on the PGex2T vector were analysed to confirm the presence of the ID1, ID2, ID3, ID2-HLH, ID2–δHLH and E47 inserts. The reactions contained 1 µl of each restriction enzyme, 1 µg of plasmid DNA and 3 µl of the MULTI-CORE buffer (Promega) in a final volume of 30 µl. The DNA was left to digest for 1 hour at 37°C. The sample products were mixed with 6x agarose gel DNA loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol] to give a final 1x loading buffer concentration and analysed by 1% (w/v) agarose gel

electrophoresis. Electrophoresis was allowed to proceed in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) containing 0.1 µg/ml ethidium bromide (Sigma) and DNA was visualised under ultraviolet (UV) light.

2.5 Expression of recombinant glutathione-S-transferase (GST) fusion proteins

E. coli BL21 Rosetta competent cells were transformed for plasmid propagation. Competent cells were stored at -80°C and thawed on ice for transformation. 50 µl of thawed cells and 20 ng of plasmid DNA were mixed by agitation. The cells were incubated for 20 minutes on ice and heat-shocked at 42°C for 30 seconds. The cells were recovered by the addition of 500 µl SOC medium (LB-Broth, 0.05% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$) and incubated at 37°C for 30 minutes. 150 µl of cells were plated onto LB-agar plates (LB-Broth, 2% agar, 100 µg/ml ampicillin) and incubated at 37°C overnight. Single colonies were picked and grown overnight in 10 ml of LB broth containing 100 µg/ml ampicillin at 37°C in an orbital shaker. The 10 ml culture was used to inoculate 500 ml of LB broth containing 100 µg/ml ampicillin, and the culture was grown at 37°C in an orbital shaker until reaching an OD_{595} of 0.6-0.8. An uninduced control sample of 1 ml was taken at this stage. The sample was pelleted by centrifugation at 13,000xg for 1 minute and resuspended in 50 µl 4x SDS sample buffer (150 mM Tris-HCl pH 6.8, 1% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.5% bromophenol blue). The culture was induced with 0.1 mM final concentration IPTG and grown for 2 hours at 37°C in an orbital shaker. An induced sample of 1 ml was taken, pelleted by centrifugation at 13,000xg for 1 minute, and resuspended in 4x SDS sample buffer. The large culture was harvested by centrifugation at 4,500 rpm for 20 minutes at 4°C. The pellet was snap frozen on dry ice and stored at -80°C. The induced and uninduced samples were resolved by SDS-PAGE (polyacrylamide gel electrophoresis), and the gel was stained with SimplyBlue™ SafeStain (Invitrogen), to confirm the induction of the proteins. The cells were resuspended in 20 ml 1x TBS (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl), and sonicated 10x for 30 seconds on ice using the vibra cell

sonicator (Sonics). The crude lysate was then centrifuged at 8,500xg for 20 minutes at 4°C. The clarified extract was aliquoted and snap frozen on dry ice.

2.6 *In vitro* transcription and translation of proteins in reticulocyte lysate

For *in vitro* transcription and translation of proteins the TNT reticulocyte kit (Promega) was used, according to the manufacturer's instructions. The synthesised proteins were labelled with ^{35}S-methionine and 1-2 µl of the samples were analysed by SDS-PAGE. The gels were incubated with fixing solution [10% (v/v) acetic acid, 30% (v/v) methanol] for 30 minutes at room temperature and washed with distilled H_2O for 15 minutes. The gels were incubated with autofluor (National Diagnostics) for 2 hours at room temperature on an orbital shaker. The gels were vacuum dried for 2 hours at 80°C and the radiolabelled proteins visualised by autoradiography.

2.7 GST pull down assay

GST and GST-fusion proteins were coupled to glutathione-agarose beads (Sigma). GST and GST-fusion proteins containing clarified extracts were incubated with 20 µl of packed glutathione agarose beads for 2 hours at 4°C. Following this, 1-2 µl of the *in vitro* translated protein was incubated with 20 µl packed pre-washed glutathione agarose beads in a total volume of 600 µl made up with 1x TBS, to pre-clear the *in vitro* translated protein. The recombinant GST-fusion proteins bound to the beads were washed five times to remove non-specific binding of bacterial proteins and 250 µl of pre-cleared reticulocyte lysate were added to these beads. The samples were incubated for 2 hours rotating at 4°C. The beads were washed five times with 1 ml of 1x TBS to remove non-specific binding to the recombinant proteins and 4x protein sample buffer was added to the beads. The samples were resolved by SDS-PAGE and the proteins

detected by SimplyBlue™ SafeStain (Invitrogen) in order to confirm equal loading. The rpn800e full-range rainbow molecular weight marker (GE Healthcare) was run next to the samples. Samples were destained (30% methanol, 10% acetic acid). The gels were incubated with fixing solution [10% (v/v) acetic acid, 30% (v/v) methanol] for 30 minutes at room temperature and washed with distilled H_2O for 15 minutes. The gels were incubated with autofluor (National Diagnostics) for 2 hours on an orbital shaker, to enhance the radio-labelled signal, vacuum dried and exposed to autoradiographic film overnight at -80°C. Pull-down assays using HeLa nuclear extracts were also carried out. 1-2 µl of *in vitro* transcribed and translated protein were added to the beads as well as 200 µg of HeLa nuclear extracts in a total volume of 500 µl in 1x TBS. The incubation took place for 2 hours rotating at 4°C. The beads were washed five times with 1x TBS and mixed with an appropriate volume of protein sample buffer. The samples were analysed by western blotting.

2.8 Preparation of protein extracts for western blots and co-immunoprecipitations (co-IPs)

Whole cell protein extracts were prepared from cells cultured in 10 cm dishes or 6-well plates, when cells reached a confluency of approximately 80%. The procedure was performed on ice rapidly and all solutions and plasticwear were kept cold to maintain cell activity and avoid protein degradation. The maintenance medium was aspirated and the culture cells were washed three times with ice-cold PBS. The cells were scraped with a plastic spatula 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 phenylmethylsulfonyl fluoride (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 microfuge tubes. 150 µl of buffer was used for each well of the 6-well plates and 500 µl of buffer for each 10 cm dish. The lysates were passed three times through a 26G needle

and centrifuged at 13,000xg for 10 minutes at 4°C. The supernatants were aliquoted and snap frozen on dry ice. The whole cell extracts were stored at -80°C.

2.9 Determination of protein concentrations

The protein concentration of samples was determined using Bradford's reagent (BioRad) diluted 1 in 5 with distilled H_2O. The colour reaction produced by the mixing of the reagent with protein was quantified by the absorbance at 595 nm using a spectrophotometer. The values measured were indicative of the amount of protein in each sample. The standard curve for each experiment was constructed by measuring the absorbance at 595 nm of 1, 2, 3, 4, 5, 6, 8, 10, 12 and 14 µg of BSA in 1 ml of Bradford's reagent. For each sample, absorbance readings were performed in triplicate and compared to the standard curve to determine the protein concentration.

2.10 Adenovirus titre immunoassay analysis

To calculate the viral titre, the QuickTitre^{tm} Adenovirus Titre Immunoassay kit (Cell Biolabs) was used. HEK 293 cells were harvested and resuspended in DMEM culture medium at 2.5x 10^5 cells/ml. In each well of a 24-well plate 1 ml of the resuspended cells were incubated at 37°C, 5% CO_2 for 1 hour. Following that, a 10-fold serial dilution of the viral sample in medium was performed. The infected cells were incubated at 37°C for 2 days. After two days, the culture medium was aspirated and the infected cells were fixed to the plates by the addition of 0.5 ml ice-cold methanol. The cells were incubated for 20 minutes at -20°C and washed three times with 1x PBS, each wash for 5 minutes. The cells were blocked with 1% BSA in PBS for 1 hour at room temperature. 250 µl of 1x anti-Hexon antibody were added to each well and the cells were incubated for 1 hour at room temperature. The cells were washed with 1x PBS three times, each wash for 5 minutes. 250 µl of 1x secondary antibody were added to

each well and the cells were incubated for 1 hour at room temperature. The cells were washed five times with 1x PBS, each wash for 5 minutes. Freshly made 1x DAB substrate solution was added to each well, and the cells were incubated for 10 minutes at room temperature on an orbital shaker. Infected cells appeared dark brown. To calculate the positive cells (dark brown colour) five separate fields were selected using a light microscope. The number of positive cells and the viral titre, in infectious units/ml, were calculated.

2.11 Separation of proteins by SDS-polyacrylamide gel electrophoresis

50 µg of protein extracts were resolved by denaturing SDS 7.8% or 12% polyacrylamide (National Diagnostics) minigels (375 mM Tris pH 8.8, 0.1% SDS), with 4% polyacrylamide stacking gels (125 mM Tris pH 6.8, 0.1% SDS) according to molecular weight by electrophoresis. Prior to loading, samples were boiled for 3 minutes in 1x protein sample buffer (62.5 mM Tris pH 6.8, 0.5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue). Electrophoresis was performed in 1x SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris, pH 8.3), at an initial voltage of 75 V while the dye moved through the stacking gel; the voltage was then increased to 160 V until the bromophenol blue dye moved to the bottom of the gel.

2.12 Western blot analysis

Following SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane (BioRad) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell System. Transfer was carried out in 1x transfer buffer (76.8 mM glycine, 10 mM Tris pH 8.3, 20% methanol) at 100 V for 1 hour or overnight at 4°C. Nitrocellulose membranes were stained with Ponceau S (Sigma) to ensure transfer of the proteins to the

membrane. Membranes were washed with 1x TBS for 10 minutes and blocked in milk buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 4% skimmed milk powder (Marvel) for 30 minutes at room temperature. Membranes were incubated with primary antibodies for 3 hours at room temperature or overnight at 4°C (see table 2.2 for the antibody dilutions used). Excess primary antibody was washed three times for 5 minutes in PBS containing 0.5% Tween-20. Following the washes, membranes were incubated for 1 hour at room temperature in the presence of the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in milk buffer) (Dako). Membranes were washed three times with western wash buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20) for 5 minutes, followed by two washes for 15 minutes. Membranes were washed for 5 minutes with 1x TBS (2.5 mM Tris-HCl pH 7.6, 15 mM NaCl) and antibodies were detected using the enhanced chemiluminescence method (ECL, Amersham), following the manufacturer's instructions.

2.13 Co-immunoprecipitation

Antibodies for immunoprecipitation experiments were bound to either protein-A sepharose or protein-G sepharose beads (Sigma). For each immunoprecipitation, 25 µl of packed protein beads were used. The beads were washed five times using 1 ml for each wash of low salt microextraction buffer (LS-MEB) (150 mM NaCl, 50 mM NaF, 20 mM HEPES pH 7.8, 25% glycerol, 1 mM DTT, 0.5 mM (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) prior to incubation with 5 µl of anti-ID1, anti-ID2, anti-ID3, anti-E47 and anti-Brf1 (128); the final volume was made up to 50 µl with LS-MEB and the antibodies were incubated with the beads by rotating for 2 hours at 4°C. Following binding, the beads were washed five times with LS-MEB to remove excess antibody. 300 µg of HeLa protein extracts (Computer Cell Culture, Mons, Belgium) were added to the beads and left to incubate for 1 hour at 4°C. The beads were washed five times with LS-MEB and 50 µl of 4x protein sample buffer were added. Samples were heated at 95°C for 3 minutes and analysed by SDS-PAGE and western blotting.

Table 2.2: Antibodies used for western blots

Protein	Antibody	Dilution	Type	Company
ID1	C-20	1:1000	Polyclonal	Santa Cruz
ID2	C-20	1:1000	Polyclonal	Santa Cruz
ID3	C-20	1:1000	Polyclonal	Santa Cruz
E47	N-649	1:1000	Polyclonal	Santa Cruz
TFIIB	C18	1:1000	Polyclonal	Santa Cruz
Actin	C-11	1:5000	Polyclonal	Santa Cruz
Brf1	128	1:1000	Serum	In House
Brf1	330	1:1000	Serum	In House
TBP	MTBP-6	1:250	Monoclonal	In House
HA	F-7	1:1000	Monoclonal	Santa Cruz

2.14 RNA extraction

Total cellular RNA was extracted from cells grown in 10 cm culture dishes using TRI reagent (Sigma). The maintenance medium was aspirated and the cells were washed four times with ice-cold PBS. The cells were scraped into 1 ml or 500 µl of TRI reagent, for 10 cm dishes or per well of 6-well plates respectively, and transferred into microfuge tubes. The samples were incubated for 10 minutes at room temperature in order to achieve dissociation of nucleoprotein complexes prior to the addition of 200 µl of chloroform to each sample. The samples were mixed by vortexing for 1 minute, incubated for 10 minutes at room temperature and centrifuged at 13,000xg for 15

minutes at 4°C. Samples separated into three phases: a lower red organic phase containing the protein, a white intermediate phase containing the DNA and an upper colourless aqueous phase containing the RNA. The RNA-containing phase was carefully transferred into a clean microfuge tube and 0.5 ml of isopropanol was added to the samples to precipitate the RNA. The tubes were mixed by inverting, incubated at room temperature and centrifuged at 13,000xg for 10 minutes at 4°C. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 13,000xg for 5 minutes at 4°C. The supernatant was aspirated and the pellets were air dried for 20 minutes at room temperature. The RNA was resuspended in 20 µl of RNase-free water and the samples were heated at 50°C to facilitate resuspension. RNA concentration was determined by spectrophotometry at 260 nm, using the following equation: 1 OD unit at 260 nm = 40 µg/ml of RNA. The samples were stored at -80°C.

2.15 cDNA preparation

2 µg of RNA were added to 200 ng of hexanucleotide primers (Roche) and made up with RNase-free water to a final volume of 20 µl. Samples were incubated at 80°C for 10 minutes for primer annealing. The tubes were transferred on ice and 8 µl of 5x First Strand Buffer (Invitrogen), 4 µl of 0.1 M DTT (Invitrogen), 2 µl of 10 mM dNTP mix (Promega) and 1 µl (200 U) of Superscript II Reverse Transcriptase (Invitrogen) were added to initiate transcription. The reverse transcription reaction was placed for 50 minutes at 42°C. The reaction was terminated by heating the samples for 15 minutes at 70°C to inactivate the enzyme. The cDNA samples were stored at -20°C.

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

The PCR reactions were performed using the DNA Engine Dyad Peltier Thermal Cycler (BioRad). Each PCR reaction had a final volume of 20 µl and contained 2 µl of cDNA with 20 pmol of primers. The reaction contained 0.5 U Taq DNA polymerase (Promega), 1x Taq DNA polymerase buffer (Promega) containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP plus 1.8 µCi of [α^{32}P] dCTP (Amersham). Primer cycling parameters and sequences are listed in table 2.3. The PCR products were diluted 1:1 with formamide loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) and resolved on 7% polyacrylamide (National Diagnostics) sequencing gels containing 7 M urea and 1x TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0). Gels were pre-run at 40 W for 30 minutes in 1x TBE and 2 µl of sample was loaded after being heated at 95°C for 2 minutes and quenched on ice. Electrophoresis was performed for 1 hour at 40 W and the gels were vacuum-dried at 80°C for 1 hour before being exposed to autoradiography film to detect the radiolabelled products. In some cases, PCR products were detected using SYBR Gold Nucleic Acid Gel Stain (Invitrogen). In this case, the above final 20 µl PCR reaction mixture, the 1.8 µCi of [α^{32}P] dCTP was replaced with distilled H_2O. 5 µl of Orange G loading dye (Sigma) were added to each 20 µl PCR product sample and mixed. The samples were resolved on 5% polyacrylamide (National Diagnostics) gels. 6 µl of sample was loaded for 30 minutes and electrophoresis was performed in 1x TBE at 150 V. The gels were stained with 1x SYBR Gold Stain for 30 minutes, protecting the stain solution from light by placing it in the dark. The gels were visualised under UV light.

Table 2.3: Primers and cycling parameters for RT-PCR

Transcript	Primers 5'-3' Forward Reverse	Cycle number	Product length	PCR conditions (denaturing; cycling; final elongation)
ARPP P0	5' GCACTGGAAGTCCAACTACTTC 3' 5' TGAGGTCCTCCTTGGTGAACAC 3'	18-20	266 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min
ID1	5' CGCATCTTGTGTCGCTGAAG 3' 5' GGTGACTAGTAGGTGTGCAG 3'	27-29	228 bp	95°C for 2 min; 95°C for 1 min, 53°C for 30 s, 72°C for 1 min; 72°C for 5 min
ID2	5' ATGAAAGCCTTCAGTCCCGT 3' 5' TTCCATCTTGCTCACCTTCTT 3'	25-27	189 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min
ID3	5' TCAGCGCTTCCTCATTCTTT 3' 5' AAGGCACGCCTCTTTATTCA 3'	28-30	154 bp	95°C for 2 min; 95°C for 1 min, 59°C for 30 s, 72°C for 1 min; 72°C for 5 min
E2A	5' TGTGCCAACTGCACCTCAA 3' 5' GGGATTCAGGTTCCGCTCTC 3'	22-24	116 bp	95°C for 2 min; 95°C for 1 min, 62°C for 30 s, 72°C for 1 min; 72°C for 5 min
5S rRNA	5' GGCCATACCACCCTGAACGC 3' 5' CAGCACCCGGTATTCCCAGG 3'	18-20	107 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min

tRNA Leu	5’ GAGGACAACGGGGACAGTAA 3’ 5’ TCCACCAGAAAAACTCCAGC 3’	25-27	88 bp	95°C for 3 min; 95°C for 30 s, 68°C for 30 s, 72°C for 30 s; 72°C for 5 min
tRNA Tyr	5’ AGGACTTGGCTTCCTCCATT 3’ 5’ GACCTAAGGATGTCCGCAAA 3’	25-27	84 bp	95°C for 3 min; 95°C for 1 min, 65°C for 30 s, 72°C for 15 s; 72°C for 1 min
tRNA Arg	5’ GGCTCTGTGGCGCAATGGATA 3’ 5’ TTCGAACCCACAACCTTTGAATT 3’	25-30	74 bp	95°C for 2 min; 95°C for 30 s, 66°C for 30 s, 72°C for 15 s; 72°C for 5 min
tRNA Sec	5’ GGATGATCCTCAGTGGTC 3’ 5’ GGTGGAATTGAACCACTC 3’	25-27	74 bp	95°C for 3 min; 95°C for 30 s, 54°C for 30 s, 72°C for 30 s; 72°C for 5 min
7SL	5’ GTGCCGCACTAAGTTCGGCATC 3’ 5’ TATTCACAGGCGCGATCCCACTACTGAGATC 3’	10-20	150 bp	95°C for 2 min; 95°C for 20 s, 62°C for 30 s, 72°C for 30 s; 72°C for 10 min
7SK	5’ CGATCTGGCTGCGACATCTG 3’ 5’ CGTTCTCCTACAAATGGAC 3’	14-16	248 bp	95°C for 3 min; 95°C for 30 s, 57°C for 30 s, 72°C for 30 s; 72°C for 10 min
MRP	5’ CGTGCTGAAGGCCTGTATC 3’ 5’ GGTGCGCGGACACGCAC 3’	25-30	232 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; 72°C for 5 min

U6 snRNA	5′ GCTCGCTTCGGCAGCACATATAC 3′ 5′ TATCGAACGCTTCACGAATTTGCG 3′	18-20	96 bp	95°C for 3 min; 95°C for 1 min, 60°C for 30 s, 72°C for 1 min; 72°C for 5 min
Pre-rRNA	5′ GCTTGGGTCTGTCGCGGT 3′ 5′ CACCTCGGGGAAATCGGGA 3′	23-25	151 bp	95°C for 3 min; 95°C for 1 min, 65°C for 30 s, 72°C for 15 s; 72°C for 1 min

2.17 RNA Pol III *in vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 15 µg of HeLa nuclear extracts (Computer Cell Culture, Mons, Belgium) to provide the basal pol III transcription components. This was supplemented with the addition of 250 ng of plasmid DNA to supply a specific pol III template and reactions were carried out in a 25 µl volume with a final concentration of 12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM $MgCl_2$, 0.28 mM EDTA, 1.2 mM DTT, 10% (v/v) glycerol, 1 mM creatine phosphate, 0.5 mM each of rATP, rCTP and rGTP and 10 µCi [α^{32}P] UTP (400 mCi/mmol) (Amersham). The transcription components were assembled on ice, and the reaction was performed in a waterbath at 30°C for 1 hour. Transcription was terminated with the addition of 250 µl of 1 M ammonium acetate/0.1% SDS containing 20 µg of yeast tRNA, which acts as a stabiliser for synthesised RNA. In order to remove protein and DNA, phenol-chloroform extraction of the samples was performed by addition of 250 µl of a 25:24:1 ratio of PhOH/$CHCl_3$/IAA. The samples were vortexed, microcentrifuged at 13,000xg for 5 minutes and 200 µl of the upper aqueous layer was transferred to a new microfuge tube containing 750 µl of 96% ethanol, and samples were mixed thoroughly. To precipitate the RNA, the samples were inverted several times and placed at -20°C overnight before being centrifuged at 13,000xg for 30 minutes to pellet the precipitated

RNA. The supernatant was removed and the pellet was washed with 750 µl of 70% ethanol. The samples were centrifuged and the pellets were dried by heating at 50°C for 5-10 minutes. 4 µl of formamide loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol FF) were added to each sample, which was vortexed for 1 hour to ensure the RNA was fully redissolved. From each sample, 1.5 µl was loaded on a pre-run 7% polyacrylamide sequencing gel consisting of 7 M urea and 1x TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0) after being heated at 95°C for 2 minutes and quenched on ice. Electrophoresis was carried out at 40 W for 1 hour in 1x TBE, and gels were vacuum-dried and exposed to autoradiography film to detect the radiolabelled transcripts.

2.18 Small interfering RNA (siRNA)

2.18.1 siRNA targeting

Commercially available siRNAs (Santa Cruz Biotechnology), which had been validated, were acquired for the knockdown of Oct-1, ID1, ID2, ID3 and E2A. siRNAs targeting Oct-1 (Oct-1 siRNA (h): sc-36119, Santa Cruz Biotechnology), ID1 (ID1 siRNA (h): sc-29356, Santa Cruz Biotechnology), ID2 (ID2 siRNA (h): sc-38000, Santa Cruz Biotechnology), ID3 (ID3 siRNA (h): sc-38002, Santa Cruz Biotechnology) and E2A (E2A siRNA (h): sc-35245, Santa Cruz Biotechnology) were used to reduce the expression of their targets.

2.18.2 Transient transfections using Lipofectatime 2000

Lipofectamine 2000 (Invitrogen) was used to perform transfections according to the manufacturer's instructions. 10 µl siRNA were used per reaction and added to

Optimem (Gibco) medium to a final volume of 100 µl. 2.5 µl Lipofectamine 2000 were added to 97.5 µl Optimem. The mixtures were incubated for 10 minutes at room temperature, combined and incubated at room temperature for 25 minutes. In the meantime, cells that that had reached about 70-80% confluency on 6-well plates were washed twice with Optimem and 800 µl of Optimem were added to each well. To each well, 200 µl siRNA/Lipofectamine 2000 mixture were added. The 1 ml final volume was left in each well for 6 hours, and then replaced with 2.5 ml of DMEM culture medium. Fresh medium was added after 24 hours and the cells were harvested 48 hours after transfection, unless stated otherwise.

2.19 Chromatin immunoprecipitation assay

Cells were grown in 10 cm tissue culture dishes until reaching a confluency of about 80%. One 10 cm dish was used per immunoprecipitation. Formaldehyde was added to the culture medium to a final concentration of 1% in order to cross-link the DNA-protein complexes. The reaction was allowed to proceed for 10 minutes at 37°C. To stop the crosslinking, glycine was added at a final concentration of 0.125 M and the dishes were transferred to ice for harvesting. The cells were harvested in the plating media/formaldehyde/glycine mix and pelleted by centrifugation at 500xg for 5 minutes at 4°C. The cell pellets were resuspended/washed in ice-cold PBS and centrifuged at 500xg for 5 minutes at 4°C. The last step was repeated twice. At this stage the cell pellets could be frozen on dry ice and stored at -80°C.

Cell pellets were resuspended/washed with ice-cold PBS/0.5% NP-40 and centrifuged at 500xg for 5 minutes at 4°C. The supernatant was removed and 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. After the incubation period, the cells were pelleted by centrifugation at 1,500 rpm for 5 minutes at 4°C and washed with 40 ml PBS/1% NP-40. The cells were resuspended in 40 ml low salt buffer (0.5% NP-40, 10 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl) and placed on ice for 30 minutes. Following the incubation, the cells were centrifuged at 500xg for 5 minutes at 4°C. The cells were

resuspended in 1 ml of low salt buffer and passed through a 26G needle four times. The suspension was made up to a final volume of 2.7 ml by the addition of low salt buffer. 300 µl of 20% sarcosyl were added to the suspension in order to lyse the nuclei. The suspension was transferred to a sucrose cushion and centrifuged at 4,000xg for 10 minutes at 4°C. The supernatant was removed and the pellet resuspended in 3 ml TE. A second cushion was used and the suspension was centrifuged again at 4,000xg for 10 minutes at 4°C. The resulting pellet containing the genomic DNA and cross-linked proteins was further resuspended in 2 ml TE (10 mM Tris pH 8.0, 1 mM EDTA), and the DNA was sheared into smaller fragments by sonication (10x 30 seconds, duty cycle 30%). 0.2 ml of 11x NET Buffer (1.56 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM Tris-HCl, pH 7.4) were added to the 2 ml sonicated sample, which was centrifuged at 13,000xg for 5 minutes. The supernatant was aliquoted evenly in microfuge tubes, and 10% of the aliquoted sample was used as input control. The amount of antibody added per aliquot (Table 2.5) was 25 µl for the in house made antibodies or 5 µg for the commercial antibodies. The microfuge tubes were rotated overnight at 4°C.

The following day, 50 µl of protein A or G sepharose beads (Sigma) (beads were washed five times with 1x NET buffer) were added to each tube and incubated rotating for 2 hours at 4°C. The beads were recovered on polypropylene columns (Pierce), washed twice with 10 ml ice-cold RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) buffer, twice with ice-cold 10 ml LiCl buffer (10 mM Tris-HCl, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0) and twice with ice-cold TE. The beads were incubated with 400 µl TE/1% SDS at room temperature and the protein-DNA complexes were eluted into microfuge tubes. Proteinase K (0.125 mg/ml) was added to the eluted sample in order to degrade the antibodies and proteins. The eluted material was incubated overnight at 42°C.

The DNA was extracted twice using 400 µl phenol/chloroform/isoamylalcohol (25:24:1) and once with 400 µl of chloroform. To precipitate DNA, 1 ml ethanol and 40 µl of 3 M sodium acetate were added and the tubes were mixed by inversion and left at -20°C overnight. The following day, the samples were centrifuged at 13,000xg for 20 minutes to pellet the precipitated DNA. The supernatant was removed and the pellets were washed with 1 ml of 75% ethanol. The samples were centrifuged again at 13,000xg

for 5 minutes and air dried before adding 40 µl of TE to resuspend the DNA. Samples were kept at 4°C and analysed by PCR. The primer sequences and antibodies for ChIP assays are displayed in table 2.4 and table 2.5 respectively.

Table 2.4: Primers and cycling parameters for ChIP assays

Transcript	Primers 5'-3' Forward Reverse	Cycle number	Product length	PCR conditions (denaturing; cycling; final elongation)
7SL set 1	5' CCGTGGCCTCCTCTACTTG 3' 5' TTTACCTCGTTGCACTGCTG 3'	26-28	171 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 3 min
7SL set 2	5' CGTCACCATACCACAGCTTC 3' 5' CGGGAGGTCACCATATTGAT 3'	26-28	194 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 3 min
7SL set 3	5' GTTGCCTAAGGAGGGGTGA 3' 5' TCTCTTGAGAGTCCAAAATTAA 3'	26-28	174 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 3 min
7SL set 4	5' TTTTTGACACACTCCTCCAAGA 3' 5' ATCTGGTCAAAGCAACATACACTG 3'	26-28	150 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 3 min
7SL set 5	5' TGCCTCCAGATAAAACTGCTC 3' 5' ACCCCACTAGAACCCTGACA 3'	26-28	156 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 3 min

ARPP P0	5’ GCACTGGAAGTCCAACTACTTC 3’ 5’ TGAGGTCCTCCTTGGTGAACAC 3’	18-20	266 bp	95°C for 2 min; 95°C for 1 min, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min
5S rRNA	5’ GGCCATACCACCCTGAACGC 3’ 5’ CAGCACCCGGTATTCCCAGG 3’	18-20	107 bp	95°C for 3 min; 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min
7SL	5’ GTGCCGCACTAAGTTCGGCATC 3’ 5’ TATTCACAGGCGCGATCCCACTACTGAGATC 3’	10-20	150 bp	95°C for 2 min; 95°C for 20 s, 62°C for 30 s, 72°C for 30 s; 72°C for 10 min
tRNA Leu	5’ GAGGACAACGGGGACAGTAA 3’ 5’ TCCACCAGAAAAACTCCAGC 3’	25-27	88 bp	95°C for 3 min; 95°C for 30 s, 68°C for 30 s, 72°C for 30 s; 72°C for 5 min
tRNA Tyr	5’ AGGACTTGGCTTCCTCCATT 3’ 5’ GACCTAAGGATGTCCGCAAA 3’	25-27	84 bp	95°C for 3 min; 95°C for 1 min, 65°C for 30 s, 72°C for 15 s; 72°C for 1 min
tRNA Arg	5’ GGCTCTGTGGCGCAATGGATA 3’ 5’ TTCGAACCCACAACCTTTGAATT 3’	25-30	74 bp	95°C for 2 min; 95°C for 30 s, 66°C for 30 s, 72°C for 15 s; 72°C for 5 min
U6 snRNA	5’ GTTTCGTCCTTTCCACAAG 3’ 5’ TTCTTGGGTAGTTTGCAG 3’	18-20	116 bp	95°C for 3 min; 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 5 min

Table 2.5: Antibodies for ChIP assays

Protein	Antibody	Type	Company
TBP	MTBP-6	Monoclonal	In House
RPC 155	1900	Serum	In House
Brf1	128	Serum	In House
TFIIIC 220	Ab7	Serum	In House
TFIIA	FL-109	Polyclonal	Santa Cruz
TFIIB	C18	Polyclonal	Santa Cruz
TAF_I48	M19	Polyclonal	Santa Cruz
ID1	C-20	Polyclonal	Santa Cruz
ID2	C-20	Polyclonal	Santa Cruz
ID3	C-20	Polyclonal	Santa Cruz
E47	N-649	Polyclonal	Santa Cruz

Chapter 3

Activation of RNA Polymerase III Transcription by ID1, ID2 and ID3

3.1 Introduction

3.1.1 The role of ID proteins in tumourigenesis

High expression levels of ID protein family members are associated with several types of human tumours. In particular, overexpression of ID proteins is linked with disease severity and poor prognosis. ID proteins contribute to tumourigenesis by inhibiting differentiation, promoting proliferation and affecting invasiveness (Lasorella *et al.*, 2001; Perk *et al.*, 2005; Fong *et al.*, 2003). ID protein deregulation in addition to affecting the proliferation of tumour cells is also implicated in tumour progression (Fong *et al.*, 2004; Perk *et al.*, 2005). Some of these features are the potential of tumour cells to metastasise and become involved in the formation of new blood vessels (Minn *et al.*, 2005).

Elevated protein levels of ID1 correlate with poor prognostic outcome in breast cancer patients and when ID1 was ectopically expressed in breast cancer cells this led to invasiveness of the basement membrane (Schoppmann *et al.*, 2003; Desprez *et al.*, 1998). In addition, expression of ID1 in prostate tumours increases with tumour grade (Ouyang *et al.*, 2002a). Also, high levels of ID1 expression correlate with poor histological grade in ovarian tumours and aggressive clinical behavior (Schindl *et al.*, 2001). ID2 has been identified as a prognostic variable in neuroblastoma patients, where high ID2 expression is associated with a poor outcome (Lasorella *et al.*, 2002). High levels of ID2 correlate with Ewings sarcoma EWS-ETS fusion protein in fibrosarcoma cells (Fukuma *et al.*, 2003). Studies have also revealed that ID3 is linked to human tumourigenesis. ID3 has been demonstrated to be overexpressed in human ovarian cancer cells and in small cell lung cancer (Shepherd *et al.*, 2008; Kamalian *et al.*, 2008). When ID proteins are ectopically expressed, this leads to inhibition of differentiation in a number of cell types, such as muscle and erythroleukaemia cells (Jen *et al.*, 1992; Shoji *et al.*, 1994). Overexpression of ID1 in SCp2 epithelial cells causes increased proliferation and when ID1 is targeted using antisense oligonucleotides, this reduces cell proliferation and inhibits invasiveness (Fong *et al.*, 2003). ID proteins are also thought

to be involved in a process known as oncogenic mimicry, where overexpression of ID proteins can regulate the activity of a target gene. The oncogenic acute myeloid leukaemia - eight-twenty-one (AML-ETO) fusion protein is formed due to a chromosomal translocation and results in acute myeloid leukaemia. It has been shown that AML-ETO binds to E-proteins and inhibits their function (Zhang *et al.*, 2004). It is therefore possible that ID proteins mimic the AML-ETO function and this promotes carcinogenesis.

3.1.2 ID mouse models

ID function is essential in mice for development and mouse models have been used to understand the role of IDs in cancer. It has been shown that overexpression or loss of function of ID proteins affects cancer progression in mouse models (Yokota *et al.*, 1999; Morrow *et al.*, 1999; Lyden *et al.*, 1999; Kim *et al.*, 1999). When ID1 was overexpressed in the intestinal epithelium of mice, three of the seventeen mice developed intestinal adenomas (Wice & Gordon, 1998). In another study, overexpression of ID1 in T cells led to the development of T cell lymphoma, suggesting an oncogenic role for the ID1 protein (Kim *et al.*, 1999). ID1 overexpression arrests T cell development, an effect which is more severe than the one observed in E2A and HEB knockout mice (Bain *et al.*, 1997). Because ID1 inhibits the function of E-proteins, it is possible that it interferes with E-proteins which are expressed in T cells. Overexpression of E47 can rescue the T cell deficiency exhibited in ID1 overexpressed mice (Kim *et al.*, 1999). All combinations of double knockout embryos of ID1, ID2 and ID3 are not viable and die before birth (Lyden *et al.*, 1999; Benezra R, unpublished observations cited in Ruzinova & Benezra, 2003). The double knockouts of ID1-ID3 in mice display vascular malformations and premature withdrawal of neuroblasts from the cell cycle and inappropriate expression of neural markers (Lyden *et al.*, 1999). In $ID1^{+/-}$ $ID3^{-/-}$ mice, the endothelial vasculature of tumour xenographs was affected, which led to failure in tumour growth and metastasis (Lyden *et al.*, 1999). Crosses of ID1- and ID3- mice lacking 1-3 copies of the four ID1 and ID3 alleles were carried out with phosphatase and

tensin homologue $(PTEN)^{+/-}$ tumour-prone strains, which develop lymph node hyperplasia, uterine carcinomas and prostate neoplasias (Ruzinova *et al.*, 2003). However, the partial loss of IDs had little effect on the tumours of this model.

Transgenic mice that overexpressed ID2 were generated, with five out of six founder lines developing aggressive T cell lymphomas (Morrow *et al.*, 1999). The hyperproliferation is similar to the growth of transformed cells, however, the cells are not monoclonal, indicating that the expanded T cells in the tumourous ID2 mice arise from multiple independent transformation processes. Therefore, overexpressing the ID2 protein has an important role in T cell development and oncogenesis. Also, studies have demonstrated that the ID2 knockout mice display the most severe phenotype compared to the other ID proteins. Knocking out ID2 in mice lead to ~25% perinatal mortality and the surviving ID2-null mice displayed retarded growth (Yokota *et al.*, 1999). The ID2-null mice lacked lymph nodes and Peyer's patches, had fewer natural killer cells, fewer Langerhan's cells and a lactation defect was observed in female mice (Hacker *et al.*, 2003; Yokota *et al.*, 1999; Kusunoki *et al.*, 2003; Mori *et al.*, 2000).

3.1.3 ID role in *Drosophila*

The role of ID proteins has been mostly studied in mouse and humans which consist of four members (ID1-ID4) and in *Drosophila* whose extramachrochaetae (emc) gene encodes a transcription factor which is similar to an ID protein (Garrell *et al.*, 1990; Ellis *et al.*, 1990). The emc protein contains an HLH domain without the basic region, which is involved in interaction with DNA. Emc forms heterodimers with bHLH proteins preventing them from binding DNA (Jan & Jan, 1993). Studies have demonstrated that emc is required in developmental processes in *Drosophila* during wing morphogenesis (Garrell *et al.*, 1990; Ellis *et al.*, 1990).

Loss of function (LOF) and gain of function (GOF) mutants have demonstrated that the emc protein is a negative regulator of the achaetae-scute bHLH proteins which are required in neurogenesis and sex determination (Garrell *et al.*, 1990; Ellis *et al.*, 1990). Emc null alleles are embryonic lethal. Studies using clones of LOF and GOF alleles of emc have revealed that emc is involved in cell proliferation and vein

differentiation (García-Alonso & García-Bellido, 1988). Emc appears to interact with genes involved in regulating *Drosophila* wing development. Proteins of the Ras signalling pathway (torpedo, vein, veinlet and gap) cooperate with emc during cell proliferation and act antagonistically during vein differentiation (Baonza & García-Bellido, 1999). Baonza and García-Bellido proposed two models in which emc and the genes of the Ras signalling pathway interact during cell proliferation and differentiation. In the first model, mutants of emc and genes of the Ras pathway have smaller wings, due to a smaller number of cells, than normal wings in both cases (of LOF and GOF mutants of emc with LOF mutants of the Ras pathway) indicating a possible co-operation of emc and genes of the Ras signalling pathway in cell proliferation. In the second model, the extraveins phenotype of the GOF mutant of the Ras signalling pathway is enhanced by interaction with the LOF alleles of emc, and is suppressed with the GOF mutants of emc, suggesting that emc and the Ras pathway operate antagonistically in vein differentiation.

Also, a strong genetic interaction between emc and components of the Notch signalling pathway has been demonstrated, indicating a possible functional interaction between them (Baonza *et al.*, 2000). Notch encodes a transmembrane protein that functions as a receptor in several developmental processes during embryonic and adult life (Artavanis-Tsakonas *et al.*, 1995). Some of the phenotypes in the wings of *Drosophila* caused by emc are similar to those observed in Notch mutants. The emc protein expression levels are higher in areas where Notch is activated. Studies have demonstrated that the expression of emc at the dorso/ventral border relies on the activity of the Notch signalling pathway (Baonza *et al.*, 2000). In vein differentiation and wing formation, emc is thought to be regulated by Notch and cooperates with Notch-downstream genes. Since the Ras and Notch signalling pathways are crucial in these processes, these studies could give insight into how ID proteins function in human molecular signalling pathways.

3.1.4 Role of ID proteins in the cell cycle

ID proteins are involved in negatively regulating cell differentiation as well as positively affecting the cell cycle. When ID proteins are expressed ectopically in mammalian cell lines they can stimulate cell growth (Lister *et al.*, 1995; Desprez *et al.*, 1995). Also, the use of antisense oligonucleotides and antibody-microinjection methods to inhibit ID protein expression and function have revealed a positive regulatory role for ID proteins in the G0 to S phase transition in the cell cycle (Hara *et al.*, 1994; Peverali *et al.*, 1994). On the other hand, when E47 was overexpressed in mammalian cell lines cells were arrested in the G1 phase of the cell cycle (Peverali *et al.*, 1994). ID proteins are present at low levels in quiescent cells, although after mitogenic stimulation ID expression is increased within 1-2 hours, similarly to other early response genes (Norton, 2000). ID protein expression is maintained throughout the G1 phase and is upregulated when cells enter the S phase of the cell cycle (Hara *et al.*, 1994; Norton *et al.*, 1998).

ID proteins can affect the cell cycle by interacting with a number of proteins such as the RB (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996), E47 (Zheng *et al.*, 2004) and ETS proteins (Figure 3.1) (Yates *et al.*, 1999). Studies have shown that the ID2 protein interacts with the RB protein, and that ID2 can reverse the cell cycle arrest caused by the overexpression of the tumour suppressor RB protein and the related ‘pocket’ proteins p107 and p130 (Iavarone *et al.*, 1994). More information on the interaction of ID2 with RB is presented in chapter 4.

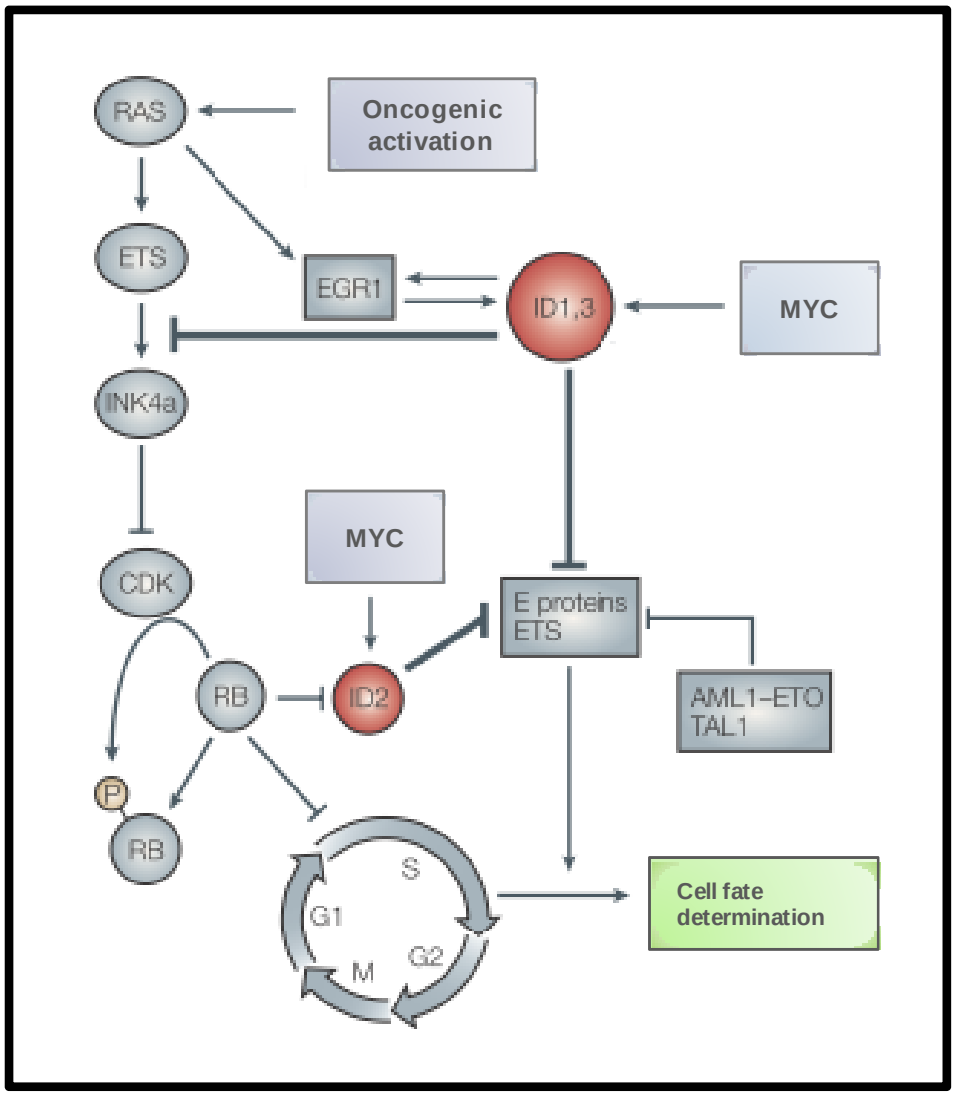

Figure 3.1: Model for ID function in cell cycle progression.

ID1, ID2 and ID3 family proteins act by inhibiting the E-proteins and ETS family members. ID1 and ID3 indirectly inactivate the RB protein by suppressing the expression of cyclin-dependent kinase inhibitors such as INK4a, also known as p16, which leads to the phosphorylation of RB. ID2 directly interacts with RB and interferes with its cell cycle regulatory functions (adapted from Perk *et al.*, 2005).

The ID1, ID2 and ID3 proteins are positive regulators of cell growth and negative regulators of differentiation (Ruzinova & Benezra, 2003). Studies have suggested that several human tumour types depend, at least in part, on ID expression for increased proliferation and other phenotypic features of malignancy (Kleeff *et al.*, 1998; Lin *et al.*, 2000). Therefore, it is important to investigate other changes resulting from the overexpression of ID proteins. It has been shown that pol III transcripts are present in increased levels in ovarian tumours compared to the normal tissues from the same individual patients (Winter *et al.*, 2000). Moreover, the overexpression of pol III transcripts has been demonstrated in breast, cervical and thyroid cancers with the same types of carcinomas observed when ID1, ID2 or ID3 are overexpressed (Chen *et al.*, 1997a,b; Lasorella *et al.*, 2001). Studies have revealed that oncoproteins such as c-Myc, CK2 and ERK are likely to affect cell growth by regulating pol III activity (Gomez-Roman *et al.*, 2003; Johnston *et al.*, 2002; Felton-Edkins *et al.*, 2003a,b). Pol III-transcribed genes are essential for cell growth and their expression becomes downregulated in differentiating cells (White, 2001; Alzuherri & White, 1998). Given that pol III activity and the above ID family members are required in the same processes, it would be interesting to investigate whether the expression of ID proteins would have an effect on pol III transcription. The following experiments presented in this chapter were performed to determine if indeed this is occurring. The results here demonstrate that pol III transcription is regulated by the ID1, ID2 and ID3 proteins.

3.2 Results

3.2.1 Knockdown of endogenous ID1, ID2 or ID3 by siRNA reduced the levels of pol I and pol III transcripts

To investigate the role of ID1, ID2 and ID3 in the regulation of pol III transcription *in vivo*, ID1, ID2 or ID3 were knocked-down using small interfering RNA. HeLa cells were transfected with siRNA targeting ID1, ID2 or ID3 and the control Oct-1. The cells were treated for 72 hours, harvested and protein and RNA extracts were analysed. RNA was analysed by RT-PCR in order to confirm the knockdown of ID1, ID2 or ID3 RNA levels in comparison to the control (Figure 3.2, A; Figure 3.4, A; Figure 3.6, A). Western blotting showed that ID1, ID2 or ID3 proteins levels decreased compared to the control (Figure 3.2, B; Figure 3.4, B; Figure 3.6, B).

To ensure the specificity of ID1, ID2 and ID3 siRNA the expression levels of their related proteins was investigated. Western blot analysis revealed that in ID1 siRNA-transfected HeLa cells the related proteins ID2 and ID3 and the associated E47 remained at the same levels (Figure 3.2, B). Knockdown of ID2 by siRNA targeting HeLa cells established the specificity of ID2 siRNA with the levels of the ID1, ID3 and E47 protein expression remaining unaltered (Figure 3.4, B). Also, it was found that in ID3 siRNA-transfected cells the knockdown effect was specific and the expression levels of ID1, ID2 and E47 were not affected (Figure 3.6, B). Therefore, specific silencing of a particular ID protein did not result in the silencing or overexpression of another related ID family member or the associated E47 protein.

The next step was to investigate if the knockdown of protein and RNA levels of the ID family members would have an effect on pol III transcription. RT-PCR analysis demonstrated that the knockdown of ID1, ID2 or ID3 resulted in reduced levels of pol III-transcribed 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, 7SL RNA, 7SK RNA, MRP RNA and U6 snRNA compared to the control siRNA transfection (Figure 3.3; Figure 3.5; Figure 3.7). Moreover, the knockdown of ID2 or ID3 decreased the levels of the pol I transcript, precursor rRNA (pre-rRNA) (Figure 3.5; Figure 3.7). These effects are specific, since

the pol II-transcribed acidic ribosomal phosphoprotein P0 (ARPP P0) mRNA does not change due to ID1, ID2 or ID3 knockdown. In conclusion, these results here suggest that all three ID family members analysed can have a positive effect on pol III activity *in vivo*, with ID2 or ID3 regulating pol I transcription as well.

RNA interference is a system for post-transcriptional gene silencing that can be applied to the study of gene function. siRNAs target the homologous mRNA transcripts for destruction. In the case of sequence identity between the siRNA and random mRNA transcripts, RNAi can lead to the suppression of non-targeted genes, a phenomenon known as off-target effects. Computational studies have shown that off-target effects mediated by RNAi in the *Homo sapiens*, *Schizosaccharomyces* and *Caenorhabtitis elegans* genomes range from 5 to 80% in each organism (Qiu *et al.*, 2005). It was also demonstrated that the optimal length for siRNAs was 21 nt in order to achieve high target efficiency and decreased off-target effects. When the siRNA length increased this led to higher chances of non-specific targeting (Qiu *et al.*, 2005). dsRNAs are also implicated in the induction of the innate immune response (Sledz *et al.*, 2003). The use of siRNAs which are longer than 23 bp can have an effect on cell viability and cause an interferon response, demonstrated by an upregulation of the dsRNA receptor TLR3, in a cell type-dependent manner (Reynolds *et al.*, 2006). The length threshold for siRNA stimulation of the interferon response varies in different cell lines (Reynolds *et al.*, 2006). The transfection of 21 bp ds siRNA used for gene silencing leads to interferon mediated activation of the Jak-Stat signalling pathway and the induction of interferon stimulated genes (Sledz *et al.*, 2003). These results are due to the protein kinase PKR, since this kinase becomes induced by siRNA and INF-β activation. The stimulation of interferon and other signalling pathways demonstrates that siRNAs also have targets other than specifically silenced target genes (Sledz *et al.*, 2003).

Furthermore, off-target effects can occur due to seed-sequence-dependent binding (Jackson *et al.*, 2006). Microarray experiments have demonstrated that the use of siRNA leads to the silencing of several off-target transcripts (Jackson *et al.*, 2003; Scacheri *et al.*, 2004). Analysis of the off-target transcripts showed sequence complementarity with the 5’ end of the siRNA guide strand (Lin *et al.*, 2005). This region of the guide strand has analogy to the seed region at the 5’ end of a microRNA

that is involved in gene silencing (Lai, 2002). It has been shown that the sequence complementarity to the seed region of the siRNA is an important determinant of the regulation of unintended target transcripts (Jackson *et al.*, 2006). The interpretation of results using siRNA to knockdown expression of genes should be carefully evaluated since off-target effects represent a real concern. Strategies could be used to identify off-target genes as false positives and remove them from regulated transcripts. These off-target effects should be taken into account, since the use of RNAi technology has moved towards therapeutic applications.

The ID family members have been reported to be involved in the regulation of cell growth (Barone *et al.*, 1994). Antisense oligonucleotides against ID1, ID2 and ID3 were used to determine the effect on growth induction. DNA synthesis, measured by BrdU incorporation was decreased upon addition of the above antisense oligonucleotides in NIH 3T3 fibroblasts (Barone *et al.*, 1994). When all three oligonucleotides were added together the inhibition on cell proliferation was even greater. To determine whether the effect from adding antisense oligonucleotides was reversible, the oligonucleotides were removed which led to an increase of BrdU incorporation and after 12 hours the recovery was complete. This demonstrated that the treatment of cells with these oligonucleotides was not toxic and the reduction in DNA synthesis could be reversed. Also, depletion of ID1 and ID2 using an antisense oligonucleotide strategy in TIG-3 cells resulted in inhibition of cell proliferation (Hara *et al.*, 1994). Cells were labelled with [^{3}H] thymidine at 6 hour intervals and the radioactivity incorporated into acid-insoluble material was monitored. In cells treated with the antisense oligonucleotides, DNA synthesis was reduced by half compared to the TIG-3 cells treated with the control oligonucleotide (Hara *et al.*, 1994). These results suggest that G1 progression and entry into S phase was reduced by the depletion of the ID1 and ID2 protein levels. Furthermore, in order to investigate the role of ID expression in mammary epithelial cell proliferation, ID1 was silenced using an siRNA approach (Swarbrick *et al.*, 2005). In MCF-7 cells treated with siRNA against ID1, DNA synthesis was reduced to about one-third within 24-48 hours post transfection. Flow cytometry analysis detecting both parameters such as BrdU/DNA content demonstrated

that siRNA against ID1 led to the accumulation of cells in G1 phase (Swarbrick *et al.*, 2005). Therefore, downregulation of ID1 resulted in impaired proliferation.

Other studies have demonstrated that the knockdown of ID1 and ID2 in the RIE-1 (rat intestinal epithelial 1) cells induces apoptosis (Cao *et al.*, 2009). siRNA to target ID1, ID2 and ID3 gene expression was used. The efficiency of siRNA knockdown of both protein and RNA levels was measured by western blotting and Q-PCR. The targeting of ID2 by siRNA lead to the induction of apoptosis, with the effects being greater when compared to ID1, whereas ID3 siRNA did not show induction of apoptosis. Apoptotic cells were stained with Annexin V-FITC and the RIE-1 cells were analysed by flow cytometry (Cao *et al.*, 2009). In further experiments the knockdown of ID1 and ID3 by siRNA resulted in decreased proliferation, however, the loss of ID2 did not have an effect on cell proliferation in the LNCaP and DU145 prostate cancer cell lines (Asirvatham *et al.*, 2006). The results were further investigated by FACS (Fluorescence-Activated Cell Sorting) analysis. It was demonstrated that in the ID2 siRNA transfected LNCaP cells there was an increase in the percentage of cells undergoing apoptosis (Asirvatham *et al.*, 2006). These results demonstrate that ID2 is involved in cell survival. Since ID proteins are involved in cell proliferation and cell survival the biological consequences of knocking down ID proteins should be further investigated. A possible explanation for the effects demonstrated upon knockdown of ID proteins on pol III transcription could be indirect effects attributed to the decrease in cell proliferation. The reduction of cell growth could then result in the decrease of pol III activity. However, chromatin immunoprecipitation assays and co-immunoprecipitation experiments in the next chapter reveal a possible direct effect of ID proteins on pol III transcription. Therefore, it is possible that the ID proteins are able to control cell proliferation and cell survival by directly affecting pol III transcription, since pol III products are involved in protein synthesis and cell growth.

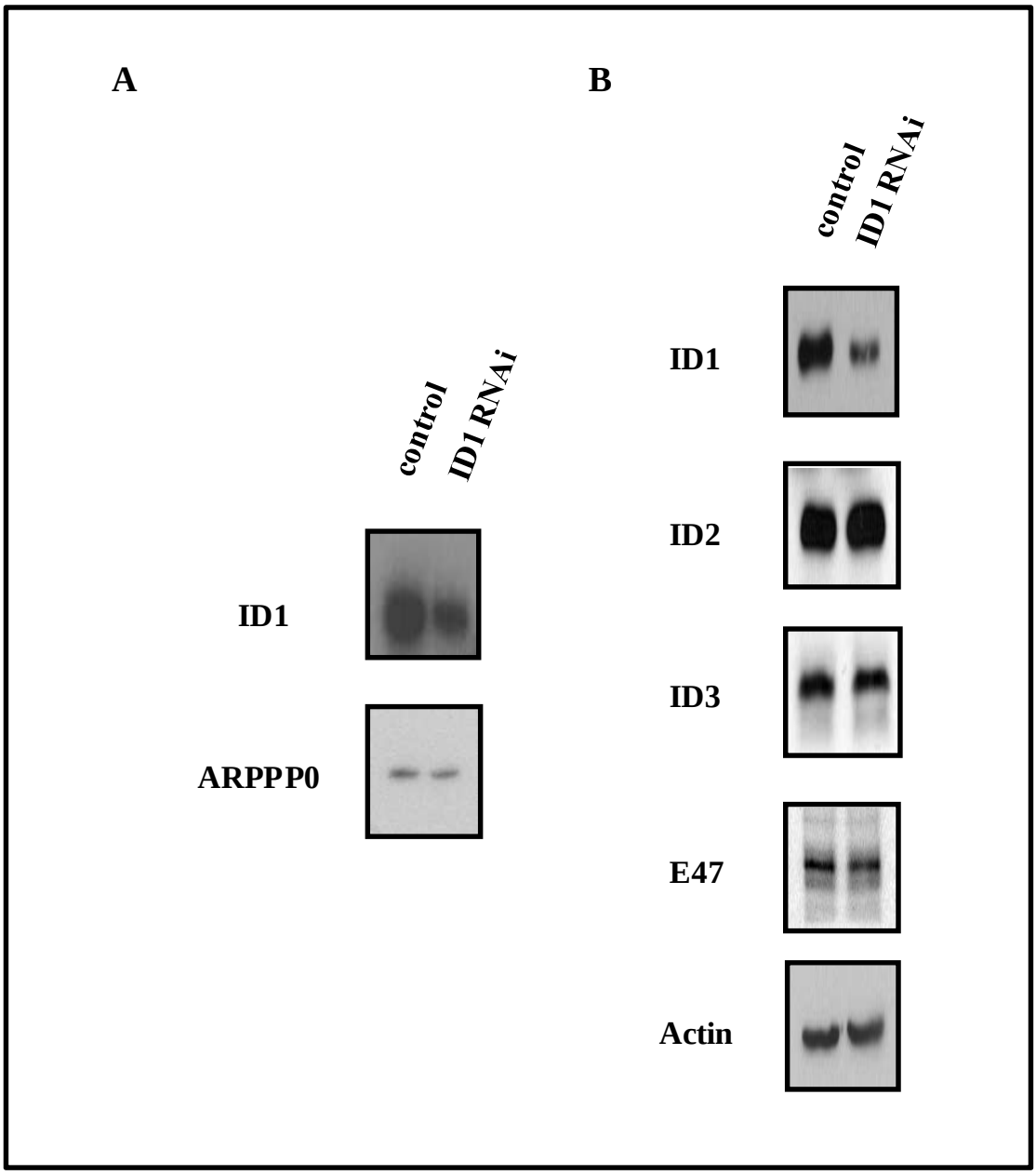

Figure 3.2: Knockdown of endogenous ID1 by siRNA.

HeLa cells were transfected with ID1 siRNA and siRNA targeting Oct-1 was used as control. **A.** RT-PCR analysis for ID1 depletion by siRNA. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control. **B.** Western blot analysis of ID1, ID2, ID3 and E47 expression, following transfection with siRNA against ID1. Actin was used as loading control.

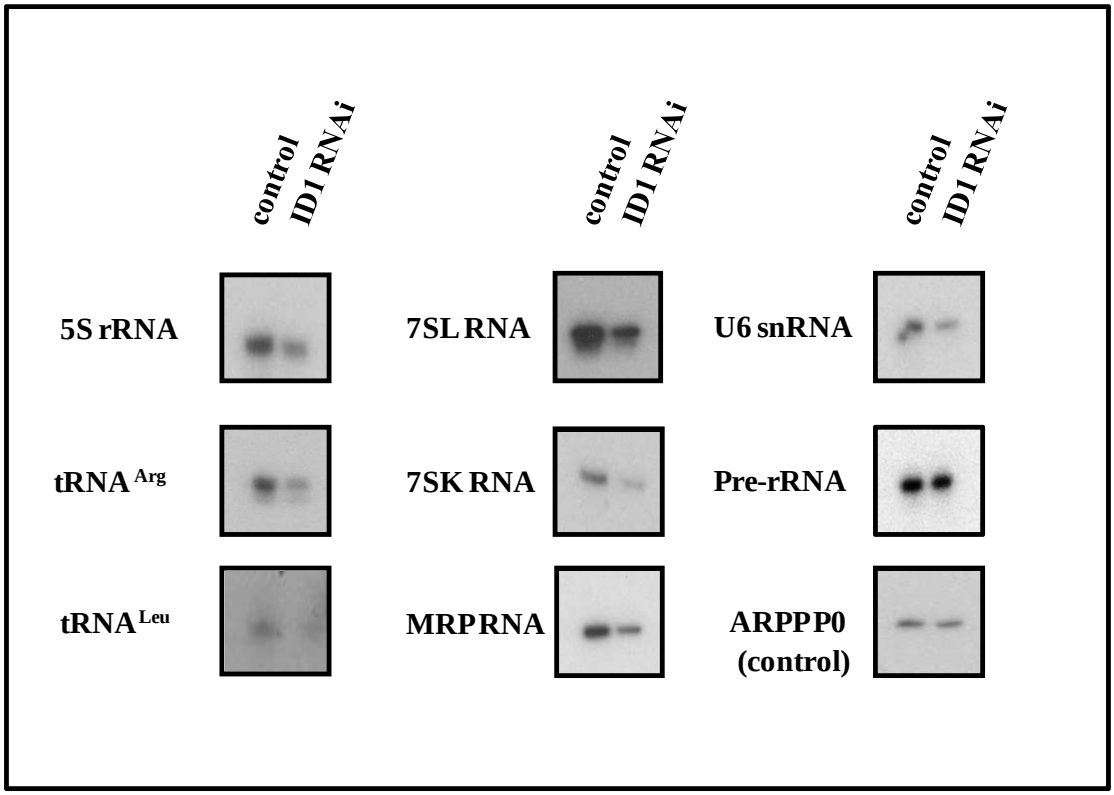

Figure 3.3: Knockdown of ID1 by RNAi reduces the levels of pol III transcripts.
Effects after ID1 knockdown by siRNA in HeLa cells, 72 hours post-transfection. RNA was harvested and analysed by RT-PCR for the expression of the pol III transcripts 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, 7SL RNA, 7SK RNA, MRP RNA and U6 snRNA. The pol I transcript pre-rRNA was also analysed. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control.

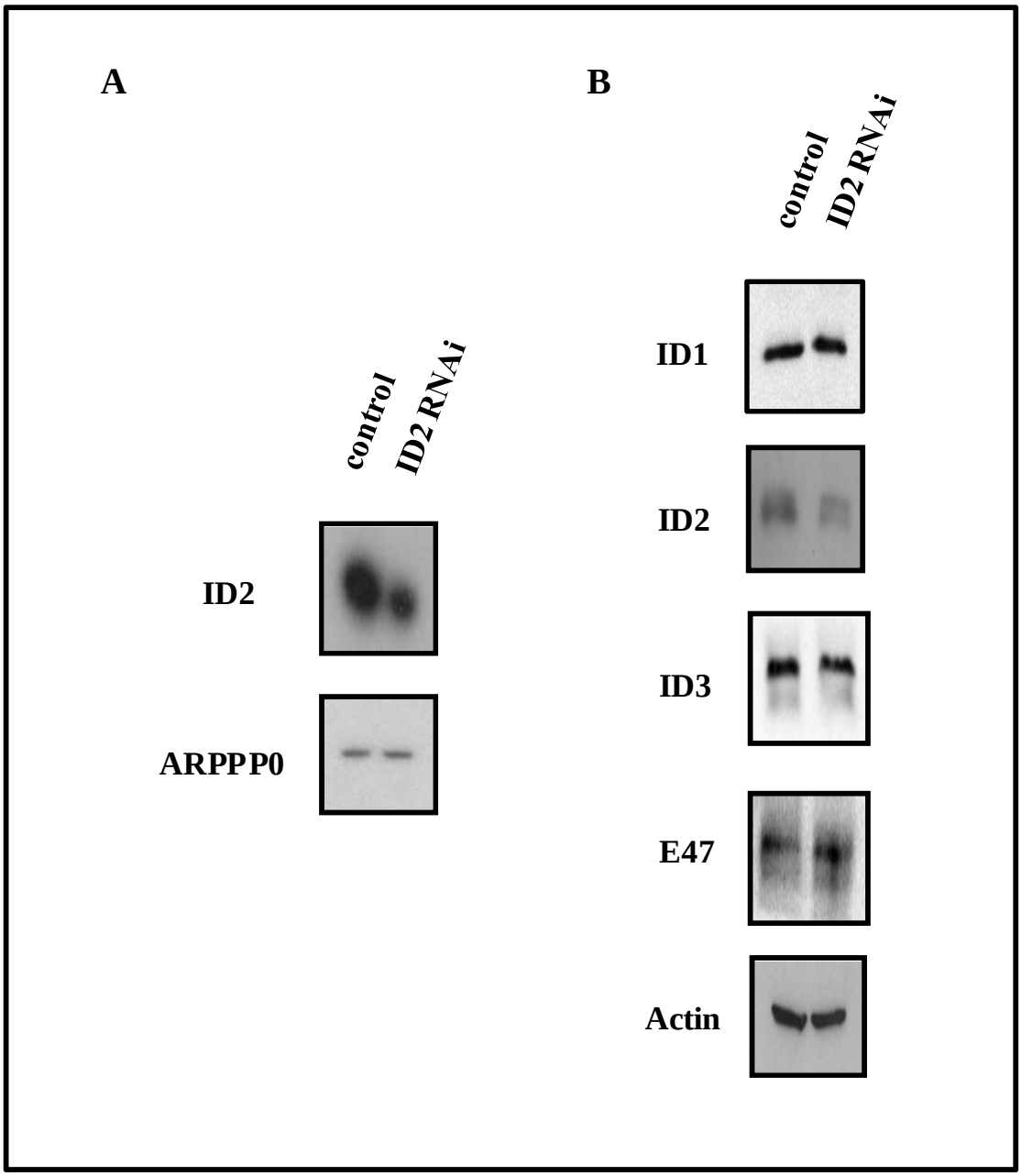

Figure 3.4: ID2 knockdown by siRNA.

HeLa cells were transfected with ID2 siRNA or the control Oct-1 siRNA and harvested 72 hours later. **A.** RT-PCR for ID2 depletion by siRNA. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control. **B.** Western blot analysis of ID1, ID2, ID3 and E47 expression, following transfection with siRNA against ID2. Actin was used as loading control.

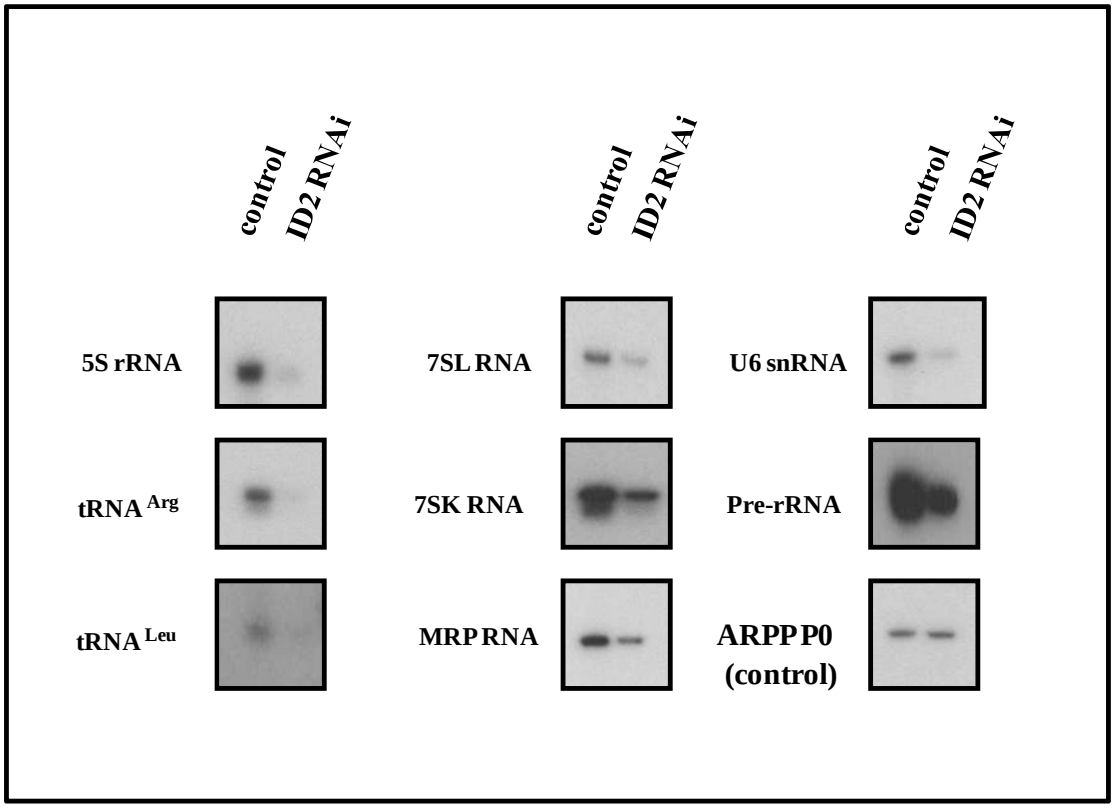

Figure 3.5: Knockdown of endogenous ID2 by RNAi decreases the levels of pol I and pol III transcripts.

Effects of ID3 siRNA knockdown in HeLa cells 72 hours post transfection. RNA was harvested and analysed by RT-PCR for the expression of the pol III transcripts 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, 7SL RNA, 7SK RNA, MRP RNA and U6 snRNA. The pol I transcript pre-rRNA was also analysed. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control.

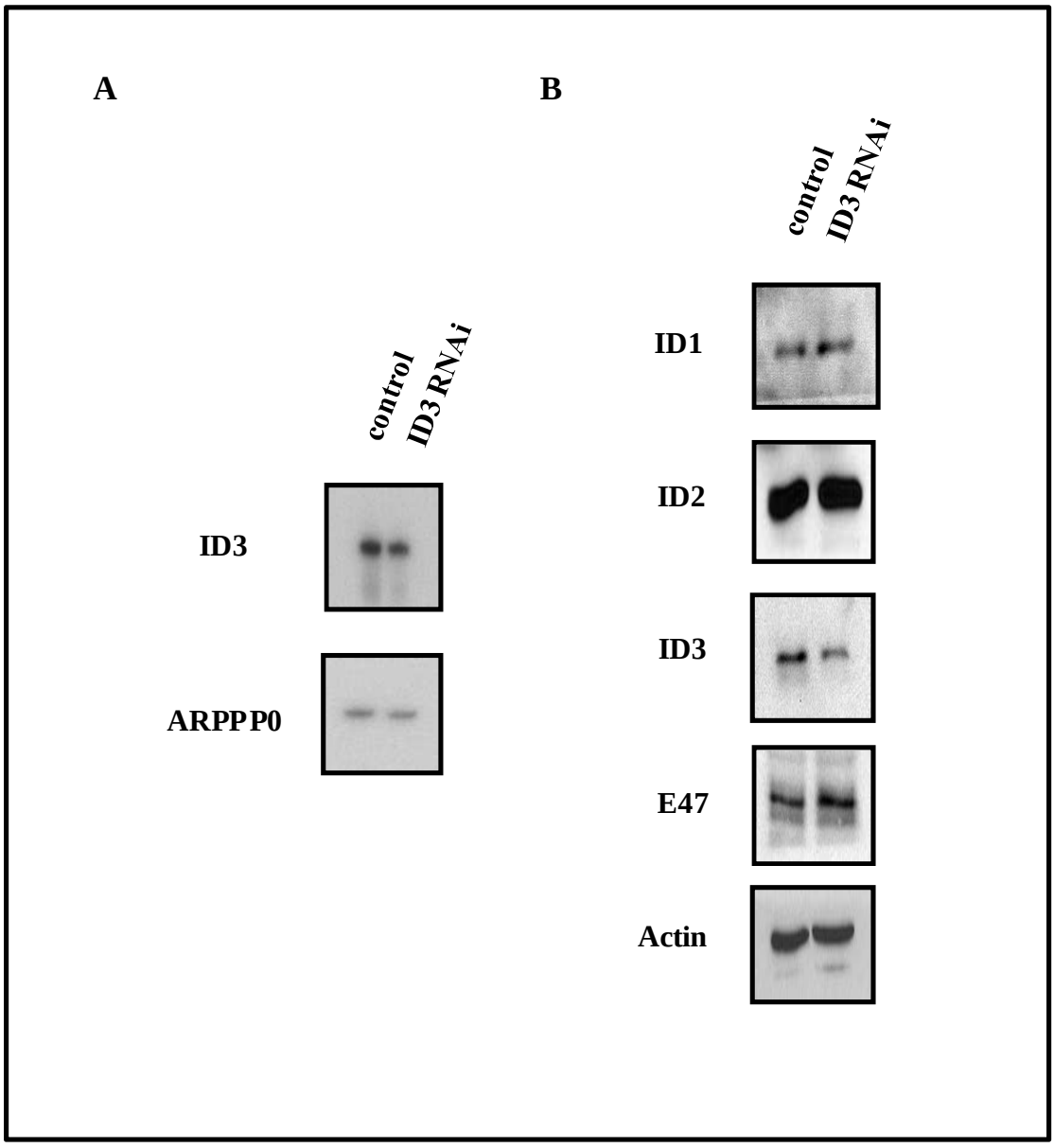

Figure 3.6: Knockdown of ID3 by siRNA.

HeLa cells were transfected with ID3 siRNA or the negative control Oct-1 siRNA and harvested 72 hours later. **A.** RNA was analysed by RT-PCR for ID3 depletion by siRNA. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control. **B.** Western blot analysis of ID1, ID2, ID3 and E47 expression, following transfection with siRNA against ID3. Actin was used as loading control.

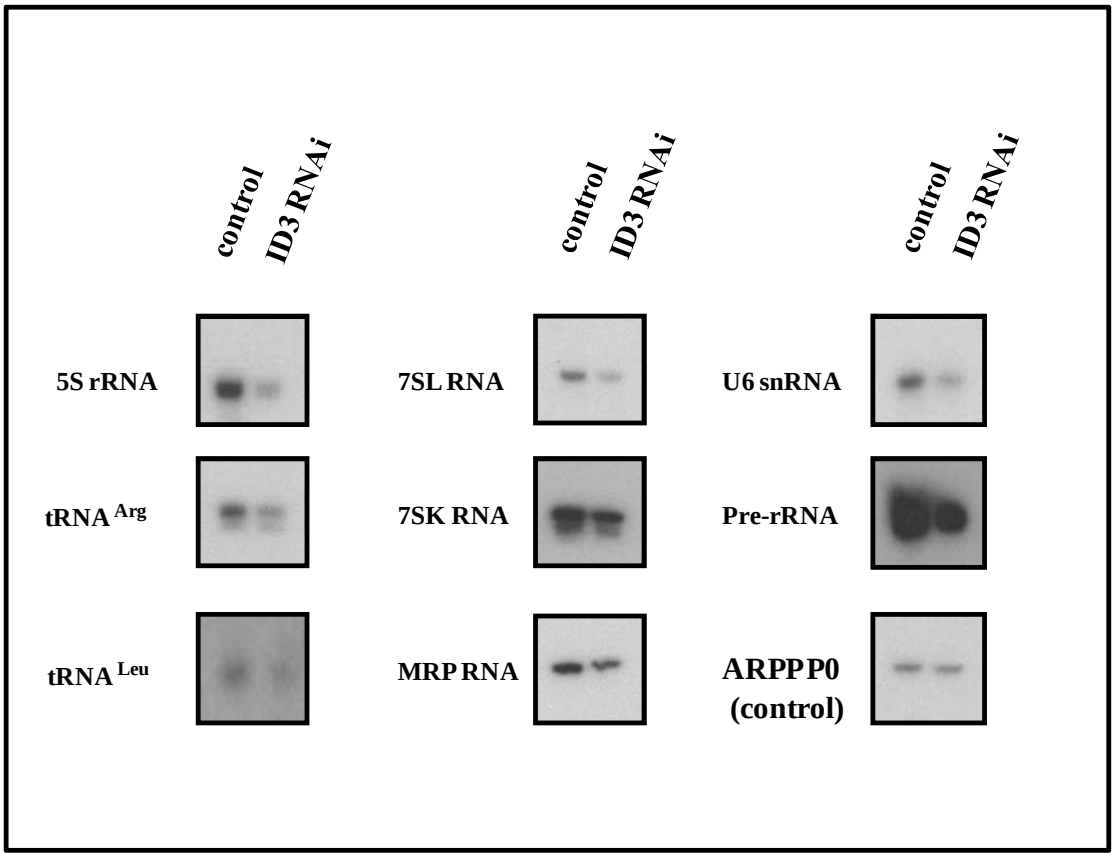

Figure 3.7: Knockdown of ID3 by RNAi diminishes the levels of pol I and pol III transcripts.

HeLa cells were transfected with ID3 siRNA and harvested 72 hours after transfection. RNA was analysed by RT-PCR for the expression of 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, 7SL RNA, 7SK RNA, MRP RNA and U6 snRNA. RT-PCR analysis was also performed for the pol I transcript pre-rRNA. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control.

3.2.2 Overexpression of ID2 raises the levels of pol III transcripts

Since ID2 overexpression can stimulate cell growth (Iavarone *et al.*, 1994), it would be interesting to determine whether ID2 can upregulate pol III transcription *in vivo* in human cells using adenoviral infection methods to achieve ID2 expression. HeLa cells were infected with the parental virus adenovirus-cytomegalovirus 5' internal ribosomal entry green fluorescent protein (Ad-Vector) or with an adenovirus expressing ID2 (Ad-ID2). Cells were infected with the above adenoviruses for 30 hours and then harvested. Western blot analysis demonstrated that infection of cells with the ID2 adenovirus resulted in overexpression of the ID2 protein levels compared to the control vector (Figure 3.8, A). This is due to a specific effect since actin was used as loading control and the levels in both lanes remained the same. RNA was analysed by RT-PCR and revealed that infected ID2 cells resulted in an increase in the RNA levels of ID2 compared to the control vector (Figure 3.8, B). The ARPP P0 mRNA was used as loading control and was the same in both conditions suggesting that this effect is specific. To examine the role of the increased protein and RNA levels of ID2 on pol III transcripts, RT-PCR experiments were performed. The results showed that the levels of pol III-transcribed $tRNA^{Leu}$, 5S rRNA, $tRNA^{Tyr}$, MRP RNA, $tRNA^{Leu}$ and U6 snRNA are raised compared to the control vector. The effect is specific, as the pol II-transcribed ARPP P0 does not change in response to ID2 overexpression. These data demonstrate that overexpression of ID2 induces pol III transcription.

To investigate the effect of ID2 on cell proliferation and in cell cycle progression, ID2 has been overexpressed in the human osteosarcoma cell line U2OS (Iavarone *et al.*, 1994). The human ID2 cDNA was cloned into a pMEP4 vector which has a metalloprotein promoter regulating the expression of ID2. The pMEP4(ID2) was then transfected into the U2OS cell line. The U2OS cell line expresses functional p53 and RB therefore it can be employed to study cell proliferation. To demonstrate the effect of ID2 overexpression on cell proliferation two expression vectors were compared, the pMEP4(ID2) vector and the control pMEP4. ID2 overexpression led to

the shortening of the cell doubling time (Iavarone *et al.*, 1994). Also, the saturation density of cells where ID2 was overexpressed was higher than that of cells transfected with the control vector. To determine the effect of ID2 overexpression on cell cycle progression FACS analysis was employed. Flow cytometry was used to monitor cells labelled with BrdU. The ID2 expressing population demonstrated an increase in the number of cells in S phase and a decrease in the number of cells in G1 phase (Iavarone *et al.*, 1994). In another study, the neuroblastoma SK-N-SH cell line was used to determine the effect of ID2 overexpression (Rothschild *et al.*, 2006). ID2 was overexpressed in the SK-N-SH cell line using an adenovirus expression system and compared to cells infected with a control vector. Cells were infected and labelled with BrdU. BrdU incorporation assays demonstrated that infection with adeno-ID2 lead to increased entry of cells into S phase within 20 hours (Rothschild *et al.*, 2006). Collectively these results demonstrate that ID2 expression can affect the growth potential of cells.

An important feature of the tumour suppressors RB and p53 is their ability to inhibit cell proliferation (Huang *et al.*, 1988; Baker *et al.*, 1990). It has been demonstrated that the SAOS-2 cell line does not have functional RB and p53 and the expression of these two suppressors in these cells leads to decreased proliferation of SAOS-2 cells (Diller *et al.*, 1990; Qin *et al.*, 1992). In order to investigate whether the effect of enhancement of cell proliferation upon ID2 expression in U2OS cells was due to regulation of RB or p53, ID2 was co-expressed with RB and p53 in SAOS-2 cells (Iavarone *et al.*, 1994). The expression of RB in SAOS-2 cells inhibited the colony formation of these cells by 63%-73%. In contrast, when ID2 was co-expressed this resulted in a reduction of colony formation by about only 15%-30% (Iavarone *et al.*, 1994). However, when p53 was expressed in SAOS-2 cells, ID2 co-expression was unable to affect the suppression of cell growth exhibited by p53. From previous work, p53 has been demonstrated to be involved in the regulation of ID proteins (Qian & Chen, 2008; Wilson *et al.*, 2001). ID1, ID2 and ID3 protein expression levels correlate with p53 expression in human colorectal adenocarcinomas (Wilson *et al.*, 2001). ID protein levels are increased in p53 knockout mice (Wilson *et al.*, 2001). In addition, ID1 has been found to be downregulated by DEC1. Interestingly, DEC1 has been found to be

targeted by p53. The DEC1 protein is present at the promoter of the ID1 gene and this leads to the suppression of ID1 expression (Qian & Chen, 2008). The HEK 293 cell line used in this thesis expresses functional RB. HeLa cells have been shown to express the E7 oncoprotein, which binds and inactivates RB (Gage *et al.*, 1990). Two mechanisms have been proposed to be involved in the inactivation of RB by E7. The first one involves the competition with E2F for the binding to RB. This is proposed since the RB-E2F binding complex is lacking in cell lines tested (Chellappan *et al.*, 1992). *In vitro* experiments have also demonstrated that E7 can inhibit the association of E2F with RB (Chellappan *et al.*, 1992). The second mechanism of inactivation of RB by E7 is by the destabilisation of RB. The expression of E7 decreases the levels of RB in several cell lines and this is due to posttranslational events (Berezutskaya *et al.*, 1997; Boyer *et al.*, 1996). It has been demonstrated that the E7 oncoprotein induces degradation of the RB protein through the ubiquitin proteasomal pathway (Boyer *et al.*, 1996). In the case of HeLa cells p53 is expressed in low levels, which are altered in function as a consequence of association with the HPV E6 oncoprotein (Scheffner *et al.*, 1991). Whereas the HEK 293 cells express functional p53. In the future, in addition to HeLa cells another cell line could be used which expresses functional p53 and RB, in order to repeat these experiments and correlate these results to the presence of p53 and RB. The ID2 protein has been demonstrated to bind to the RB protein family members and suppress their function. In addition, it has been shown that ID2 competes with RB for TFIIIB and this could be one mechanism by which ID2 stimulates pol III transcription (Gomez-Roman N, unpublished observations). Furthermore, the overexpression of ID2 in fibroblasts derived from RB knockout mice still resulted in induction of pol III transcription, although this was lower than in wild-type control cells, suggesting that ID2 can also activate pol III transcription by an RB-independent mechanism (Gomez-Roman N, unpublished observations). In the future, experiments should be performed on pol III transcription to study the biological response on cell growth and apoptosis after depletion or overexpression of ID proteins. FACS analysis should be used to determine the biological consequences such as cell cycle progression and cell viability upon depletion or overexpression of ID proteins.

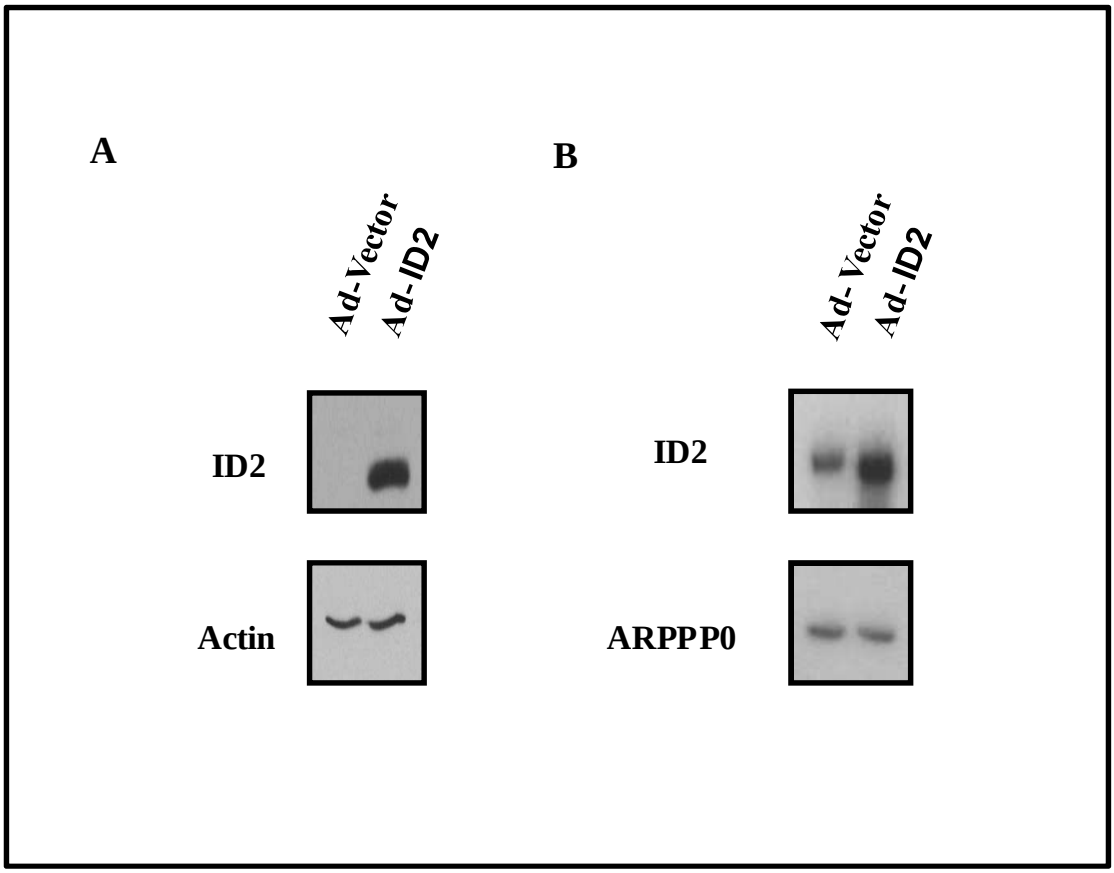

Figure 3.8: ID2 overexpression by adenoviral infection.

HeLa cells were infected using an ID2 adenovirus expressing vector or control vector. **A.** Western blotting analysis of ID2 after cells were infected for 30 hours with adeno-ID2 (Ad-ID2) or adenovirus vector (Ad-Vector). Actin was used as control. **B.** RT-PCR analysis for the overexpression of ID2 mRNA. mRNA from the pol II-transcribed ARPP P0 gene was used as loading control.

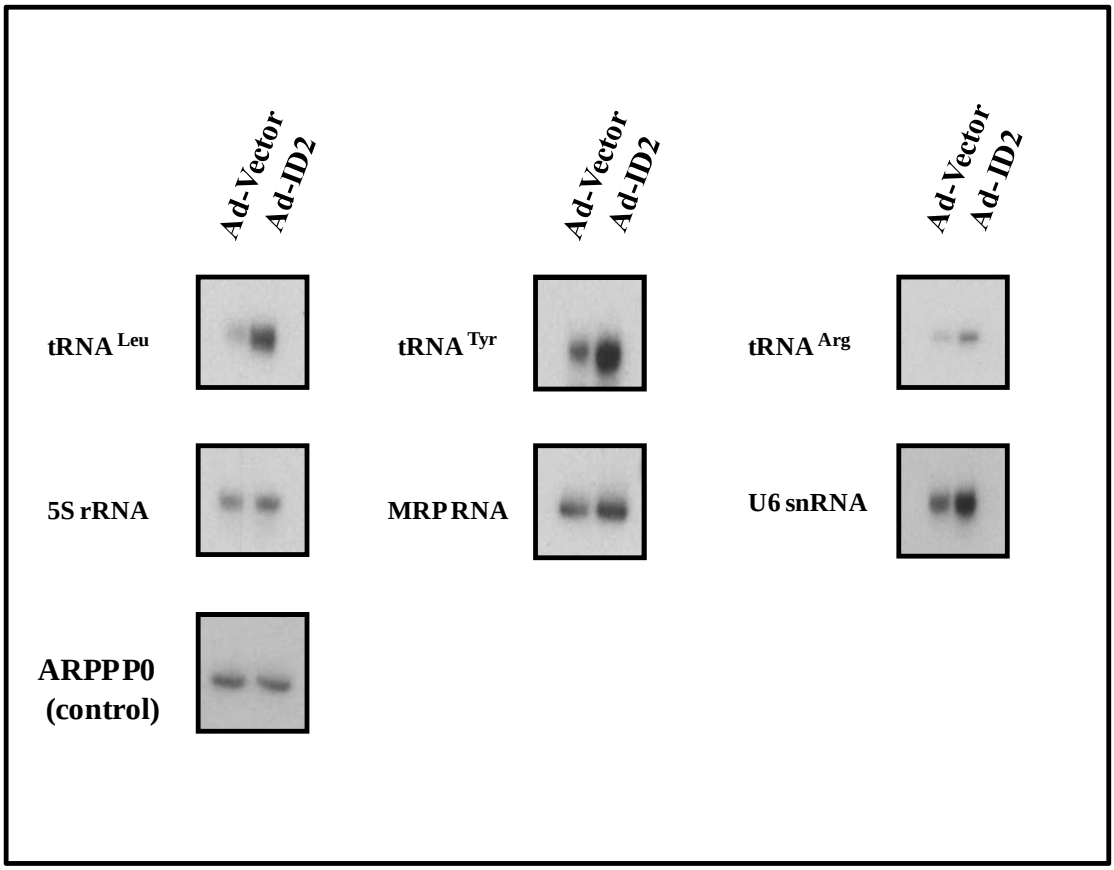

Figure 3.9: Overexpression of ID2 by adenoviral infection raises the levels of pol III transcripts.

Effects from using an ID2 expressing vector in HeLa cells. Cells were harvested 30 hours post infection with Ad-ID2 or Ad-Vector. RNA was harvested and analysed by RT-PCR for the expression of the pol III transcripts $tRNA^{Leu}$, 5S rRNA, $tRNA^{Tyr}$, MRP RNA, $tRNA^{Leu}$ and U6 snRNA. The ARPP P0 mRNA was used as control.

3.2.3 ID2 stimulates pol III transcription *in vitro*

Since previous experiments shown in figures 3.5 and figure 3.9 have demonstrated that ID2 regulates pol III transcription *in vivo* when using RNAi or adenoviral infection, it would be interesting to investigate if this is the case *in vitro*. To further confirm that endogenous ID2 stimulates pol III transcription, HeLa nuclear extracts were depleted using an antibody against ID2. *In vitro* transcription was performed using the adenovirus VA1 gene as template. As a negative control the pol II-specific factor TFIIB was used, whereas, as positive control nuclear extracts were depleted of TBP, since this protein is required for pol III transcription. Given that ID2 depletion *in vivo* by RNAi was shown to reduce pol III activity in HeLa cells, depletion of ID2 *in vitro* is likely to affect pol III activity. HeLa extracts depleted of TBP demonstrate a reduction in VA1 transcription compared to the control TFIIB-depleted extracts (Figure 3.10). The extracts depleted from ID2 using an anti-ID2 antibody show decreased VA1 transcription (Figure 3.10). Therefore these data suggest that endogenous ID2 can stimulate pol III activity *in vitro*. Further experiments should be done in order to determine the effect of the immunodepletion of ID2 on pol III transcription. Immunodepletion of ID1 and ID3 could also be performed to investigate the effects of these proteins on pol III transcription. Control experiments should be included such as the use of an undepleted control. Also, a secondary control that does not affect pol III transcription should be used. In order to eliminate the possibility that the effect on pol III transcription shown after immunodepletion of ID2 was not due to non-specific immunodepletion of other proteins, pre-immune serum against ID2 should be used as a control. Rescue with addition of ID2 could also be done as a control. Furthermore, western blot analysis should be done in order to assess the specificity and efficacy of immunodepletion. Detection of the levels of ID2, TFIIB and TBP proteins compared to the undepleted controls should be performed by western blotting to measure the efficacy of the immmunodepletion of these proteins. The above experiments should be repeated in order to assess the reproducibility of the experiments and to quantify the levels of immunodepletion of the above proteins and the effect on pol III transcription using the ImageJ program.

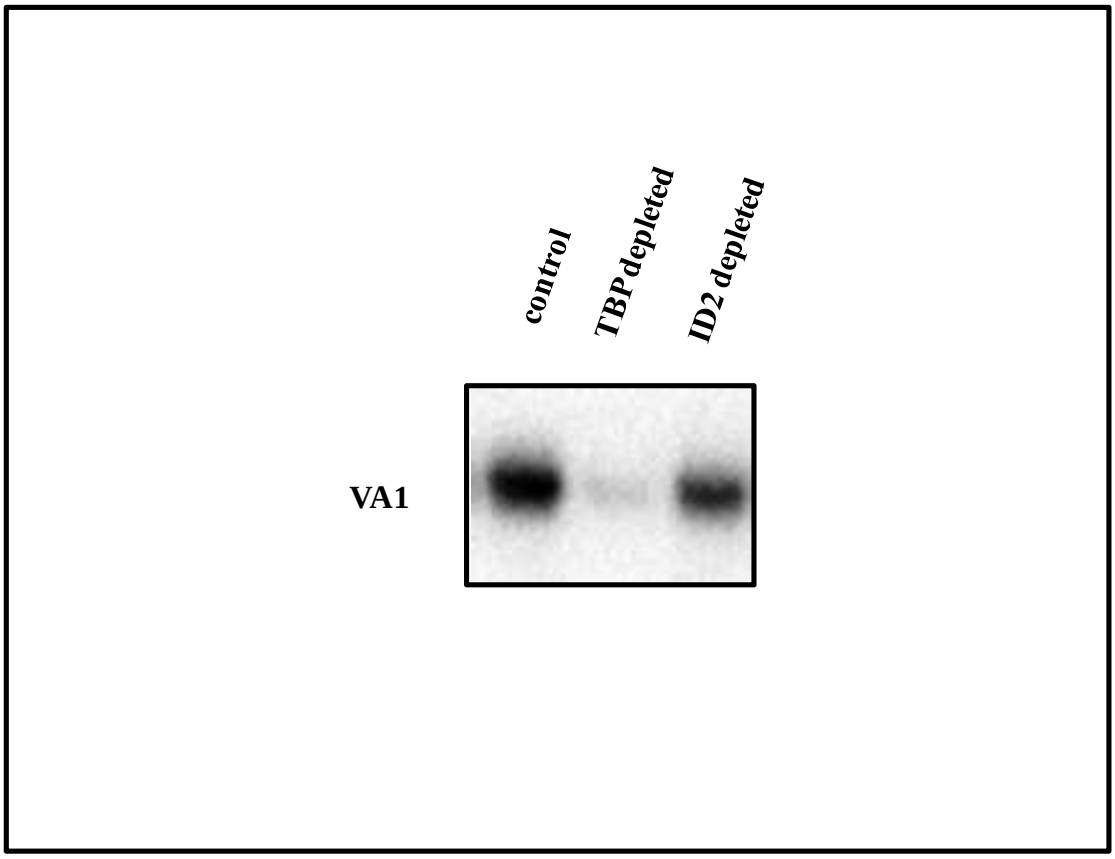

Figure 3.10: ID2 immunodepletion reduces pol III activity.

In vitro transcription of the VA1 gene was analysed in HeLa nuclear extracts. Samples were immunodepleted for 2 hours on ice with antibodies against the TFIIB control (C-18), TBP (mTBP-6) or ID2 (C-20).

3.3 Discussion

The ID1, ID2 and ID3 family members are known to function in the regulation of cell growth by impinging on cell proliferation, cell fate determination and angiogenesis (Norton *et al.*, 1998; Israel *et al.*, 1999). One of the ID members, ID2, is a direct functional target of the RB suppressor protein and deregulated expression of ID proteins may lead to proliferative progression of tumours (Lasorella *et al.*, 2000). Therefore, it is possible that tumour cells promote the expression of ID2 to inhibit the RB pathway which is implicated in cancer (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). Also, the ID1 and ID3 genes might be involved in the recruitment of blood vessels in human tumourigenesis (Lyden *et al.*, 1999). The disruption of ID1 and ID3 resulted in the failure to support the growth of tumours and metastasis in mice, suggesting a role for these proteins in neoangiogenesis, which is an important process required for tumour growth. Overexpression of ID proteins stimulates an increased rate of cell proliferation in erythroleukaemia and myoblast cells in addition to inhibiting cell differentiation (Lister *et al.*, 1995; Atherton *et al.*, 1996). So, ID proteins function by positively affecting cell growth and pol III transcripts are required for maintaining cell growth. Therefore, it is likely that positive regulators of cell growth could induce pol III transcription.

In order to determine if ID1, ID2 or ID3 are able to regulate pol III activity in human cells *in vivo* an RNAi approach was used. HeLa cells were transfected with siRNA targeting ID1, ID2 or ID3 and this treatment resulted in successful knockdown of RNA and protein levels of ID1, ID2 or ID3 compared to the controls (Figure 3.2; Figure 3.4; Figure 3.6). Previous studies have shown that the knockdown of ID family members transfected with siRNA was specific. Silencing of one of the ID1, ID2 or ID3 members by siRNA did not result in repression or overexpression of other ID family members in prostate cancer cell lines (Asirvatham *et al.*, 2007). Furthermore, knockdown of the ID1 gene by siRNA was specific with the expression of the other ID proteins remaining the same compared to the control siRNA treated cells (Zheng *et al.*, 2004). To investigate the efficiency and specificity of ID1, ID2 or ID3 siRNA transfected cells, the levels of

their targets were determined. Western analysis revealed that the knockdown of ID1 by siRNA did not alter the protein levels of other ID family members and the associated E47 protein (Figure 3.2, B). Similarly, the ID2 or ID3 knockdowns were efficient and this depletion did not lead to the silencing or overexpression of their related proteins and the associated E47 protein (Figure 3.4, B; Figure 3.6, B). These data demonstrate that the knockdown of ID family members is specific and suggest the absence of any regulatory cross-talk between the ID members or the E47 expression levels.

Oct-1 has some limitations as a control since it is a basal transcription factor which enhances SNAPc/TFIIIB recruitment although it is not essential for transcription. It is present on type III promoters such as those of the U6 and 7SK pol III genes. Oct-1 removal causes a delay of 30 minutes for the onset of transcription. This might be a cause for concern because Oct-1 is involved in transcription of type III genes (Hu *et al.*, 2003). In siRNA experiments knocking down ID proteins, additional controls to which the knockdown of ID proteins and the effects on pol III transcripts should be compared should be used. These include a mock control and non-silencing controls. Non-silencing controls refer to siRNA which does not target any protein sequence, but which is able to activate the siRNA pathway. This is important because a non-silencing control does not target a protein and should avoid non-specific targeting due to the use of siRNA. Also, a mock control using only lipofectamine 2000 without adding siRNA should be employed to investigate whether there is an effect mediated by the transfection reagent on the cells. Other techniques for the knockdown of ID proteins should be used in the future, in order to further validate the effects observed here. RT-PCR was used here, which has some limitations. Firstly, it provides a semi-quantitative measurement of the levels of transcripts. In the future, Q-PCR could be used. The advantages of Q-PCR are its large dynamic range being able to detect $>10^7$ fold differences in expression, and its sensitivity, potentially being able to detect even a single copy (Bustin *et al.*, 2005; Please see Introduction section, 9^{th} sentence of 1^{st} paragraph). Q-PCR gives the Ct (threshold cycle) values, which are compared by relative quantitation of samples. In the future, western blot experiments could be performed to detect the expression levels of the other ID protein family members, ID1 and ID3, and also of the associated E47 protein upon overexpression of ID2. Studies have demonstrated that when the ID2 or E47 proteins are

overexpressed the protein levels of each other are not affected (Rothschild *et al.,* 2006). Also, immunofluorescence could be used in order to determine where ID and E47 proteins are localised in the cell and whether they co-localise.

The following step involved the determination of whether these depletions would have an effect on pol III transcription. The results demonstrated that the expression of the pol III transcripts 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, 7SL RNA, 7SK RNA, MRP RNA and U6 snRNA was reduced in comparison to the controls (Figure 3.3; Figure 3.5; Figure 3.7). Furthermore, the ID2 or ID3 knockdown but not the ID1 depletion resulted in the decrease of the pol I transcript pre-rRNA (Figure 3.3; Figure 3.5; Figure 3.7). Possible explanations for this difference in the effect of this knockdown could be that in the case of the ID1 knockdown a more efficient depletion of ID1 protein and RNA levels might result in reduced expression of the pol I transcript pre-rRNA. Another possibility might be that although the family members share similarity in the HLH domain, differences in the rest of the sequence of ID members may be important for their interaction with factors of the pol I machinery. However, more studies are required to determine the role of ID members in the regulation of pol I transcription. These results demonstrate that the depletion of ID family members can regulate pol I and pol III transcription and indicates a role for IDs as activators in human cells *in vivo*. To investigate the effect of expressing ID2 on pol III transcription in human cells in *vivo*, HeLa cells were infected with adenoviruses expressing the ID2 or the control vector. Infection with the ID2 adenovirus resulted in increased expression of ID2 protein and RNA compared to the control (Figure 3.8). Moreover, the levels of the pol III transcripts $tRNA^{Leu}$, 5S rRNA, $tRNA^{Tyr}$, MRP RNA and U6 snRNA were raised after overexpression of ID2 in comparison to the control vector (Figure 3.9). From these results, it can be concluded that in both cases, knockdown or overexpression, ID family members demonstrated a positive effect on pol III activity.

The results in this chapter demonstrated that ID members can regulate pol III transcription *in vivo* indicating a positive role for this family in pol III activity under both siRNA and adenoviral expression conditions. To determine whether the above is true *in vitro*, nuclear extracts were depleted of ID2. In HeLa extracts the depletion of ID2 resulted in reduction of pol III transcription, in comparison to the pol II specific

factor TFIIB, used as control (Figure 3.10). These data indicate that the endogenous ID2 can positively regulate pol III transcription *in vitro*.

The results in this thesis demonstrate that both the knockdown and overexpression of ID proteins affect pol III transcription. In the next chapter ID proteins are shown to associate with the pol III transcription apparatus. ID proteins appear to possibly interact directly with the basal transcription factor Brf1. However, indirect effects mediated by knockdown or overexpression of ID proteins cannot be excluded. The knockdown of proteins by siRNA can lead to off-target effects mediated by the induction of the interferon response and by seed region sequence complementarity (Sledz *et al.*, 2003; Jackson *et al.*, 2006). Experiments using siRNA against the widely used control GFP have shown that other unintended genes are targeted in the cell lines tested (Tschuch *et al.*, 2008). Since GFP is a widely used negative control for siRNA experiments, genome wide expression analysis was carried out to detect the complete set of unintended target genes. The results demonstrated that even siRNAs without physiological targets can lead to off-target effects (Tschuch *et al.*, 2008). The knockdown of ID proteins can also lead to biological consequences such as the induction of apoptosis (Cao *et al.*, 2009; Asirvatham *et al.*, 2006). Induction of apoptosis could activate signalling pathways, which can indirectly lead to regulation of pol III transcription. Overexpression of ID proteins at high levels that are not physiological may not represent a real situation in cells. However, it may reflect an example of what may happen in cancer where ID proteins are expressed in high levels in many tumour samples (Lin *et al.*, 2000; Schindl *et al.*, 2003; Wilson *et al.*, 2001). Since ID proteins are shown to potentially affect cell growth by interacting with the RB protein family as well as with the E-protein transcription factors and ETS binding proteins, the possible indirect effects mediated by the overexpression of ID proteins should be taken into account (Iavarone *et al.*, 1994; Perk *et al.*, 2005; Yates *et al.*, 1999). Since ID proteins could affect pol III transcription indirectly by interacting with the tumour suppressor RB, which has been demonstrated to sequester TFIIIB and result in the repression of pol III transcription (Sutcliffe *et al.*, 2000). Also, in this thesis in chapter 5 E47 is shown to repress pol III transcription. Therefore, it is possible that ID proteins, which are known to function by sequestering E-proteins, could indirectly affect pol III transcription by forming transcriptionally

inactive complexes with E-proteins. In addition, the increased or decreased growth rate of cells could provide a possible explanation for the effects mediated by the overexpression or knockdown of ID proteins on pol III transcription. The increased or decreased growth rate of cells could be the result of indirect, and not direct, effects caused by the alteration of ID protein levels. In order to try to avoid indirect effects caused by the knockdown or overexpression of ID proteins, cell lines that contain functional p53 and RB that represent a more physiological state should be used instead of the HeLa cell line used. Also, the specificity and efficiency of the siRNA used to target ID proteins could be improved to avoid off-target effects that can lead to indirect effects. Furthermore, *in vitro* transcription assays should be performed investigating the effect on more pol III transcripts, since the results obtained from these experiments might be less likely to be affected by indirect consequences.

In conclusion, the data presented in this chapter suggest that the ID1, ID2 and ID3 family members activate pol III transcription, indicating a possible role through which these transcription factors may regulate cell growth. Since preliminary experiments expose an effect of ID proteins on pol I activity, it would be interesting to determine whether these proteins can also regulate pol I transcription.

Chapter 4

ID1, ID2 and ID3 Association with the RNA Polymerase III Transcription Machinery

4.1 Introduction

4.1.1 ID protein interaction with the RB family

ID proteins are important proliferative factors in a number of cell types (Hara *et al.*, 1994; Lasorella *et al.*, 2002). Serum induction results in rapid expression of ID proteins with high levels being maintained throughout S phase (Hara *et al.*, 1994; Lasorella *et al.*, 2000). Several studies have suggested that G_1 progression relies on the activity of ID proteins on their targets (Norton, 2000; Roberts *et al.*, 2001; Yates *et al.*, 1999). In particular, IDs are functional targets of the RB tumour suppressor protein and the related proteins p107 and p130 (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996; Lasorella *et al.*, 2000). Overexpression of ID2 in osteosarcoma U2OS cells promoted proliferation, with a larger population of cells in the S phase compared to controls (Iavarone *et al.*, 1994). The tumour suppressor RB regulates the cell cycle by binding and inactivating transcription factors such as E2F-DP1, whose activity is required for G_1 to S phase progression (Harbour *et al.*, 1999). Only the unphosphorylated active form of RB can bind to transcription factors, whereas the phosphorylated form is inactive. The ID2 protein, but not ID1 or ID3, can bind to the unphosphorylated form of RB *in vitro* and *in vivo* (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). The binding of ID2 to RB and the related proteins p107 and p130 is dependent on the HLH domain of ID2. The HLH domain of ID2 associates with the pocket domain of RB, which is required for growth inhibition. A mutant lacking the HLH domain was unable to inhibit the growth suppression caused by RB. ID2 was able to reverse the growth inhibition caused by RB when these were co-transfected in the osteosarcoma cell line Saos-2 that lacks RB. Also, overexpression of ID2 in U2OS cells reversed the cell cycle arrest induced by cyclin-dependent kinase inhibitors p16 and p21, by inactivating RB (Lasorella *et al.*, 1996). Knockout experiments of ID2 and RB in mice demonstrated a genetic interaction between ID2 and RB during development. Specifically, the embryonic lethality of RB-null mice can be rescued by elimination of ID2 (Lasorella *et al.*, 2000).

In addition to binding RB directly to inhibit its function, ID proteins may affect the activity of RB indirectly. ID1, and possibly other ID family members, can inhibit the expression of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ by binding and sequestering E47, which mediates its expression (Prabhu *et al.*, 1997). This reduces the suppression from $p21^{Cip1/Waf1}$ and allows cyclin E/CDK2 to phosphorylate and inactivate RB, which leads to activation of genes needed to promote the G_1 to S progression. It has also been demonstrated that $p57^{kip2}$, which is the only CDK inhibitors required for normal development, is a target of both ID2 and E47 (Rothschild *et al.*, 2006). The ID2 protein inhibits activation of $p57^{kip2}$ and leads to progression into S phase with the RB protein reversing this activity, whereas E47 induces $p57^{kip2}$ mediated cell cycle arrest and prevents progression into S phase. Studies have demonstrated that ID1 expression can downregulate $p16^{INK4a}$ which leads to RB phosphorylation (Ouyang *et al.*, 2002b). The inactivation of the $p16^{INK4a}$/RB pathway by ID1 may be required for induced cell proliferation in prostate cancer cells. The ETS family transcription factor members ETS1 and ETS2 have been shown to interact with and activate the $p16^{INK4a}$ gene (Ohtani *et al.*, 2001). ETS family members are negatively regulated by the ID family of HLH proteins, with ETS2 binding directly to ID1 *in vitro*. ID1 can reduce the activation of $p16^{INK4a}$ by ETS2. Therefore, ETS2 and ID1 have antagonistic effects on $p16^{INK4a}$.

4.1.2 ID cellular localisation and regulation by bHLH proteins

Appropriate subcellular localisation is important for the proper function of several proteins. Some proteins are constitutively nuclear, whereas others are actively imported into and exported out of the nucleus, by a signal dependent or independent mechanism (Mattaj & Englmeier, 1998; Gorlich & Kutay, 1999). Shuttling of large proteins between the cytoplasm and the nucleus is accomplished through nuclear pore complexes mediated by nuclear export signals (NES) and nuclear localisation signals (NLS) present on these proteins. The NES and NLS are identified by export and import receptors (Macara, 2001; Suntharalingam & Wente, 2003). However, small proteins with molecular weights of less than 40kDa can freely pass through the nuclear pore (Mattaj & Englmeier, 1998; Macara, 2001). The molecular weights of the ID protein

family members range from 13kDa to 20kDa which suggests that they may enter and exit the nucleus by passive diffusion. However, other mechanisms that regulate the subcellular localisation of ID proteins have emerged (Deed *et al.*, 1996; Trausch-Azar *et al.*, 2004; Kurooka & Yokota, 2005; Lingbeck *et al.*, 2005; Makita *et al.*, 2006).

ID proteins have been reported to be distributed both in the cytoplasm and nucleus under physiological conditions in growing cells (Jen *et al.*, 1992; Iavarone *et al.*, 1994). When ID3 was transiently expressed in COS cells and in the absence of the E47 protein it localised in the cytoplasm/perinuclear region (Deed *et al.*, 1996). However, when E47 and ID3 were co-transfected, the ID3 protein was translocated to the nucleus, whereas E47 was not sequestered into the cytoplasm. Similarly to other early response genes, ID3 protein levels are rapidly turned over with a short half-life ($t_{1/2}$) of 1 hour, whereas the E47 protein has a longer half-life of 24 hours. After co-transfection of ID3 and E47 the half life of the ID3 protein was extended to 3 hours and that of the E47 protein was reduced to 16 hours. In order to investigate the effect of nuclear localisation on the stability of ID3, co-transfection experiments of ID3 with a truncated form of the E12 protein, E12r, were carried out. The E12r protein was able to heterodimerise with ID3, however ID3 was not able to localise to the nucleus. The half life of the co-transfected E12r was reduced, similarly to E47, from 18 to 12 hours and the half life of ID3 was extended to about 4 hours (Deed *et al.*, 1996). Therefore, heterodimerisation and not nuclear localisation is thought to mediate for these effects on protein stability. To conclude, E47 can act as a nuclear chaperone and can regulate the availability of ID proteins. Other studies have also confirmed that E47 and E12 can regulate cellular localisation and degradation of ID proteins (Lingbeck *et al.*, 2005).

Myogenic determination (MyoD) is a bHLH protein that is a key regulator of muscle differentiation and forms complexes with ID1. MyoD is another example of a bHLH protein that modulates the degradation of an ID family member (Trausch-Azar *et al.*, 2004). Mutagenesis to the NLS motif of ID1 ($ID1^{NLS}$) resulted in enhanced localisation of the protein to the cytoplasm and increased the rate of degradation ($t_{1/2}$ ~0.5 hour) compared to wild type ID1 ($t_{1/2}$ ~1 hour). Co-transfection of MyoD with ID1 or $ID1^{NLS}$ resulted in increased localisation of ID1 or $ID1^{NLS}$ to the nucleus and also reduced the rate of degradation of ID1 or $ID1^{NLS}$. The converse, however, does not

occur, as the degradation and localisation of MyoD was unaltered by expression of ID1 (Trausch-Azar *et al.*, 2004). In another study, the shuttling of ID2 between the nucleus and cytoplasm was investigated (Kurooka & Yokota, 2005). A functional NES was revealed in the C-terminal region of the ID2 protein which is required for cytoplasmic localisation. Inhibiting nuclear export led to accumulation of ID2 to the nucleus without the co-expression of E-proteins. Also, the HLH domain of ID2 is important for exhibiting NLS activity. Other findings have established that the ID1 proteins, in addition to the ID2 proteins, have a functional NES (Makita *et al.*, 2006).

4.1.3 ID protein degradation by the ubiquitin-proteasomal pathway

The expression of the ID proteins is regulated at various levels, one of which is the post-translational level, through the ubiquitin-proteasome pathway (Bounpheng *et al.*, 1999b; Fajerman *et al.*, 2004). ID proteins are short-lived proteins with a half-life of 20-60 min, depending on the cell type (Deed *et al.*, 1996; Bounpheng *et al.*, 1999b). Heterodimerisation of ID proteins with bHLH proteins stabilises the ID proteins, which become less susceptible to degradation (Bounpheng *et al.*, 1999b; Trausch-Azar *et al.*, 2004; Lingbeck *et al.*, 2005). ID3 interacts with Jun activating binding protein 1 (JAB1), which is related to factors present in the 19S complex of the 29S proteasome and ID1 interacts with the S5a proteasome subunit (Bounpheng *et al.*, 1999b; Anand *et al.*, 1997). ID1, ID2 and ID3 protein degradation can be blocked by treating cells with inhibitors of the 26S proteasome (Bounpheng *et al.*, 1999b). The inhibitors of the 26S proteasome, lactacystin or Z-L3VS, lead to an increase in the accumulation of ID1, ID2 and ID3 proteins. Also, when chloroquine, an inhibitor of lysosomal proteolysis, was used to treat cells it did not have an effect on the levels of ID proteins, suggesting that ID proteins are not degraded by this pathway. It has been demonstrated that ID proteins can be modified by ubiquitination (Bounpheng *et al.*, 1999b). In order to identify the domain of the ID proteins that is targeted by the 26S proteasome, ID1 deletion mutants of the HLH domain, amino terminus and carboxyl terminus were tested in cells treated with the Z-L3VS inhibitor. The ID1 deletion mutants showed a higher accumulation,

implying that the mutants can also be targeted for degradation by the 26S proteasome (Christy BA, unpublished observations cited in Bounpheng *et al.*, 1999b).

Other studies have demonstrated that the ID1 and ID2 proteins are degraded by the ubuiquitin proteasome system following ubiquitination at its N-terminal residue (Fajerman *et al.*, 2004; Trausch-Azar *et al.*, 2004). Deleting the first 15 N-terminal residues of ID2 resulted in stabilisation of the protein, indicating that this particular domain possibly acts as a substrate for the E3 ubiquitin ligase. A mutant fibroblast cell line, ts20tg, with a temperature-sensitive defect in the E1 ubiquitin activating enzyme, was used to investigate if ID proteins are degraded by the ubiquitin-proteasomal pathway (Bounpheng *et al.*, 1999b). The mutant cell line grows normally at 35°C; however, when the temperature is raised to 39°C, the cells grow more slowly. Blocking the function of the E1 ubiquitin activating enzyme causes proteins that are degraded by the ubiquitin pathway to accumulate (Chowdary *et al.*, 1994). Transfection of ts20tg cells with ID cDNAs and increasing the temperature to 39°C led to the accumulation of ID1, ID2 and ID3 proteins.

4.1.4 Phosphorylation of ID proteins

Protein modification is a significant step in the regulation of protein function. Phosphorylation sites for protein kinase A, protein kinase C, cell division cycle 2 (cdc2) kinase and CK2 have been found in the ID family proteins (Nagata *et al.*, 1995). Cyclin-dependent kinases are thought to be involved in the regulation of the cell cycle, with cyclin E-CDK2 being important for G_1 to S phase transition (Resnitzky & Reed, 1995). Studies have identified ID2 as a substrate for cyclin E-CDK2, and also for cyclin A-CDK2 (Hara *et al.*, 1997; Matsumura *et al.*, 2002). Phosphorylation of a serine residue (Ser5) of ID2 by CDK2 inhibits ID2 from disrupting the formation of E-box DNA-binding complexes (Hara *et al.*, 1997). Phosphorylation of ID2 occurs during the late G_1 phase, together with the activation of cyclin E-CDK2. Furthermore, during late G_1 the levels of ID2 expression and the E-box complex were both found to be maximal, in serum-stimulated fibroblasts. This is surprising, but, an explanation could be that the

phosphorylated ID2 is not inactivated but changes its specificity for another protein which is involved in the G_1 to S transition.

To investigate the biological importance of the ID2 Ser5 phosphorylation site, vectors expressing ID2 and an ID2-S5A mutant which cannot be phosphorylated were transfected into cells. The ID2-S5A mutant exhibited a 50% reduction in colony formation, perhaps because phosphorylated ID2 is important for cell cycle progression from late G_1 to S phase (Hara *et al.*, 1997). In a later study, cell cycle regulated phosphorylation of ID3 was implicated in inhibiting homo- or heterodimer complex formation (Deed *et al.*, 1997). Similar to ID2, ID3 becomes phosphorylated in the late G_1 phase and contains a CDK phosphorylation site at Ser5 (Figure 4.1). The unphosphorylated ID3 can inhibit an E12 homodimer complex; however, it cannot disrupt the E12-MyoD heterodimer. Conversely, phosphorylation of ID3 at Ser5 resulted in inhibition of the E12-MyoD complex. This indicates that phosphorylation of ID proteins could result in a change of bHLH target specificity, rather than total loss of bHLH antagonism.

In other studies, the role of ID2 in smooth muscle cell (SMC) proliferation was investigated (Matsumura *et al.*, 2002). SMC proliferation is a significant component of vascular proliferative disorders. Overexpression of ID2 in SMCs increased entry into S phase and cell growth. The CDK2 phosphorylation site Ser5 of ID2 is required for p21 inhibition. It has been found that mutation of Ser5 of ID2 results in inhibition of proliferation and entry into S phase. Wild-type ID2 and mutant ID2-S5A were co-transfected into SMCs with a vector expressing a full length coding region of E47, which contained a nuclear localisation signal. It was found that the mutant ID2 was excluded from the nucleus whereas the wild type ID2 was localised in the nucleus. Therefore, the effect of CDK2 phosphorylation on ID2 may be due to regulation of nuclear transport or interaction with E47 (Matsumura *et al.*, 2002).

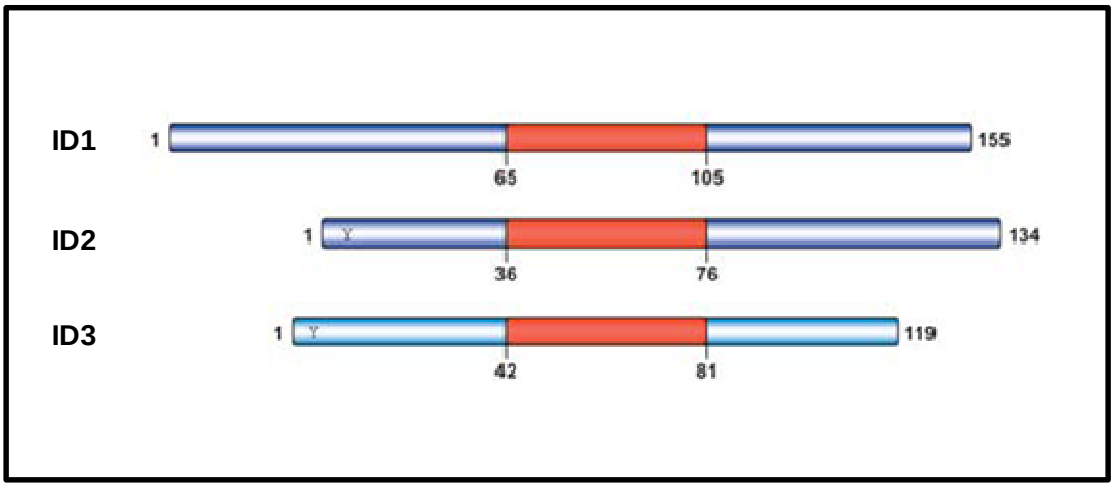

Figure 4.1: Schematic representation of the human ID1, ID2 and ID3 protein structure.

The helix-loop-helix domain region is shown in red. The ID2 and ID3 protein N-terminal domains contain the CDK2 phosphorylation site at Ser5 represented as Y. The alignment of ID proteins was performed with the Lasergene software (version 1.62d1, acquired from DNAStar Inc., Madison, WI) and visualised using the program SeqVu (version 1.1, The Garvan Institute of Medical Research, Sydney, Australia). (adapted from Hasskarl & Münger, 2002).

In the previous chapter it was suggested that ID proteins could stimulate the activation of pol III-transcribed genes. This chapter will address the possible mechanisms by which the ID family members affect pol III transcription. The localisation of ID proteins at the pol III templates *in vivo* will be investigated in human cells. Furthermore, the recruitment of ID1, ID2 and ID3 proteins to pol III-transcribed genes will be examined. The interaction of the ID members with one or more of the pol III specific factors will also be examined to provide an insight into how ID proteins regulate pol III transcription.

4.2 Results

4.2.1 IDs localise at pol III-transcribed genes *in vivo*

The results shown in chapter 3 suggest that ID1, ID2 and ID3 family members are involved in the upregulation of pol III transcription. ID proteins function by sequestering transcription factors from binding DNA, and themselves are unable to bind to DNA (Perk *et al.*, 2005; Benezra *et al.*, 1990). Therefore, one possible mechanism might be that IDs are interacting with the pol III transcription machinery and these associations with the pol III templates are required for the activation of pol III transcription. To determine whether endogenous ID1, ID2 and ID3 are found at pol III-transcribed genes *in vivo*, ChIP assays were performed using HeLa cells. ID1, ID2, ID3, TAF_I48, TBP and pol III antibodies were used to precipitate the proteins that cross-linked to DNA. TBP and pol III were utilised as positive controls, and TAF_I48 was used as the negative control. The results demonstrated that ID2 was present at the 5S rRNA and $tRNA^{Leu}$ pol III templates, with ID3 shown present on $tRNA^{Leu}$ genes, however, ID1 was not found at pol III-transcribed genes (Figure 4.2). As expected, the positive controls, TBP and pol III, were present at these templates whereas with the negative control, TAF_I48, no signal was detected. These results demonstrate that the association of endogenous IDs, mainly of ID2, with pol III-transcribed genes *in vivo* is specific. However, these experiments should be repeated in the future including serial dilutions of the inputs and stronger exposures should be taken. Also, endogenous ID1 does not appear to be present at pol III genes which may indicate that ID1 is not involved in direct regulation of pol III transcription. Presence of ID1 on pol III promoters should be determined in the future by overexpression of ID1.

Another type of cells, the HEK 293 cell line, was utilised to investigate whether ID2 was present at pol III-transcribed genes *in vivo*. Infection of cells was performed employing an adenovirus expressing ID2, with the cells harvested 30 hours after infection. ChIP experiments were performed in cells in which ID2 was overexpressed. The positive controls Brf1, TFIIIC and pol III were present at 5S rRNA, $tRNA^{Leu}$, 7SL

RNA and U6 snRNA genes as expected, whereas the negative controls TFIIA and beads gave no signal (Figure 4.3). The results revealed that ID2 was found at pol III-transcribed 5S rRNA, $tRNA^{Leu}$, 7SL RNA and U6 snRNA genes (Figure 4.3). Interestingly, E47 was also recruited to the above pol III templates. These interactions were specific and not because of over cross-linking of proteins to DNA, since no signal was present when using primers to amplify the pol II-transcribed ARPP P0 gene. Therefore, these data demonstrate that ID2 and E47 are present at pol III genes.

Furthermore, a U2OS cell line stably expressing ID3 was used to determine whether ID3 can occupy pol III templates *in vivo*. ChIP assays demonstrated that ID3 was present at 5S rRNA, $tRNA^{Leu}$, $tRNA^{Tyr}$ and 7SL RNA genes, with E47 also recruited to the 7SL template (Figure 4.4). No signal was detected when primers were used to amplify the pol II-transcribed ARPP P0 gene (Figure 4.4). These results demonstrate that the binding of antibodies to the promoter regions is specific. In conclusion, these data revealed that IDs are present at pol III-transcribed genes *in vivo*.

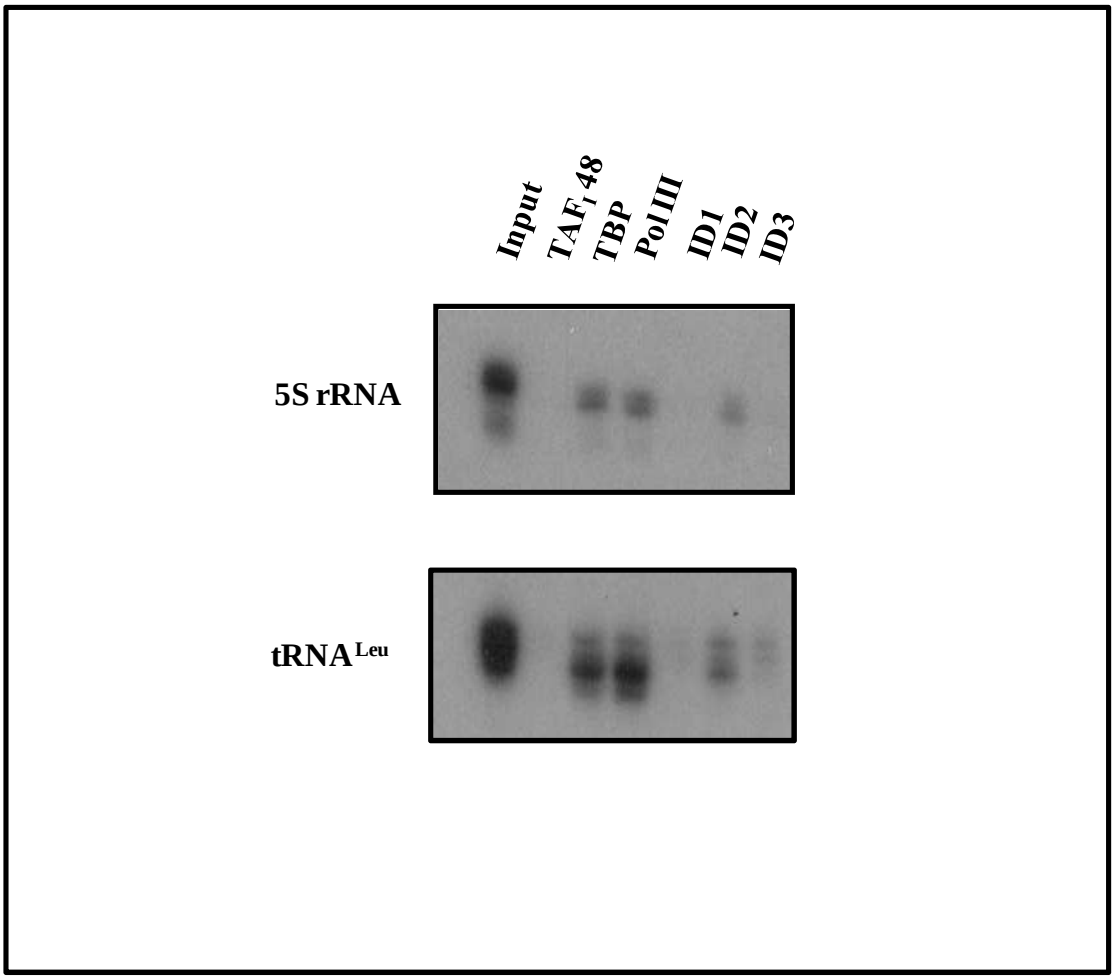

Figure 4.2: Endogenous ID occupancy at pol III-transcribed genes.

Chromatin immunoprecipitation assays were performed using HeLa cells. ID1 (C-20), ID2 (C-20) and ID3 (C-20) antibodies were used to determine the presence of IDs on pol III templates. The M19 antibody was used against the TAF_i48 as negative control. TBP (MTBP-6) and pol III (1900) antibodies were used as positive controls. Primers were designed for 5S rRNA and $tRNA^{Leu}$ genes and the immunoprecipitated DNA samples were analysed by PCR. The non-immunoprecipitated input samples were used as controls.

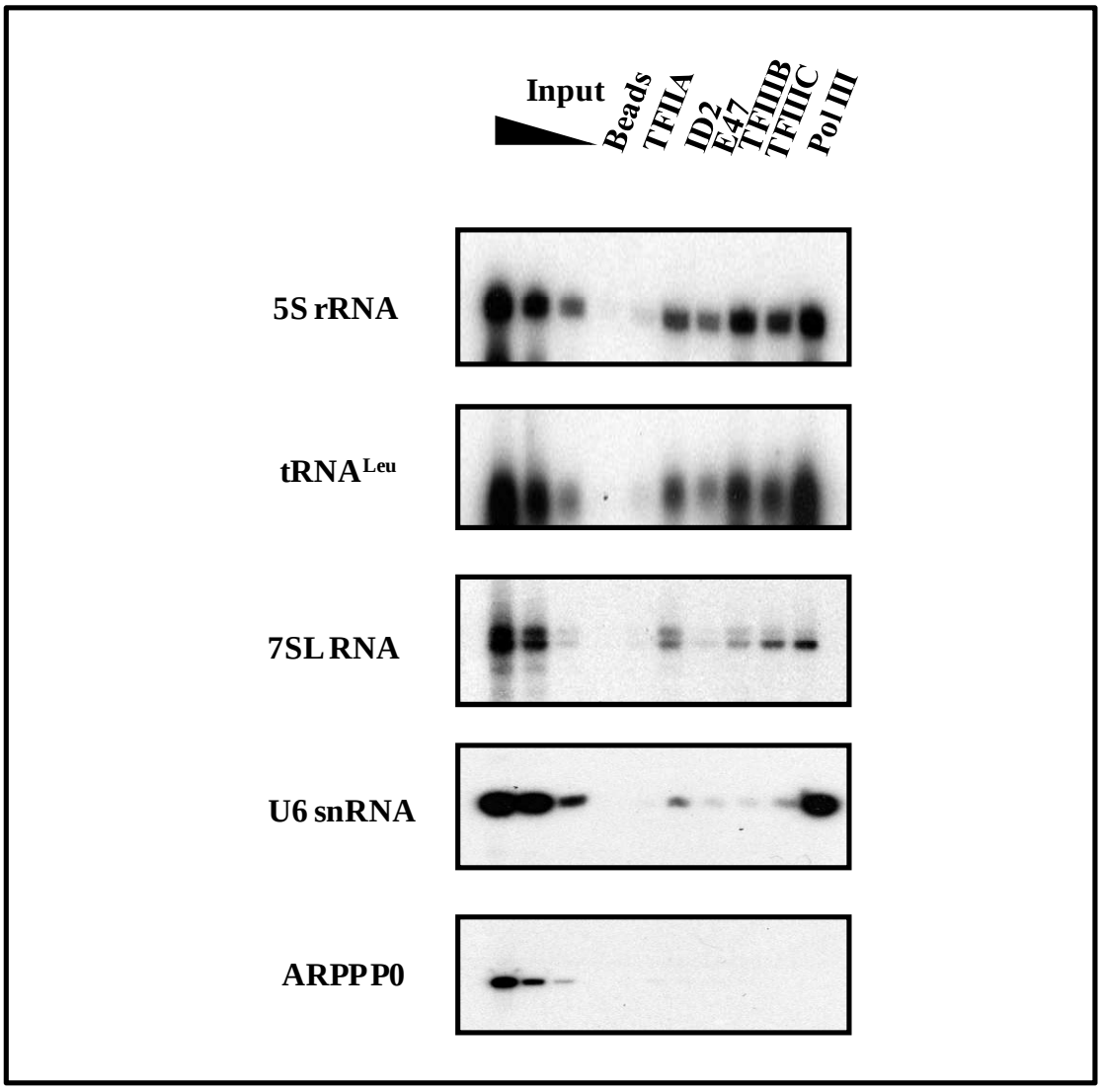

Figure 4.3: ID2 and E47 are associated with pol III-transcribed genes.

Chromatin immunoprecipitation assays were performed using HEK 293 cells harvested 30 hours after infection with the ID2 adenovirus. The TFIIA (FL-109) antibody was used together with the beads (no antibody) as negative controls. The ID2 (C-20) and E47 (N-649) antibodies were used to test whether ID2 and E47 were present at pol III-transcribed genes. The Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were used as positive controls. Primers were designed for 5S rRNA, $tRNA^{Leu}$, 7SL RNA and U6 snRNA genes and the DNA samples were analysed by PCR. Input samples were used as controls.

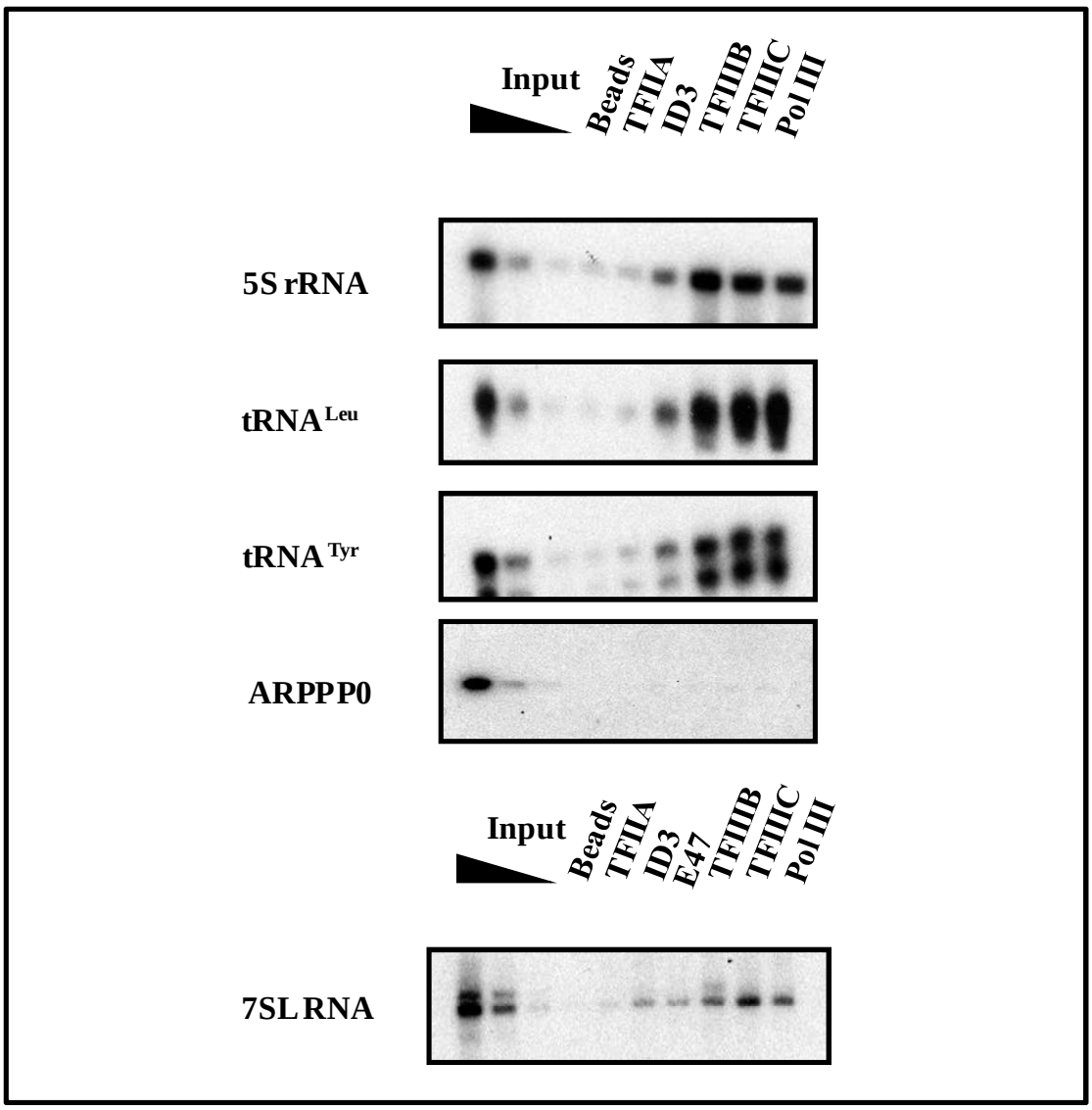

Figure 4.4: ID3 and E47 are present at pol III-transcribed genes.

Chromatin immunoprecipitation assays were performed using a U2OS cell line stably expressing ID3. The TFIIA (FL-109) antibody and beads were utilised as negative controls. The ID3 (C-20) and E47 (N-649) antibodies were used to determine whether ID3 localised at pol III-transcribed genes. The Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were utilised as positive controls. Primers designed were specific for 5S rRNA, $tRNA^{Leu}$, $tRNA^{Tyr}$ and 7SL RNA genes and the immunoprecipitated samples were analysed by PCR. The non-immunoprecipitated inputs were used as controls.

4.2.2 ID2 is present at pol III promoters and co-localises with TFIIIB

Previous experiments demonstrated that ID2 was found to localise at pol III templates (Figure 4.2; Figure 4.3). Studies using mapping ChIP assays have demonstrated that the c-Myc oncoprotein which is involved in the activation of pol III transcription, co-localises with the TFIIIB and this factor is likely to recruit c-Myc to pol III templates (Gomez-Roman *et al.*, 2003; Kenneth *et al.*, 2007). To investigate whether ID2 can localise at the promoters of pol III-transcribed genes and is associated with basal pol III factors, mapping experiments on the 7SL locus were performed. PCRs were performed using five set of primers designed for DNA regions, upstream of the pol III-transcribed 7SL gene promoter, at the gene region and downstream of the 7SL promoter region (Figure 4.5, A). Cells were infected with an ID2 expressing adenovirus. HEK 293 cells were harvested 30 hours post-infection and PCR was used for the amplification of DNA extracted from ChIP assays. The data demonstrated that the basal transcription factors TFIIIB and TFIIIC are localised at different positions on the 7SL promoter region (Figure 4.5, B). The results revealed that ID2 together with the Brf1 subunit of TFIIIB were distributed at the start of the gene exhibiting a similar pattern, with increased occupancy at the second position and displaying a decrease at the end of the gene at position 3 (Figure 4.5, C). Morover, TFIIIC and pol III are present at the second and third positions of the 7SL gene (Figure 4.5, C). Also, using this ID2 overexpressed cell line, E47 appears to be recruited to the 7SL promoter region. E47 shows an increased presence together with Brf1 at the start of the 7SL gene, and a decrease after the second position (Figure 4.5, C). These results demonstrate that ID2 co-localises with Brf1 rather than TFIIIC and it is more likely that ID2 is recruited by Brf1. To investigate the interaction of ID2 with Brf1, co-immunoprecipitation and *in vitro* transcription and translation studies are required to identify whether ID2 binds to Brf1 which could possibly lead to the recruitment of ID2 to pol III-transcribed genes.

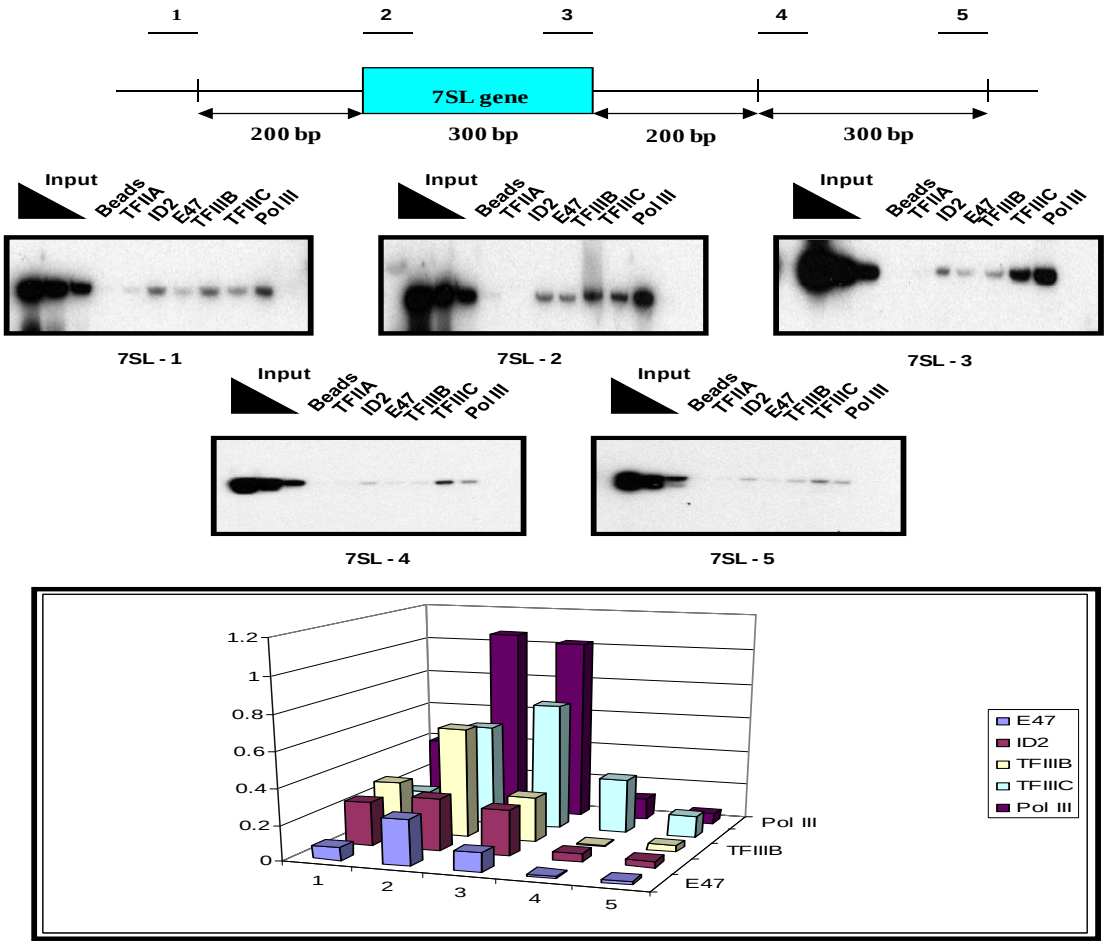

Figure 4.5: ID2 occupancy on the 7SL locus.

Chromatin immunoprecipitation assays were performed in HEK 293 cells harvested 30 hours after infection with the ID2 expressing adenovirus. **A.** The diagram of the five positions amplified by sets of primers on the 7SL locus. **B.** The ID2 (C-20), E47 (N-649), Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were used for ChIP assays, with the TFIIA (FL-109) and beads utilised as negative controls. **C**. Schematic representation of the intensity of 7SL ChIP signals. The x-axis displays the five sets of primers. The y-axis represents the ChIP signals that were normalised, by adjusting the samples accordingly against the input and the strength of signals with the TFIIA negative control. Quantification was performed using the ImageJ (version 1.42) software.

4.2.3 IDs co-immunoprecipitate with Brf1

Previous ChIP assays here have demonstrated that IDs are present at pol III-transcribed genes *in vivo* (Figure 4.2; Figure 4.3; Figure 4.4). The fact that IDs are shown to be associated with pol III templates is interesting since IDs lack a DNA binding domain (Benezra *et al.*, 1990). Therefore, it is possible that the association of IDs with the pol III templates might be through protein-protein interactions with basal factors of the pol III apparatus, and this may lead to the regulation of pol III-transcribed genes. Scanning ChIP assays revealed that ID2 can associate with the Brf1 factor at the 7SL gene promoter (Figure 4.5). These results suggested the possibility that IDs interact with the basal transcription factor TFIIIB. To investigate the interaction of ID2 with Brf1, co-immunoprecipitation studies were performed. Western blotting using HeLa extracts in which Brf1 was expressed containing an HA tag, demonstrated that HA-Brf1 co-immunoprecipitated with ID1 compared to the un-induced samples (Figure 4.6, A). In the future, additional experiments should be performed to investigate whether ID1 co-immunoprecipitates with Brf1. Moreover, western blot analysis using HeLa cells revealed that Brf1 co-immunoprecipitated with endogenous ID2 (Figure 4.6, B). In the future, control western blotting experiments should be performed using an anti-ID2 antibody as well as an anti-TFIIB antibody for the immunoprecipitation. This is important in order to demonstrate the opposite experiment that ID2 is also able to co-immunoprecipitate with Brf1 to further support these results (Figure 4.6, B). Also, using an anti-TFIIB antibody for western blotting will determine whether TFIIB can immunoprecipitate itself, and it should not co-immunoprecipitate with ID2 and Brf1 since it is used as a negative control. Also, a stable U2OS cell line expressing ID3 was used for western analysis of the immunoprecipitation and demonstrated that ID3 co-immunoprecipitated with Brf1 (Figure 4.6, C). The TFIIB antibody and beads were used as negative controls and confirmed that these associations were specific.

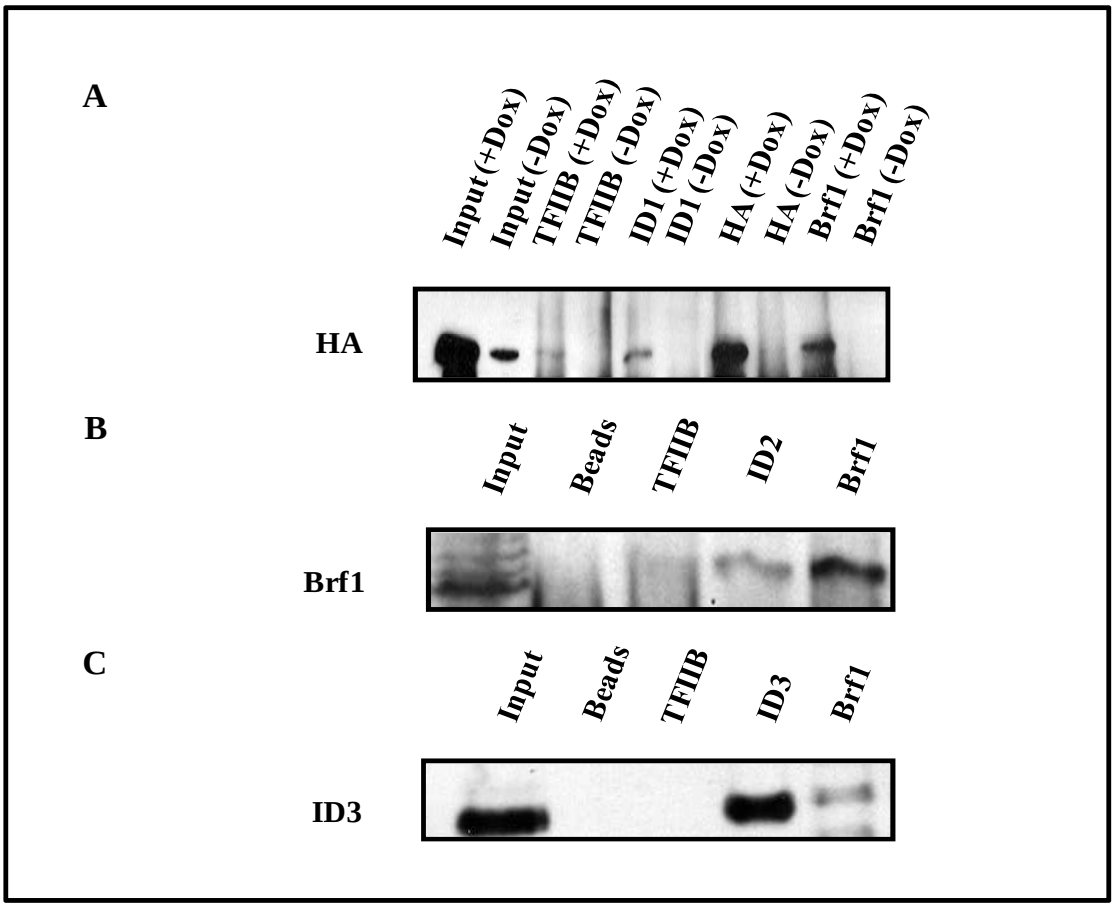

Figure 4.6: Co-immunoprecipitations reveal interactions of ID1, ID2 and ID3 with Brf1.

HeLa cells expressing HA-Brf1 as well as HeLa and U2OS cells stably expressing ID3 nuclear extracts were utilised for co-IP studies. **A.** For the HeLa HA-Brf1 TET-ON expression system, cells were induced to express HA-Brf1 for 48 hours before harvesting. The extracts were immunoprecipitated with the ID1 (C-20), HA (F-7) and Brf1 (128) antibodies. The TFIIB (C-18) antibody was used as negative control. The "+" represents induction of Brf1 compared to the un-induced "-" control. **B.** Extracts were immunoprecipitated with ID2 (C-20) and Brf1 (330) antibodies. Beads and TFIIB (C-18) were used as negative controls. **C.** Nuclear extracts were precipitated with ID3 (C-20) and Brf1 (128) antibodies. The TFIIB (C-18) and beads were utilised as negative controls. The HA (F-7) (A), Brf1 (330) (B) and ID3 (C-20) (C) antibodies were used for western blotting.

4.2.4 ID1, ID2 and ID3 proteins interact with Brf1

Co-immunoprecipitation assays revealed that ID proteins can interact with the Brf1 subunit of TFIIIB *in vivo* (Figure 4.6). The above technique, however, cannot be used to distinguish between indirect or direct interactions. Since TFIIIB is a transcription factor that has been found to be associated with regulatory proteins like the RB and p53 proteins (White *et al.*, 1996; Cairns & White, 1998), the ability of IDs to interact with TFIIIB *in vitro* was examined. To investigate whether full length ID and mutant forms of these proteins can bind to TFIIIB, *in vitro* transcription and translation experiments using ^{35}S-methionine were employed. Initially, the expression of the GST vector control and the GST-ID full length and mutant proteins was performed in *E. coli* cells. The results demonstrated that the GST-ID1, GST-ID2, GST-ID3, GST-ID2 HLH (containing only the HLH domain of ID2) and GST-ID2 δHLH (lacking the HLH domain of ID2) fusion proteins and the GST vector control were overexpressed and normalised against each other (Figure 4.7). The next step involved the *in vitro* translation of the Brf1 subunit of TFIIIB followed by its labelling with ^{35}S-methionine. The results visualised by autoradiography revealed that the GST-ID1, GST-ID2, GST-ID3, GST-ID2 HLH, GST-ID2 δHLH and GST-E47 proteins are bound by Brf1 (Figure 4.8). In the GST control no signal was detected, confirming the specificity of these interactions. From these findings it can be concluded that IDs can bind to Brf1 *in vitro*. Further experiments should be performed with additional negative controls to demonstrate the specificity of binding of ID proteins to Brf1. Other negative controls that could be used can include GST fusion proteins that are not known to interact with Brf1 such as the GST-Ras fusion protein. Another negative control could be a GST fusion protein whose expression is not induced. This assay cannot distinguish between direct or indirect interactions because other proteins were present in the protein extracts used. Other methods could be employed to analyse protein-protein interactions such as yeast two-hybrid screening, x-ray crystallography and fluorescence resonance energy transfer (FRET) techniques. Also, strategies for mapping interaction domains could be used such as creating mutants lacking various domains and regions of protein sequence as well as site-directed mutagenesis.

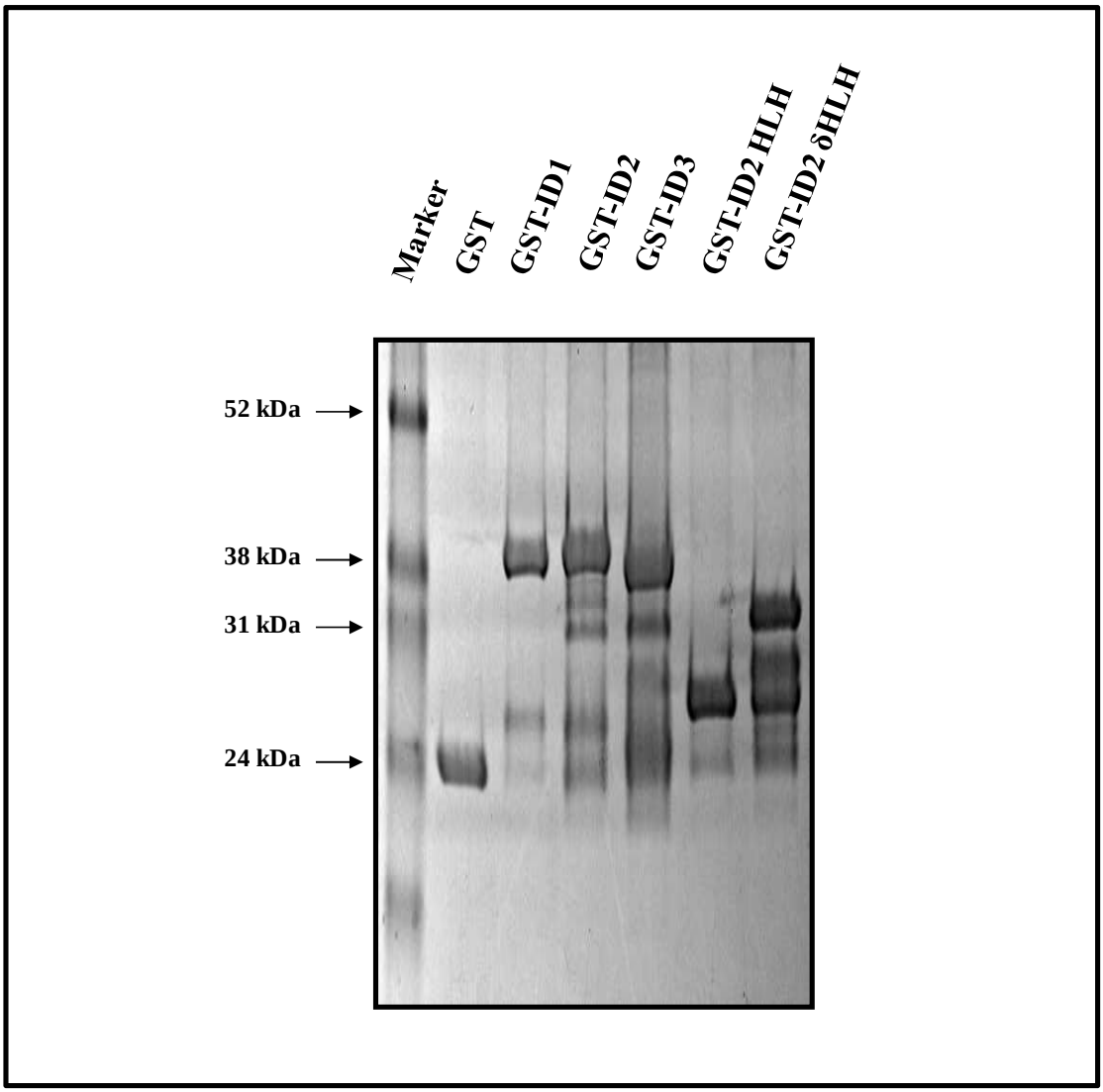

Figure 4.7: Overexpression of GST, GST-ID1, GST-ID2, GST-ID3 and GST-ID2 mutants HLH and δHLH fusion proteins.

Expression of GST, GST-ID1, GST-ID2, GST-ID3, GST-ID2 HLH and GST-δHLH fusion proteins was performed in *E. coli* cells. IPTG was used to induce the cells for 2 hours before harvesting them. The cell pellets were resuspended and sonicated. The GST and GST-ID fusion proteins were incubated with glutathione-agarose beads. Protein samples were resolved on an SDS-10% polyacrylamide gel. A full length molecular weight marker was run next to the samples.

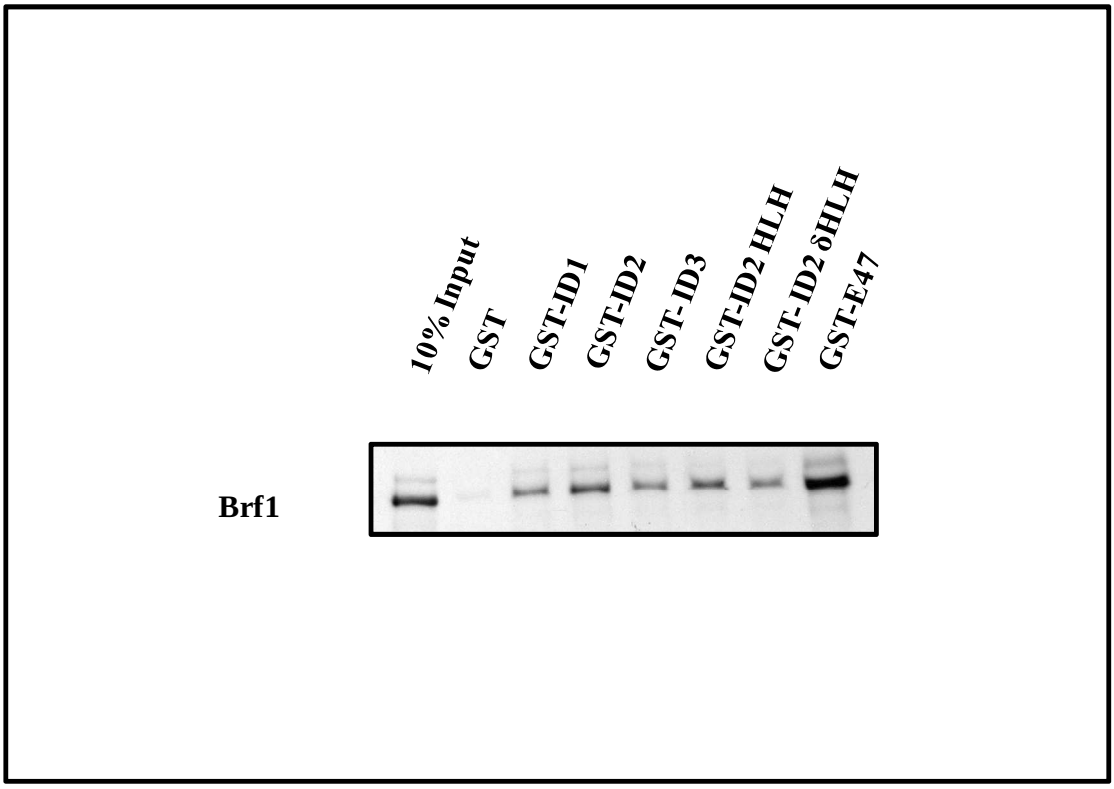

Figure 4.8: In vitro transcription/translation using ^{35}S-methionine reveals interactions of ID1, ID2, ID3 and ID2 mutants ID2-HLH and ID2 δHLH with TFIIIB.

In vitro translated Brf1 was labelled with ^{35}S-methionine and incubated with GST which was used as the negative control or with the GST-ID1, GST-ID2, GST-ID3, GST-ID2 HLH or GST-δHLH fusion proteins at 4°C with agitation. Proteins retained after washing were resolved on an SDS-7.8% polyacrylamide gel and visualised by autoradiography.

4.2.5 The Brf1 occupancy at a pol III template *in vivo* is specifically reduced in ID2 knockout cells

The interactions of the ID family members with Brf1 might be involved in the recruitment of ID proteins to the promoter of pol III templates, leading to the transactivation of pol III genes. In a previous study, ChIP assays were performed to compare the 5S rRNA gene occupancy in MycER induced and uninduced fibroblasts. The results revealed that the induction of c-Myc stimulated the recruitment of Brf1 and pol III at 5S rRNA (Kenneth *et al.*, 2007). To examine whether the occupancy of a pol III factor such as Brf1, pol III itself and E47 on 5S rRNA genes could be affected by ID2 *in vivo*, ChIP assays were performed to determine the occupancy. In order to test this hypothesis, MEF ID2 wild-type and ID2 knockout cell lines were used. Initially, the genomic DNA inputs from the two cell lines were normalised and the samples adjusted accordingly. The occupancy of Brf1, pol III and E47 was diminished at the pol III template in the ID2 knockout cell line in comparison to the wild-type MEF cell line (Figure 4.9). Interestingly, in the ID2 null cell line, there is reduced enrichment of E47 at the 5S rRNA gene compared to the wild type cell line, suggesting that ID2 might be recruiting E47 to the promoter. Therefore, the significantly decreased presence of Brf1 at the pol III template in the ID2 knockout cells reveals changes in the recruitment of this factor to the promoter region. In the future, this experiment should be repeated with the addition of controls that bind equally in ID2 wild type and ID2 knockout cells, such as a histone or TFIIIC for the ChIP assay, in order to exclude the possibility that these results do not reflect the decreased growth rate of ID2-/- cells. Also, the protein levels of E47, ID2, Pol III and Brf1 should be measured by western blot analysis. The knockdown of ID2 could result in the expression or suppression of E47, ID2, Pol III and Brf1 directly or indirectly and could also result in cells growing at a decreased rate, which could then potentially lead to altered pol III transcription.

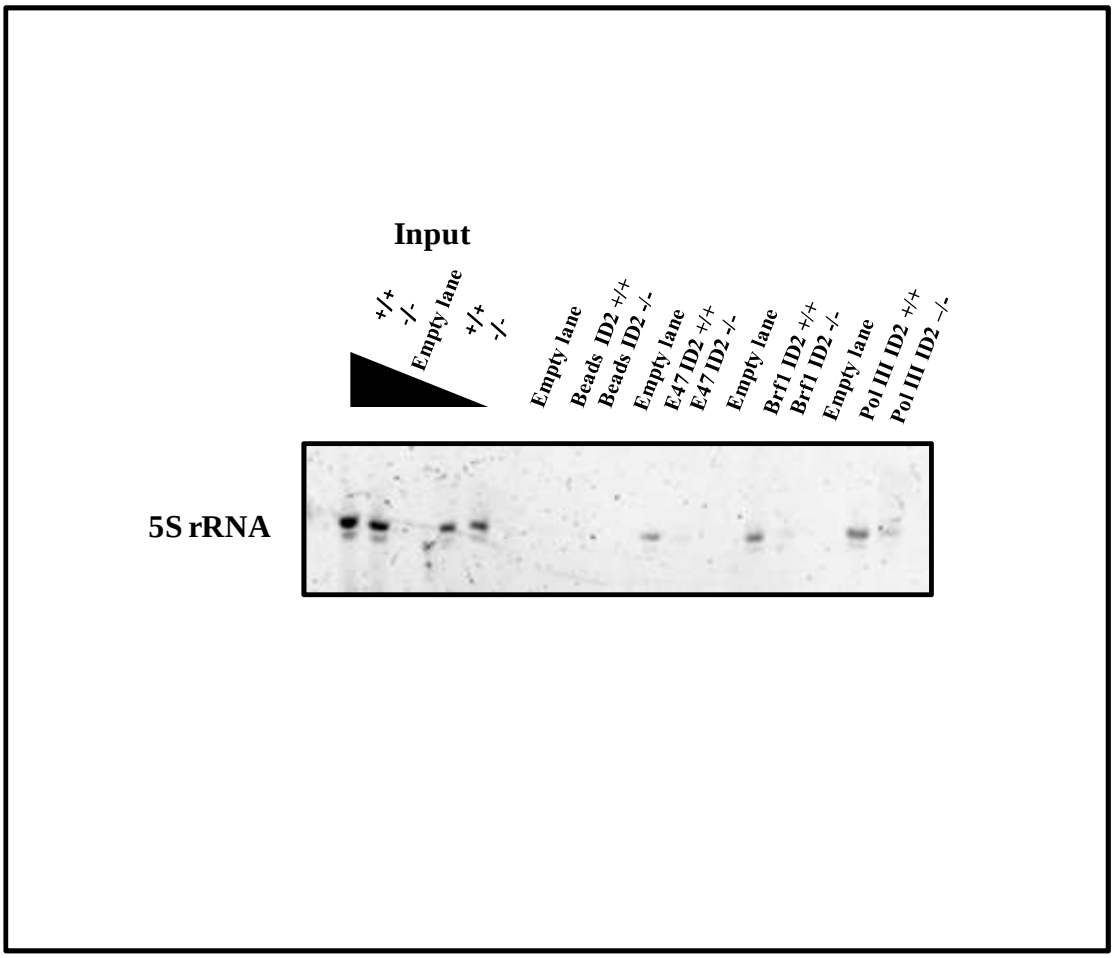

Figure 4.9: Knockout of ID2 decreases the occupancy of Brf1 on 5S rRNA genes.
Chromatin immunoprecipitation assays were performed using MEF ID2 wild-type and ID2 knockout cell lines. ChIP experiments were performed using antibodies against E47 (N-649), Brf1 (128) and pol III (1900). Beads (no antibody) were used as negative control. Samples were normalised by comparing the genomic DNA inputs. The samples were analysed by PCR performed on inputs alongside genomic DNA and primers for 5S rRNA were used. The "+/+" represents the MEF ID2 wild type compared to the "-/-" MEF ID2 knockout cell line.

4.3 Discussion

One of the mechanisms that ID proteins use is the formation of complexes with transcription factors to sequester them, inhibiting their function and capability to bind DNA (Perk *et al.*, 2005). The results presented here revealed that ID proteins are associated with pol III templates. In addition, studies have demonstrated that ID1 is involved in the stimulation of DNA binding of the transcription factor MIDA1 (mouse ID associated protein 1). ID1 is found at the MIDA1-DNA complex and interacts with MIDA1 *in vivo* and *in vitro* and increases the DNA binding activity of MIDA1 to a GTCAAGC sequence (Inoue *et al.*, 1999). MIDA1 is required for cell growth in mouse erythroleukaemia cells, and antisense oligonucleotides that block MIDA1 caused inhibition of cell growth (Shoji *et al.*, 1995). MPP11 (M-phase phosphoprotein 11) is the human orthologue of MIDA1 and overexpression of MPP11 has been observed in head and neck carcinomas. Therefore, MIDA1/MPP11 might present a modulator of the tumourigenic functions of ID1 (Resto *et al.*, 2000).

ChIP assays were performed to determine whether ID1, ID2 and ID3 could be interacting with the pol III templates to induce pol III-transcribed genes. The results revealed that in the HeLa cell line used, ID2 localised at different pol III templates such as 5S rRNA and $tRNA^{Leu}$ *in vivo* (Figure 4.2). ID3 does not appear to be present on pol III-transcribed genes as strongly as ID2, and there was no signal detected for ID1. A possible explanation could be that the antibodies used, which were not ChIP grade, cannot efficiently detect ID1 and ID3 in these assays. HEK 293 cells were also used which were infected with an ID2-expressing adenovirus. ChIP assays demonstrated that when the ID2 protein was overexpressed it exhibited a high enrichment at the promoters of pol III templates such as 5S rRNA, $tRNA^{Leu}$, 7SL RNA and U6 snRNA and was present at more pol III genes in comparison to the endogenous ID2 protein (Figure 4.3). Moreover, in this cell line, overexpressing ID2 by adenovirus infection, E47 is recruited to the same pol III templates as above. In addition, ChIP assays performed using U2OS cells stably expressing ID3, showed that ID3 localised at the promoters of the pol III templates 5S rRNA, $tRNA^{Leu}$, $tRNA^{Tyr}$ and 7SL RNA (Figure 4.4). The next step

involved mapping experiments on the 7SL gene locus, to reveal a potential pol III factor that could recruit ID2 to the class III promoters. ID2 co-localised with Brf1 displaying a similar distribution pattern at the start of the 7SL gene (Figure 4.5). Therefore, these findings suggest that Brf1 might be recruiting ID2 to pol III promoters. These data support the theory that it is TFIIIB and not a DNA sequence that recruits ID2 to pol III templates. E47 was found to correlate with Brf1 at the same regions as ID2 suggesting a possible recruitment by ID2 (Figure 4.5). In conclusion, ID proteins are localised at different pol III templates in a number of cell lines. Also, ID2 and perhaps ID1 and ID3, might be recruited through a direct interaction by Brf1 to pol III promoters, to regulate pol III transcription.

Previous studies have demonstrated that ID2 can regulate cell growth and cell cycle progression by interacting with RB and the related p107 and p130 proteins (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). The RB protein has been shown to affect the G1/S phase transition by regulating transcription of genes by pol I, pol II and pol III (White *et al.*, 1996; Larminie *et al.*, 1997; Ciarmatori *et al.*, 2001). Specifically, RB represses pol III transcription by binding and sequestering the TFIIIB factor from pol III promoters (Sutcliffe *et al.*, 2000). ID2 has been shown to interact with the RB family and inhibit its function. Furthermore, it has been revealed that ID2 competes with RB for TFIIIB and this could be one mechanism by which ID2 upregulates pol III transcription (Gomez-Roman N, unpublished observations). Also, the expression of ID2 in fibroblasts derived from RB knockout mice still resulted in activation of pol III transcription, although this was lower than in wild-type control cells, suggesting that ID2 can also stimulate pol III transcription by an RB-independent mechanism.

Since experiments described here demonstrated that ID2 and ID3 are present at the promoters of pol III templates, likely mechanisms were investigated that may be involved in this process. Given that the ID HLH proteins lack a basic region and cannot bind DNA, other possible associations were explored (Benezra *et al.*, 1990). Therefore, protein-protein interactions of ID1, ID2 and ID3 with the basal transcription factors of the pol III apparatus were examined. One of the possible targets of ID proteins is the TFIIIB factor which has been shown to interact with and be recruited to TFIIIC by the positive regulators of cell growth c-Myc and ERK oncoproteins (Gomez-Roman *et al.*,

2003; Felton-Edkins *et al.*, 2003a,b). To determine whether ID proteins can bind to TFIIIB, and also to which subunit of TFIIIB they bind, co-immunoprecipitation assays were performed. ID1 co-immunoprecipitated with the Brf1 subunit of TFIIIB *in vivo* (Figure 4.6). Moreover, endogenous ID2 interacts with Brf1 (Figure 4.6). In addition, Brf1 co-immunoprecipitated with the ID3 protein (Figure 4.6). Since this technique cannot distinguish between direct or indirect interactions, the possibility that TFIIIB was in a complex with another factor such as TFIIIC could not be eliminated. To determine whether ID1, ID2, ID3, ID2 HLH and ID2 δHLH can bind to TFIIIB, *in vitro* transcription and translation studies with ^{35}S-methionine were employed. Initially, the ID full length and mutant proteins as well as the control GST were expressed. The results revealed that the overexpression and purification of these GST proteins was successful (Figure 4.7). Next, data revealed that the ID1, ID2, ID3, ID2 HLH and ID2 δHLH proteins bind to the radio-labelled Brf1 subunit of TFIIIB (Figure 4.8). The interaction of the two mutant ID2 proteins, ID2 with and without the HLH domain, suggests that there are two or more possible binding sites of ID2 that are involved in the interaction with Brf1. Therefore, ID proteins associate with Brf1 *in vivo* and *in vitro*. Furthermore, these ID-Brf1 interactions are in agreement with ChIP data establishing that Brf1 was present at pol III genes, suggesting that Brf1 could have a role in the recruitment of IDs to the promoters of pol III templates.

The activation of Brf1 expression and therefore pol III transcription results in induction of cell cycle progression and proliferation in human and mouse fibroblasts (Marshall *et al.*, 2008). The induction of Brf1 also results in tumour formation after the injection of clones overexpressing Brf1 into mice (Marshall *et al.*, 2008). In another study, ChIP assays performed, comparing wild type and Myc null fibroblast cells, revealed that the occupancy of pol III at a tRNA template is lower in the knockout cell line (Kenneth *et al.*, 2007; Ernens *et al.*, 2006). p53 has been demonstrated to inhibit the association of Brf1 with TFIIIC and pol III. ChIP assays established that induction of p53 resulted in a reduction of Brf1 occupancy at a pol III template (Crighton *et al.*, 2003). Also, pol III occupancy was higher in the knockout p53$^{-/-}$ MEF cells compared to the wild type controls (Crighton *et al.*, 2003).

The findings that ID proteins are present at pol III-transcribed genes (Figures 4.2, 4.3 and 4.4) and form complexes with Brf1 *in vivo* and *in vitro* (Figures 4.7, 4.9) demonstrate recruitment of IDs to pol III templates. To determine whether ID2 is involved in the recruitment of Brf1, pol III and E47 on class III genes, MEF ID2 null and ID2 wild type cell lines were used. The results from ChIP experiments showed that in the ID2 knock-out cells, there was a decrease of Brf1, pol III and E47 enrichment at the promoter of the pol III template encoding 5S rRNA, in comparison to cells expressing physiological levels of ID2 (Figure 4.9). Since there is a reduction in the recruitment of Brf1 in response to ID2 knockout, it is possible that ID2 might be involved in the recruitment of Brf1 to pol III templates followed by recruitment of pol III and activation of pol III transcription. In addition, results from the decrease of the ID2 levels suggest a possible synergistic role for an ID-E47 complex that may be involved in the regulation, activation or repression of pol III-transcribed genes. The relative promoter binding ability of ID1, ID2 and ID3 could be assessed by expressing flag tagged ID1, ID2 and ID3 and chromatin immunoprecipitating using a flag antibody. If one of the IDs is present at the promoter more than the others, it can be assumed that this is not due to antibody specific issues. Chromatin immunoprecipitation experiments could be done using samples in which Brf1 has been overexpressed or depleted to assess whether Brf1 recruits IDs and E47 proteins. Also, ChIP on ChIP assays would be helpful to identify if Brf1 is present together with IDs and E47 at pol III promoters.

In conclusion, the results in this chapter reveal a role for ID1, ID2 and ID3 proteins and a possible mechanism by which ID proteins may activate pol III transcription. This function might allow ID proteins to regulate cell growth and promote cancer progression.

Chapter 5

Repression of RNA Polymerase III Transcription by E47

5.1 Introduction

5.1.1 E2A protein expression and activity

The E2A proteins were initially discovered in B cells, however northern blots, electrophoretic mobility shift assays (EMSA) and western blotting have shown that E2A is expressed in most human tissues (Vierra *et al.*, 1994; Aronheim *et al.*, 1993; Jacobs *et al.*, 1994). E2A expression varied from cell to cell, with high levels of expression in specific areas of the central nervous system, in the lung and pancreas (Rutherford & LeBrun, 1998). E2A expression levels are higher in areas of rapid cell differentiation (Rutherford & LeBrun, 1998). Owing to the large number of HLH proteins, a sub-classification system has been based on tissue distribution, dimerisation abilities and DNA binding specificities (Murre *et al.*, 1994). The class I HLH proteins are known as the E-proteins and include E47, E12, E2-5, HEB and E2-2; they are structurally similar and ubiquitously expressed (Figure 5.1, A). The class II HLH proteins, which include MyoD, TAL1/SCL (stem cell leukaemia) and LYL1 (lymphoblastic leukaemia 1), show a tissue restricted expression (Figure 5.1, B); with a few exceptions they are not capable of forming homodimers and prefer to heterodimerise with the E-proteins (Massari & Murre, 2000). Class I and class II proteins can bind to E-box sites. Class III HLH proteins include the Myc and TFE3 (transcription factor E3) proteins and contain a leucine zipper adjacent to the HLH domain (Henthorn *et al.*, 1991; Zhao *et al.*, 1993). Class IV proteins, which include the Mad and Max proteins are capable of dimerising with Myc or with each other. Another class of proteins that can heterodimerise with class I and class II proteins are the class V proteins. These include the ID and emc proteins, which lack a basic region and cannot bind to DNA (Norton, 2000). Class V proteins inhibit DNA binding of class I and class II proteins, and therefore regulate their transcriptional activity (Massari & Murre, 2000).

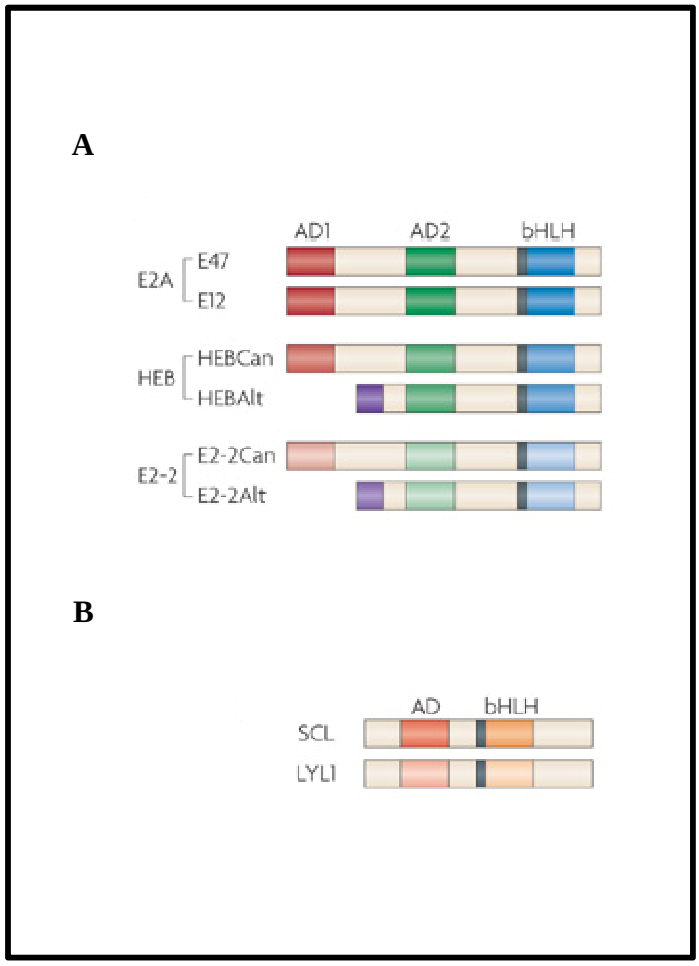

Figure 5.1: Schematic diagram of bHLH protein and their domains.

A. Schematic representation of the class I HLH proteins E47, E12, HEB and E2-2 known as E-proteins. Important domains are shown such as activation domain (AD) 1, AD2 and bHLH. Different transcription initiation sites result in canonical (Can) and alternative (Alt) forms of HEB and E2-2 proteins. **B.** Stem-cell leukaemia factor SCL also referred as TAL1 and LYL1 belong to class II HLH proteins. Shading indicates regions of homology (taken from Kee, 2009).

5.1.2 The structure of the E2A gene and protein

The crystal structure of the transcription factor E47 revealed a four-helix bundle with the two protruding helices being parallel to the DNA site (-CACCTG-) (Figure 5.2) (Ellenberger *et al.*, 1994). The E47 and E12 transcription factor proteins were initially discovered as immunoglobulin enhancer-binding proteins binding to E-boxes (Murre *et al.*, 1989). Also, they have been found at 5'-regulatory regions of lineage-restricted genes (Massari & Murre, 2000). The E47 and E12 proteins consist of a basic region, which is required for DNA binding, and an HLH structure that is critical for dimerisation. These bHLH proteins are involved in the regulation of cell growth and differentiation of cell-lineages including muscle cells and neurons (Reya & Grosschedl, 1998; Massari & Murre, 2000). The E47 and E12 proteins arise from the E2A gene through differential splicing of the exon encoding the bHLH domain. The amino acid sequences of the bHLH domains of these two proteins differ by 20% (Murre *et al.*, 1989). Although both E47 and E12 transcription factors have the ability to form heterodimers with class II HLH members, E47 and E12 have distinct biochemical properties (Chakraborty *et al.*, 1991; Lassar *et al.*, 1991). The E47 homodimer binds with relatively high affinity to DNA, however, the E12 homodimer interacts weakly with DNA (Sun & Baltimore, 1991).

The E2A gene is composed of four exons and one non-coding exon and spans about 16 kb (Hata & Mizuguchi, 2004). The promoter lacks TATA and CAAT boxes, but has possible binding sites for GATA -1/ -2 (Lowry & Atchley, 2000), CREB (Mayr & Montminy, 2001) and Sp1 (stimulating protein 1) (Briggs *et al.*, 1986). TATA-less promoters are found in a number of genes that usually have a variable transcription initiation site and are expressed ubiquitously in cells (Azizkhan *et al.*, 1993). Primer extension analysis has shown several transcription start sites in the E2A gene (Hata & Mizuguchi, 2004; Smale, 1997). A transient transfection assay using a 5'-flanking region has shown that the promoter activity can be regulated both by positive and negative ways, which is usual in the regulation of genes in eukaryotes (Ogbourne & Antalis, 1998; Hata & Mizuguchi, 2004). Deletion analysis revealed that the -257/ -238 segment plays a crucial role in the basal promoter activity of the E2A gene. Mutational

analysis of the -257/ -238 region did not show a single cis-element responsible for basal promoter activity, therefore it is possible that positive regulatory elements might interact with the E2A promoter. EMSA assays have shown that a positive regulatory segment and putative transcription factor(s) might be required for the basal activity of the E2A gene (Hata & Mizuguchi, 2004). Sequence analysis of the E2A gene comparing the human, mouse and chicken sequences showed that the sequences that are highly conserved corresponded to important functional domains such as the bHLH domain (Conlon & Meyer, 2004). The DNA binding and dimer-assembly domains of the E47 and E12 proteins are present at their C-terminal domain. The E2A gene transcriptional activation domains are mapped to the N-terminal regions (Murre *et al.*, 1989).

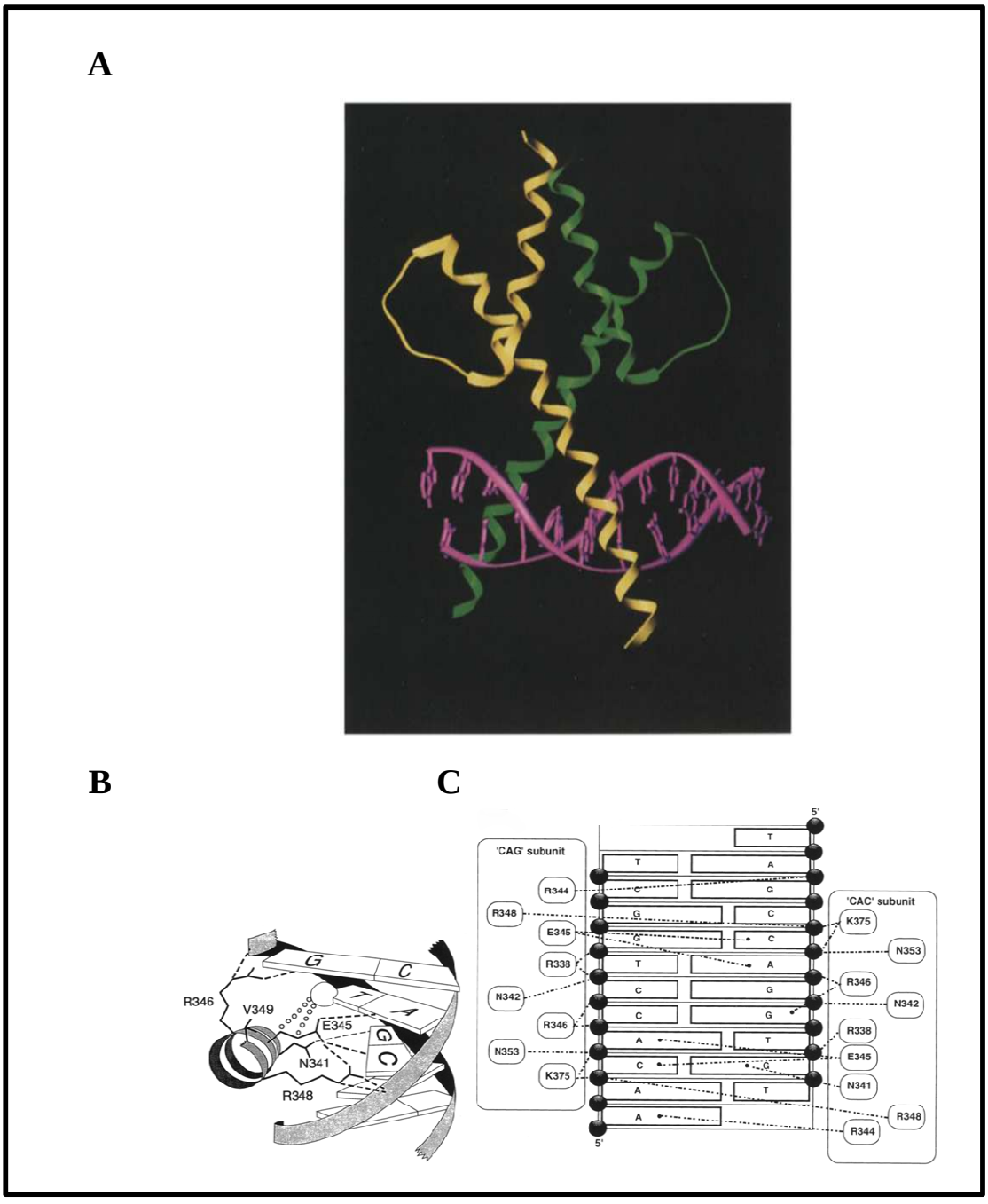

Figure 5.2: Schematic representation of the E47 crystal structure.

A. The E47 bHLH domain is a parallel four-helix bundle with two protruding helices shown in green and yellow. DNA (in red) contacts are made by the basic regions of the helices. **B.** Diagram of the E47 subunit contacts; each subunit of the E47 dimer interacts with one half of the -CACCTG- binding site. **C.** DNA base and phosphate contacts of the E47/DNA complex (taken from Ellenberger *et al.*, 1994).

5.1.3 Activation of transcription by E-proteins

E-proteins are involved in expression of B lineage-specific genes and have been found to function as transcriptional activators (Henthorn *et al.*, 1990). It has been shown that the activation domains AD1 and AD2 of E2A are involved in the regulation of target genes *in vivo* (Qiu *et al.*, 1998). When the N-terminal transactivation domain is deleted the E12 protein can no longer activate B lineage-specific gene expression in a macrophage cell line (Kee & Murre, 1998). The AD1 and AD2 domains of the E2A proteins are located in the N-terminal transactivation domain and contain the transcriptional activity (Aronheim *et al.*, 1993; Massari *et al.*, 1996). Furthermore, the AD1 and AD2 domains have been found to be functionally conserved in both mammalian cells and in the yeast *S. cerevisiae* (Quong *et al.*, 1993; Massari *et al.*, 1996).

The coactivator p300 has been shown to cooperate with tissue-specific bHLH proteins such as MyoD by binding to the bHLH domain of the protein and activating target genes (Figure 5.3) (Eckner *et al.*, 1996). Because p300 has been demonstrated to contain HAT activity it is possible that the E47 protein can recruit proteins that are involved in chromatin modification; p300 can modify chromatin structure to a locally open transcriptionally active configuration (Bannister & Kouzarides, 1996; Ogryzko *et al.*, 1996; Eckner *et al.*, 1996). The binding of MyoD to p300 is mediated by the bHLH domain of MyoD, and this was unexpected because the bHLH motif does not contain intrinsic transactivation potential (Eckner *et al.*, 1996). When p300/CREB binding protein (p300/CBP) antibodies where microinjected into myoblasts, terminal differentiation and transcriptional activity of myogenic bHLH proteins were blocked. These findings indicate that p300/CBP has an important role in cellular differentiation (Eckner *et al.*, 1996). In another study, p300 was shown to interact with the E47 protein stimulating the transcription of the insulin gene (Qiu *et al.*, 1998). However, the sequences of E47 required for this response span the activation domains AD1 and AD2 (amino acids 1 to 99 and 325 to 432). The sequence of p300 involved in the interaction and activation of E47 is between amino acids 1 to 1257, which is within the N-terminal domain of p300 (Qiu *et al.*, 1998).

Studies have identified a sequence LDFS, present in vertebrate HLH proteins and yeast HLH proteins, which is required for transactivation (Massari *et al.*, 1999). The conserved LDFS motif induces transcription by recruiting a nuclear HAT complex termed SAGA. The AD1 transactivation domain directly interacts with the SAGA complex, which is conserved throughout evolution. The SAGA complex consists of many subunits such as the TAFs (TBP-associated factors), the Ada and Spt proteins, the HAT Gcn5 and Tra1 (Grant *et al.*, 1998a,b). Activation by the LDFS domain requires functional Ada2 and Gcn5 proteins. Amino acid substitutions in the AD1 domain disrupt SAGA binding and inhibit transcriptional activation *in vivo* (Massari *et al.*, 1999). Also, in a number of yeast strains that lack a functional SAGA complex, AD1 is unable to induce transcription (Massari *et al.*, 1999). Furthermore, studies have shown that E2A is acetylated *in vitro* by p300, CBP and PCAF (p300/CBP-associated factor) (Bradney *et al.*, 2003). In conclusion, these studies indicate that E47 may in some cases induce transcription by recruiting HAT complexes to target genes.

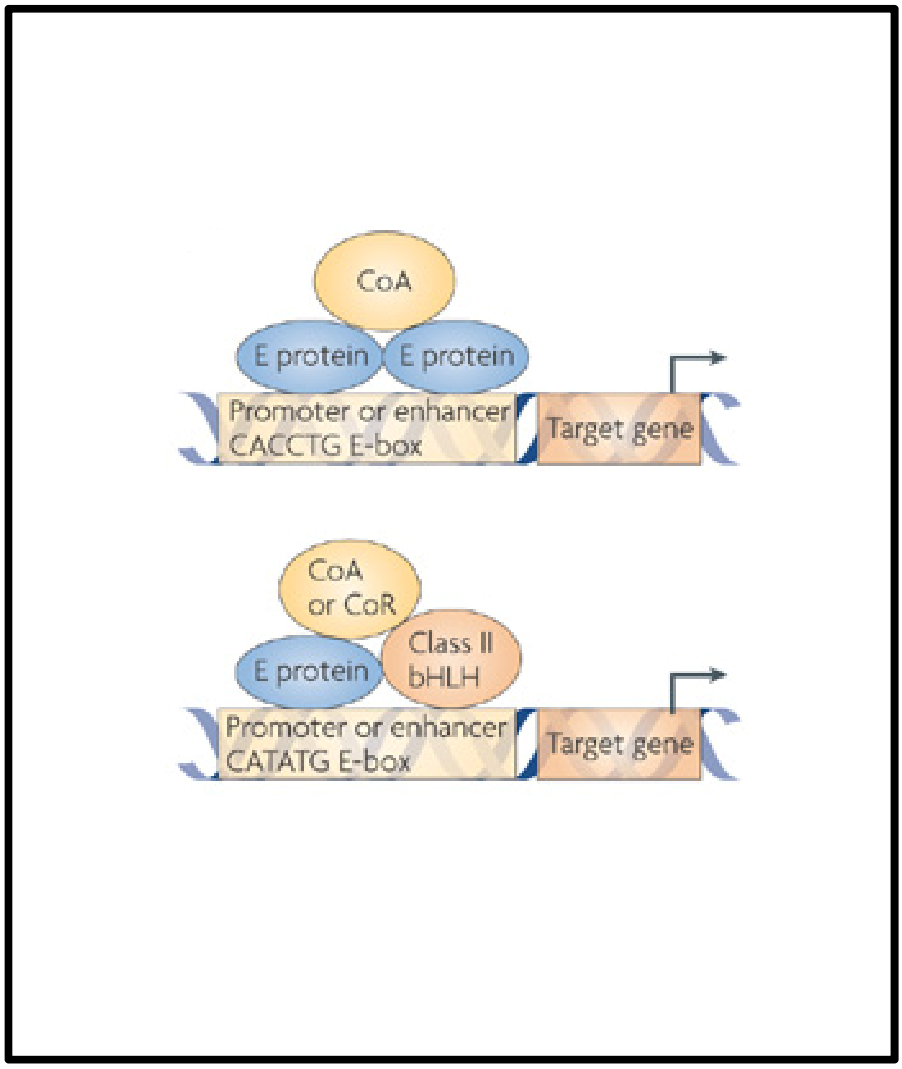

Figure 5.3: Schematic representation of E-protein dimers and their function.
E-protein homodimers are bound to target genes and act as activators with the recruitment of co-activators such as p300/CBP. E-protein class II bHLH proteins are bound to target genes and can act as transcriptional repressors by the recruitment of co-repressors like mSin3A or ETO (taken from Kee, 2009).

5.1.4 Regulation of E47 protein level by the ubiquitin-proteasomal pathway

E47 proteins are degraded through the ubiquitin-proteasome system which is a basic cellular mechanism of post-translational regulation. The degradation of a protein is a three step process that is initiated with the formation of a ubiquitin-conjugate with a ubiquitin-activating enzyme (Haas & Siepmann, 1997). Ubiquitin is moved to a ubiquitin-conjugating enzyme, which transports ubiquitin to an amino group of a lysine residue on a substrate protein. The ubiquitin-conjugating enzymes consist of 12 families, which together with several ubiquitin ligases are required for ubiquitin conjugation (Haas & Siepmann, 1997). When the ubiquitin conjugate is made, a multi-ubiquitin complex is assembled that becomes a signal for proteolysis by the 26S proteasome.

The mammalian Ubc9 (mUbc9) is required for the degradation of the E47 proteins by the ubiquitin-proteasomal pathway (Huggins *et al.*, 1999). mUbc9 interacts with E47 at the 477-530 amino acid region (Kho *et al.*, 1997). The mUbc9 sequence is highly conserved among the human, mouse and rat species (Kovalenko *et al.*, 1996) and several mUbc9 binding partners are known (Firestein & Feuerstein, 1998). The region that is rich in proline, glutamic acid, serine and threonine (PEST) residues is thought to be the E47 (478-531) degradation domain (DD). E47 proteins with a deletion mutation lacking the region, E47Δ (478-531), were more stable than the wild-type E47, exhibiting a half-life of 6 hours compared to 55 minutes (Huggins *et al.*, 1999). Also, overexpression of the full-length Ubc9 antisense construct reduces the degradation of E12 (Kho *et al.*, 1997). Deletion of the DD domain of E47 had no effect on the transcriptional activity and E-box binding of E47. Two regions that interact with Ubc9 were mapped in the E47DD, the 476-494 and 505-513 regions. The E47 (505-513) region interacts with Ubc9, but not with the Ubc5, ID3 and MyoD proteins. Substitution of the central hydrophobic residues of the E47 (505-513) region with basic residues inhibited its interaction with Ubc9. The E47 protein that lacks the second region which interacts with Ubc9 was more stable than the wild-type E47 (Huggins *et al.*, 1999).

When the E2A DD was reintroduced into the oncoprotein E2A-HLF this destabilised it, such that degradation was quicker than with the wild-type E2A-HLF

(half-life of 60 minutes compared to more than 2 hours); this hints that the DD domain can transfer a signal to a heterologous protein. The E2A-HLF-DD was stabilised by a proteasome inhibitor LLNL, which shows that the E2A-DD targets proteins for degradation through the proteasome. The E2A-DD domain is very important since every chromosomal translocation that produces a chimeric E2A-oncoprotein lacks the exon that encodes the degradation domain. The longer half-life of E2A-HLF may increase its transforming ability by allowing more protein to accumulate and activating transcription (Honda *et al.*, 1999). The prolonged half-life of a stable protein can be decreased by the addition of a degradation domain (Hochstrasser & Varshavsky, 1990; Yaglom *et al.*, 1995).

It has been shown that E47 modulates cellular localisation and degradation of the ID1 protein (Lingbeck *et al.*, 2005). Expression of E47 stabilises ID1 and causes an increase in the $t_{1/2}$ from ~1 hour to ~8 hours. Also, co-transfection of an NLS deficient ID1 ($ID1^{NLS}$) with E47 led to an altered intracellular localisation of $ID1^{NLS}$ from 27% nuclear to >95% nuclear. In another study, it was demonstrated that E47 is primarily localised in the nucleus in C2C12 myoblasts and myotubes (Sun *et al.*, 2007). Incubating cells with MG132, a proteasome inhibitor, stabilised E47, which suggests that the ubiquitin-proteasomal pathway is responsible for degradation of E47.

Notch signaling is also involved in E47 ubiquitination and degradation by MAP kinase activities (Nie *et al.*, 2003). Studies have shown that activation of Notch 1 and Notch 2 inhibits the transcriptional activity of E47, which is crucial for B and T lymphocyte development (Ordentlich *et al.*, 1998). Notch signaling is also important for lymphocyte development. In order to induce degradation of E47, Notch requires phosphorylation of E47 by p42/p44 MAP kinases. Notch-induced degradation of E47 relies on RBP-Jκ, its downstream effector, to possibly activate expression of genes involved in E47 ubiquitination (Nie *et al.*, 2003). Therefore, Notch signaling may regulate lymphocyte development by controlling E47 protein degradation.

5.1.5 E47 regulation by protein phosphorylation

E47 proteins are widely expressed and it is believed that B cell-restricted DNA binding by E47 homodimers is regulated by protein modifications. To investigate the properties of B cells that allow DNA binding by E47 homodimers, studies have concentrated on E47 phosphorylation (Sloan *et al.*, 1996). Using an N-terminal truncation of E47, it was demonstrated that the phosphorylation of two serine residues was responsible for inhibition of DNA binding by E47 homodimers *in vitro*. The two serines N-terminal of the E47Δ mutant protein are phosphorylated in several cell types; however they are hypophosphorylated in B cells. When the two serine residues were phosphorylated *in vitro* this caused inhibition of DNA binding by E47Δ homodimers. However, E47Δ-MyoD heterodimers were able to bind DNA. Therefore, hypophosphorylation may be important for the activity of E47 homodimers in B cells (Sloan *et al.*, 1996). Phosphorylation affects the activity of several transcription factors (Karin, 1994). The CREB and TCF/Elk-1 are stimulated by serine phosphorylation and the STAT proteins are activated by tyrosine phosphorylation (Gille *et al.*, 1992; Shuai *et al.*, 1993). Studies have demonstrated that Ser-514 and Ser-529, which are located N-terminal to the bHLH domain of E47, are phosphorylated by CK2 and protein kinase A (Sloan *et al.*, 1996). Phosphorylation also has a role in regulating the degradation of many proteins such as cyclin D, IκBα and c-Jun (Diehl *et al.*, 1997; Chen *et al.*, 1996; Musti *et al.*, 1997). The E47 degradation domain is rich in PEST residues, which often serve as phosphoacceptor sites (Huang *et al.*, 1998). Studies show that cdc28 phosphorylation of the cyclin 3 PEST domain reduces the half-life of Cln3 (Yaglom *et al.*, 1995). The E47 protein has several possible phosphorylation sites and deleting the E47 degradation domain permits multiphosphorylated E47 to accumulate. It is possible that hyperphosphorylation of E47Δ (478-531) leads to events that initiate E47 protein degradation (Huggins *et al.*, 1999).

5.1.6 The role of E47 in cell growth

A number of studies have indicated that E2A gene products are involved in regulating cell growth. Apart from the HLH region which is required for growth inhibition by the MyoD family, the E2A proteins also have two additional inhibitory regions that are active in the absence of the HLH domain. These regions are in the N-terminal and central region of E47/E12 and correspond to the transactivation domain of the proteins (Aronheim *et al.*, 1993; Quong *et al.*, 1993). When E47 was overexpressed in colony-forming assays, this suppressed the growth of NIH3T3 cells (Peverali *et al.*, 1994). This growth suppressive effect was counteracted by the ID proteins. However, when E47 was used that lacked the HLH domain, ID proteins could no longer reverse the growth inhibition. When E47 was overexpressed or ID proteins were neutralised, this led to inhibition of proliferation in the G_1 phase until 3-4 hours before entering the S phase. The antagonism between E47 and ID proteins appears to be involved in regulating critical G_1 progression (Peverali *et al.*, 1994). It has been shown that E2A null mice develop rapid T cell lymphomas, the B lymphocyte development is completely blocked and abnormalities appear in the αβ T cell development (Bain *et al.*, 1994; Bain *et al.*, 1997; Yan *et al.*, 1997). Disruption of the ID1 gene improves the survival rates of E2A gene-null mice, suggesting that ID1 interacts with E2A and that the unbalanced function of ID1 affects E2A null mice (Yan *et al.*, 1997). However, the E47/ID1 double knock-out mice continued to develop T cell tumours. Therefore, E2A might be involved in maintaining the homeostasis of T lymphocytes. Ectopic expression of E47 or E12 induces the death of E2A-deficient lymphomas (Engel & Murre, 1999). E2A-expressing cells were eliminated from cultures into which E47 and E12 were introduced. This was not due to an arrest in cell cycle progression, but because E2A proteins activate a programmed cell death pathway in these lymphomas. Loss of mitochondrial transmembrane potential precedes the E2A-mediated cell death. These data imply that E47 and E12 proteins can act as tumour suppressors.

Several forms of T cell acute lymphoblastic leukaemias (T-ALL) are characterised by high expression of class II HLH proteins, such as TAL1/SCL and LYL1 (Miyamoto *et al.*, 1996; Voronova & Lee, 1994). The activation of the bHLH

TAL1/SCL gene occurs by chromosomal translocation or mutation in patients with T-ALL who respond poorly to existing therapies. Studies have demonstrated that TAL1/SCL induces leukaemia by interfering with E47 and HEB target genes, which are important for thymocyte differentiation and survival (O'Neil *et al.*, 2004). The TAL1/SCL oncogene was expressed in mice in an E2A or HEB heterozygous background resulting in differentiation arrest and disease progression, therefore decreased expression of E2A and HEB plays an important role. E47/HEB heterodimers are shown to regulate cluster of differentiation 4 (CD4) expression by recruiting the p300 coactivator. In TAL1/SCL/E2A$^{+/-}$ or TAL1/SCL/HEB$^{+/-}$ thymocytes CD4 expression is decreased. Unlike ID proteins which operate by inhibiting the binding of E-proteins to DNA, TAL1/SCL represses gene transcription by recruitment of the mSin3A/HDAC1 (histone deacetylase 1) corepressor complex to the E47/HEB target CD4 and other genes (Figure 5.3) (Kee, 2009).

The AML1-ETO fusion protein occurs due to a t(8;21) chromosomal translocation and is implicated in 15% of acute myeloid leukaemia cases. The AML1-ETO fusion protein binds to E-proteins and converts them to transcriptional repressors (Zhang *et al.*, 2004). AML1-ETO inhibits E-protein mediated transcriptional activation by interactions that preclude recruitment of coactivators such as p300/CBP. The interaction of the AML1-ETO with the AD1 domain of the promoter-bound E-proteins reveals a silencing mechanism by directing an exchange of cofactors, HATs against histone deacetylases (HDACs) (Figure 5.3). Therefore, E-proteins are targets of AML1-ETO and their deregulation may play a role in leukaemia, suggesting a mechanism which is different from differentiation-inhibitory proteins.

The results discussed here demonstrate that E47 is involved in the regulation of cell growth (Pagliuca *et al.*, 2000; Zheng *et al.*, 2004). E47 negatively regulates cell growth by inhibiting cell cycle progression and promoting cell death (Rothschild *et al.*, 2006; Engel & Murre, 1999). Cells overexpressing E47 in several cell types grow at a slower rate than untreated control cells (Peverali *et al.*, 1994). Pol III transcripts are important in determining growth rate, since they are involved in protein synthesis. Studies have demonstrated that suppressor proteins such as RB, p53 and Maf1 are likely to affect the rate of cell growth by regulating pol III transcription (Sutcliffe *et al.*, 2000;

Crighton *et al.*, 2003; Goodfellow *et al.*, 2008). This chapter will address if E47 regulates pol III transcription. Since E47 is an important regulator of cell growth and also transcripts of pol III are required in the same process, it is possible that E47 expression may trigger repression of pol III activity. The following experiments were designed to assess if indeed this is occurring. The results in this chapter reveal that pol III transcription is downregulated by the expression of the E47 transcription factor protein and expose a mechanism by which E47 regulates pol III activity. In this chapter only the relationship between E47 and pol III transcription was investigated and not of other E-protein family members.

5.2 Results

5.2.1 Knockdown of E2A by RNAi raises the levels of pol III transcripts

E47 depletion has been demonstrated to promote cell growth (Zheng *et al.*, 2004). When cells were treated with siRNA against E47, this led to inhibition of expression of $p16^{INK\alpha}$ and cells displayed an extended proliferative lifespan. E2A deficient mice develop abnormalities and are prone to malignancies (Bain *et al.*, 1997; Yan *et al.*, 1997). Since pol III transcripts are required for steady cell growth, it is likely that negative regulators of this process would repress pol III transcription. To determine whether the E47 protein has an effect on pol III transcription *in vivo* in human cells, small interfering RNA was used. HeLa cells were transfected with siRNA against E2A and the control Oct-1. The cells were treated for 48 hours and harvested. Protein extracts and RNA were analysed. Western blot analysis revealed that E47 protein levels were significantly reduced compared to the control Oct-1. In addition, it was shown that in the E2A siRNA-transfected HeLa cells the associated ID1, ID2 and ID3 proteins remained at the same levels. Actin was used as a loading control (Figure 5.4, A). RT-PCR analysis demonstrated the knockdown of E2A RNA levels in comparison to the control Oct-1 with the ARPP P0 mRNA used as a loading control (Figure 5.4, B).

The next step was to identify if this knockdown of the protein and RNA levels of E47 and E2A respectively would have an effect on pol III transcription. RT-PCR analysis revealed that the knockdown of E2A has a significant effect on the levels of 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, $tRNA^{Tyr}$, 7SL RNA, 7SK RNA, MRP, and U6 snRNA (Figure 5.5). The knockdown of E2A results in an increase in the levels of these pol III transcripts compared to the control. Also, another effect of this knockdown is the increase in the levels of a pol I transcript, the pre-rRNA (Figure 5.5). These effects are specific, as the pol II transcribed ARPP P0 mRNA does not change in response to E2A knockdown. From these results it can be concluded that E2A has a negative effect on pol I and pol III transcript expression *in vivo*.

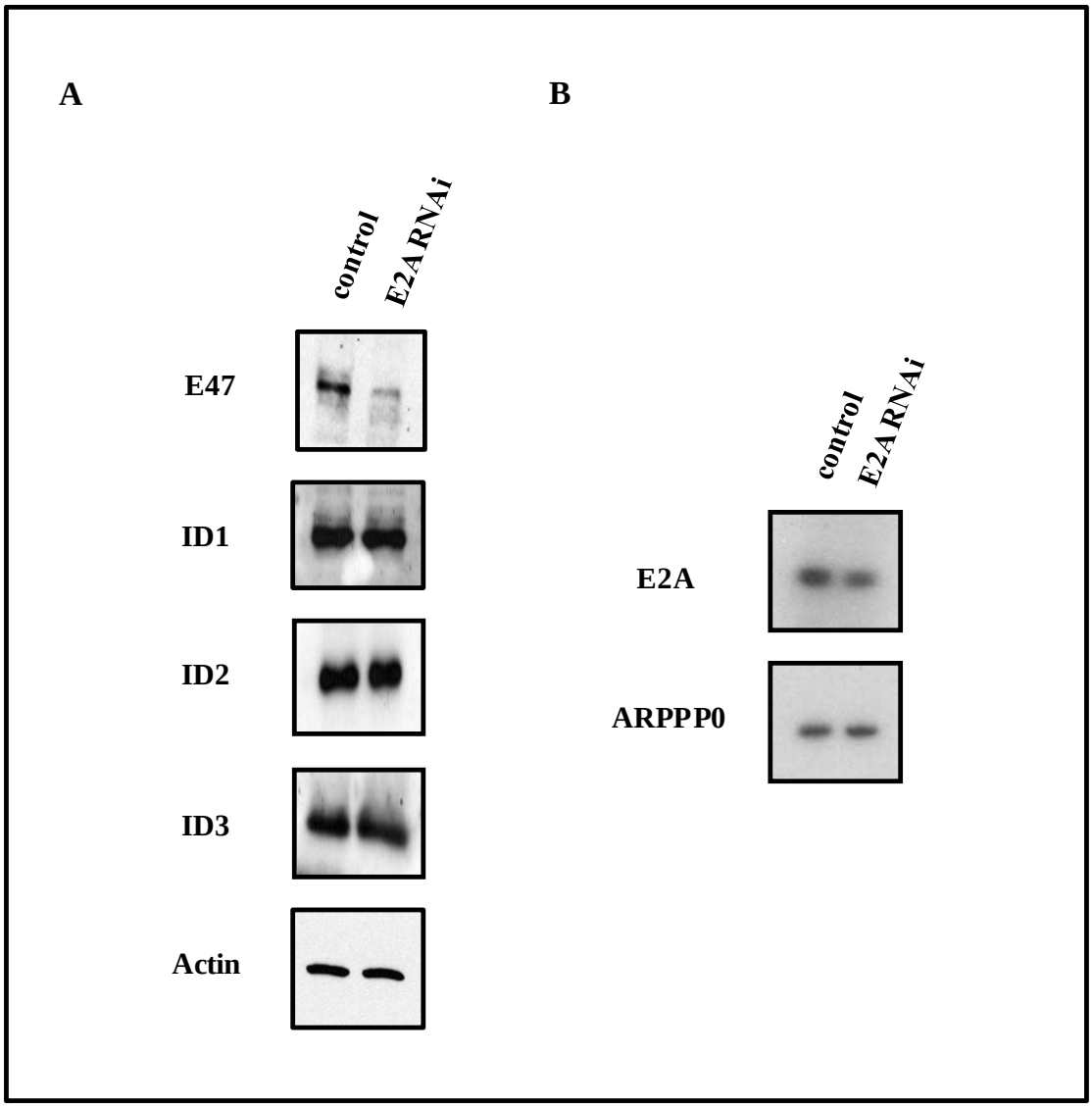

Figure 5.4: Knockdown of E47 by RNAi.

HeLa cells were transfected with E2A siRNA or the control Oct-1 siRNA. **A.** Western blot analysis of E47, ID1, ID2 and ID3 expression, following transfection with siRNA against E2A. Actin was used as the loading control. **B.** RNA was analysed by RT-PCR for E2A depletion by siRNA. The pol II-transcribed mRNA ARPP P0 was used as the loading control.

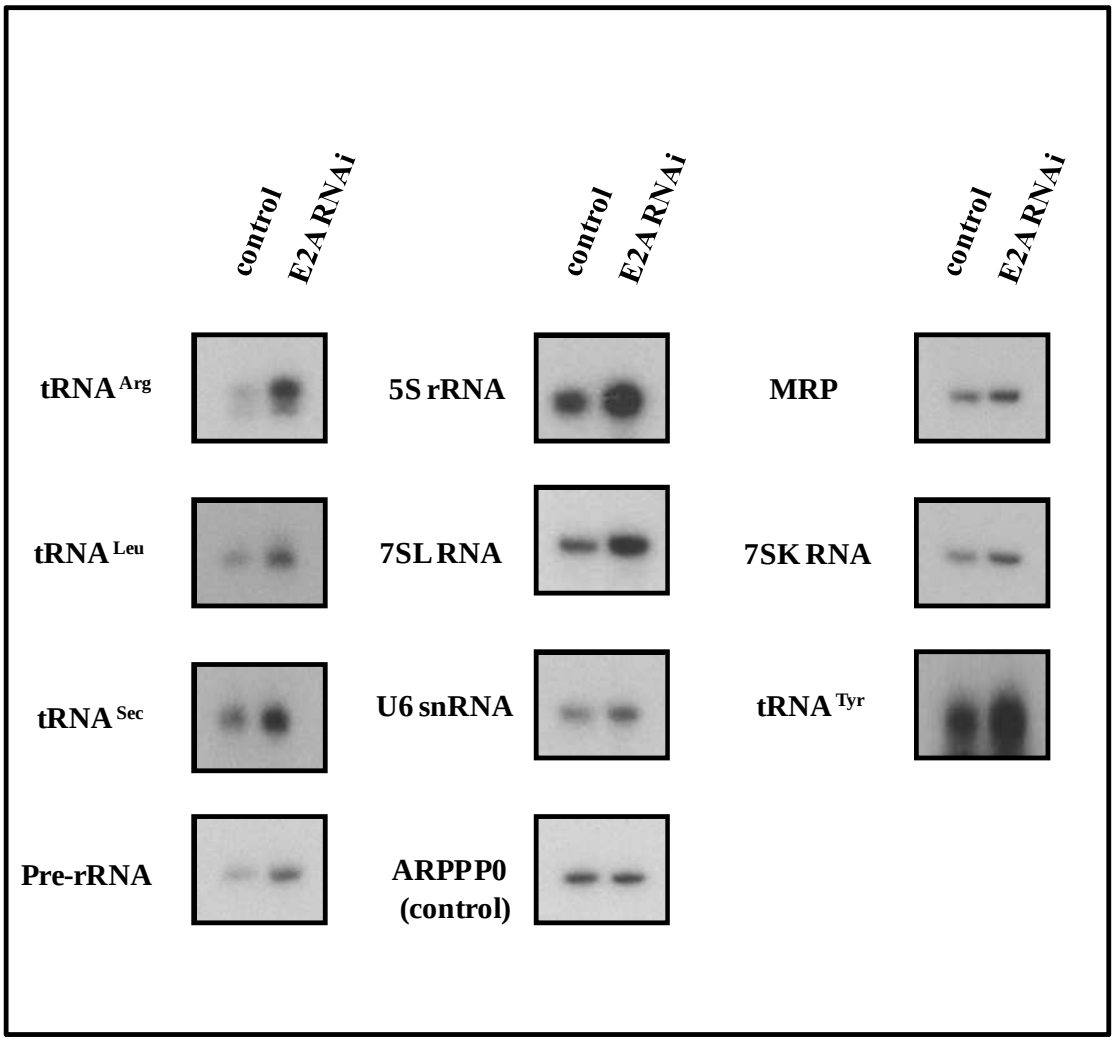

Figure 5.5: Knockdown of E47 by RNAi increases the levels of pol I and III transcripts.

Effects of the E47 knockdown by siRNA against E2A in HeLa cells after transfection for 48 hours. RNA was analysed by RT-PCR for the expression of the pol III transcripts 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, $tRNA^{Tyr}$, 7SL RNA, 7SK RNA, MRP, and U6 snRNA. The pol I transcript pre-rRNA was also analysed. The ARPP P0 mRNA was used as a control.

5.2.2 Overexpression of E47 reduces the levels of pol III transcripts

Overexpression of E47 has been found to cause growth arrest in NIH3T3 cells (Peverali *et al.*, 1994). In addition, enforced expression of E47 suppresses the cell growth efficiency of several cell lines (Pagliuca *et al.*, 2000). Ectopic expression of E47 in lymphoma cell lines derived from E2A deficient mice results in cell death of E2A-expressing cells (Engel & Murre, 1999). To investigate if E47 can downregulate pol III activity *in vivo* in human cells, adenovirus infection was used. HEK 293 cells were infected with an adenovirus expressing E47 (Ad-E47) or with the parental virus adenovirus-cytomegalovirus 5' internal ribosomal entry site green fluorescent protein (Ad-Vector). The infection was left to proceed for 24 hours and the cells were harvested. RNA and protein extracts were analysed. Western blotting reveals that infection with the E47 adenovirus results in overexpression of the E47 protein, compared to the control vector (Figure 5.6, A). This effect is specific, since actin was used as loading control and remains at the same levels in both lanes. RT-PCR analysis demonstrated that the overexpression of E47 resulted in an increase in the RNA levels of E2A compared to the control empty vector (Figure 5.6, B). The ARPP P0 loading control did not change, which suggests that the effect is specific. To investigate if increased E47 protein and E2A RNA levels would have an effect on pol III transcripts, RT-PCR experiments were performed. The results revealed that the levels of 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 7SK RNA and MRP, were reduced after overexpression of E47 (Figure 5.7). This effect is specific, since the pol II transcript encoding ARPP P0 remains unchanged. These results demonstrate that overexpression of E47 reduces pol III transcription.

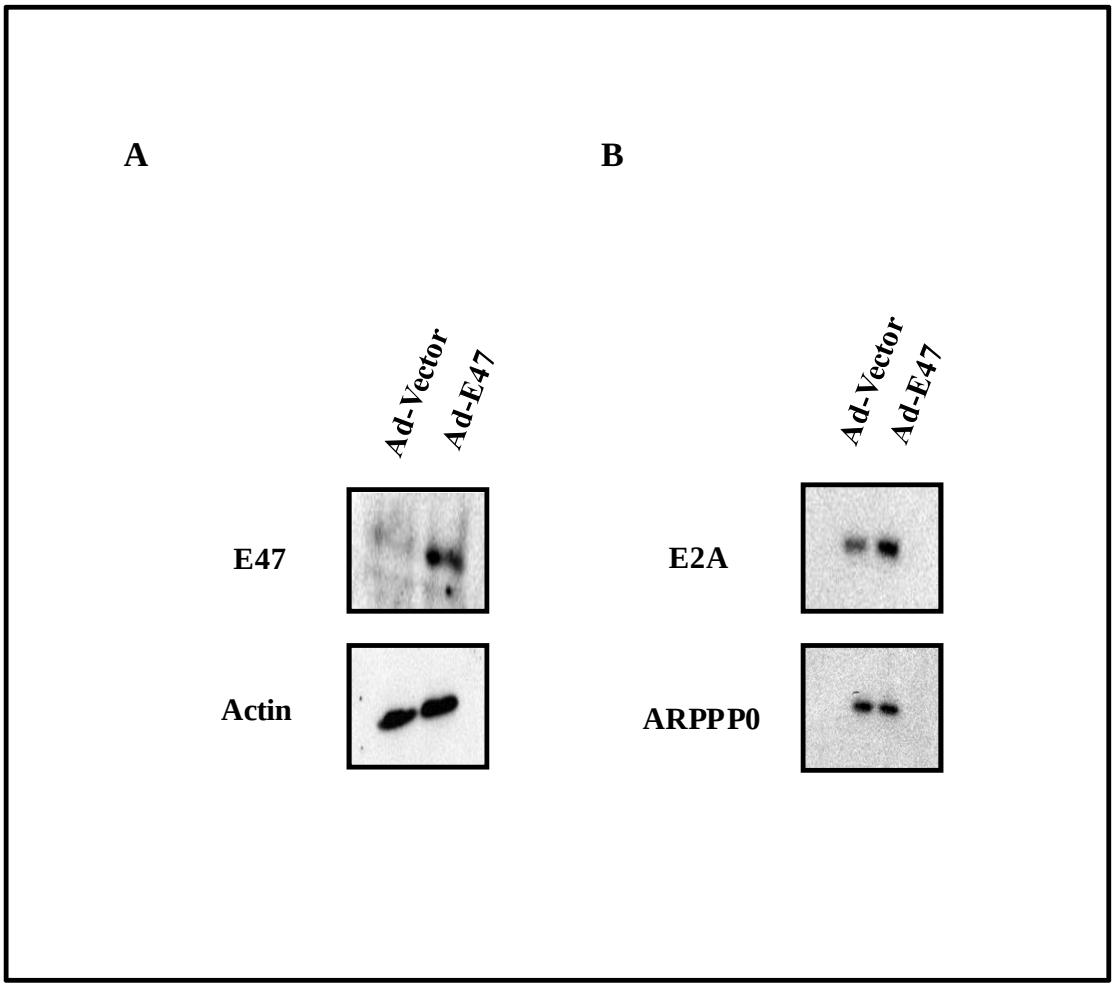

Figure 5.6: Overexpression of E47 by adenovirus infection.

HEK 293 cells were infected with an adenovirus expressing E47 or with the control vector. **A.** Western blotting of E47 with cells harvested 24 hours after infection with adeno-E47 (Ad-E47) or adenovirus vector (Ad-Vector). Actin was used as loading control. **B.** RT-PCR analysis for the overexpression of E2A. The pol II-transcribed mRNA ARPP P0 was used as control.

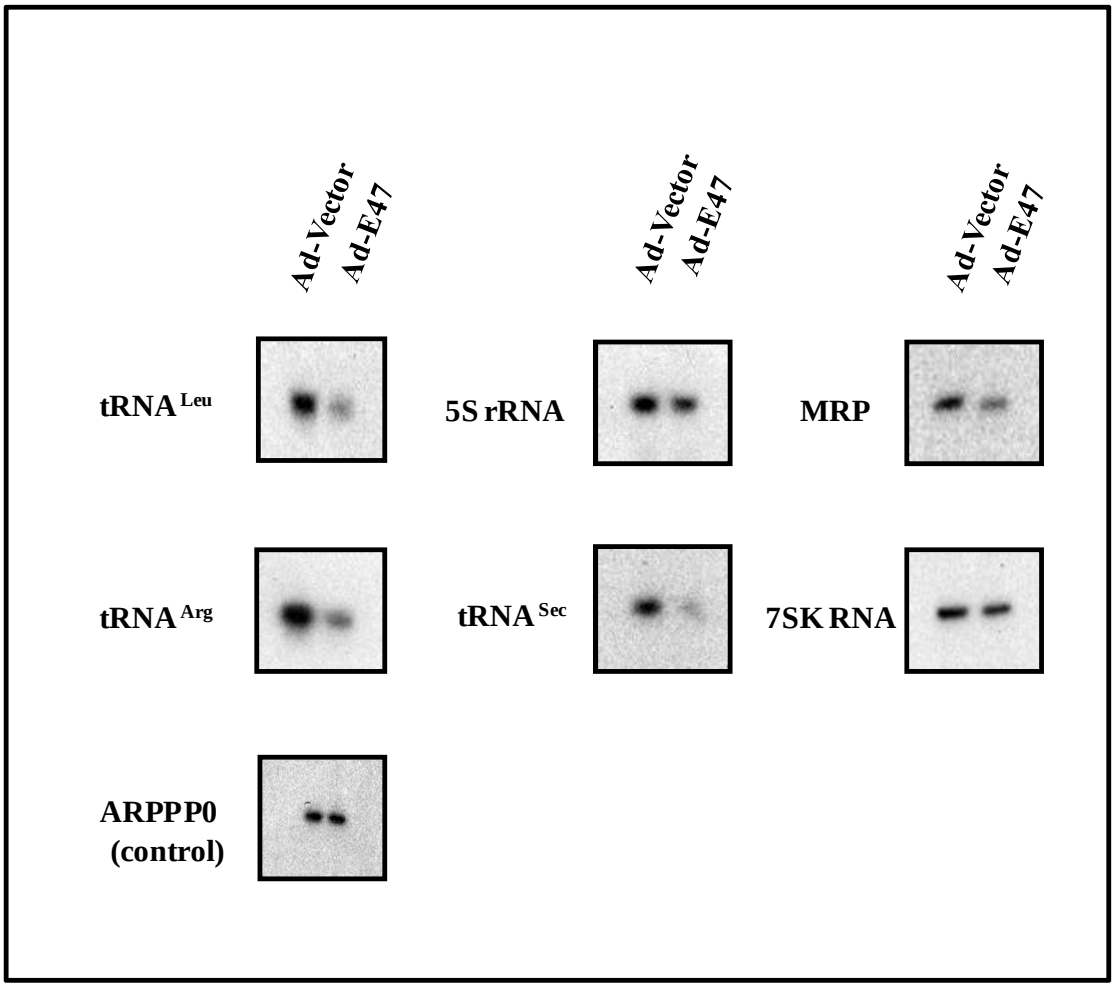

Figure 5.7: Overexpression of E47 by adenovirus infection decreases the levels of pol III transcripts.

Effects from adenoviral expression of E47 in HEK 293 cells. Cells were harvested after 24 hours infection with Ad-E47 or Ad-Vector. RNA was analysed by RT-PCR for the expression of 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 7SK RNA and MRP. The ARPP P0 mRNA was used as the control.

5.2.3 E47 localises at pol III-transcribed genes *in vivo*

E47 has been found to bind to the promoters of the p16 and p21 genes, which leads to cell growth arrest (Prabhu *et al.*, 1997; Zheng *et al.*, 2004). E47 binds to E-box sequences of these promoters and other pol II-transcribed genes (Murre *et al.*, 1989; Perez-Moreno *et al.*, 2001). However, pol III templates rarely contain E-box DNA promoter sequences which could be potentially targeted by E47. The results so far have indicated that E47 can influence pol III transcription. The effects from knocking down and overexpressing E47 imply a possible mechanism by which E47 interacts with the pol III transcription apparatus. This interaction with the pol III templates could lead to the repression of pol III activity. To investigate if endogenous E47 is present on pol III-transcribed genes *in vivo*, chromatin immunoprecipitation assays were performed in HeLa cells. Antibodies against E47, TBP, Brf1, pol III, TFIIIC, TFIIA and TFIIB were used to precipitate the proteins that were cross-linked to chromatin. TBP, Brf1, TFIIIC and pol III were used as positive controls, TFIIA, TFIIB and beads were used as negative controls. The results reveal that endogenous E47 is present at pol III-transcribed genes such as 5S rRNA and $tRNA^{Leu}$ (Figure 5.8). This experiment could be repeated in order to obtain a better exposure for figure 5.8, $tRNA^{Leu}$. The positive controls TBP, Brf1, pol III and TFIIIC are present on these templates as expected and the negative controls TFIIA, TFIIB are not detected. These data indicate that the association of endogenous E47 with pol III-transcribed genes *in vivo* is specific.

Another human cell line, HEK 293 cells, were used to investigate if E47 can occupy pol III-transcribed genes *in vivo*. Cells were infected with an adenovirus expressing E47 and harvested 24 hours post-infection. Chromatin immunoprecipitation assays were performed in cells in which E47 was overexpressed. The positive controls Brf1, TFIIIC and pol III are present at 5S rRNA and $tRNA^{Leu}$ and 7SL genes, whereas the negative control TFIIA is not detected (Figure 5.9). It was found that overexpression of E47 results in strong detection on pol III-transcribed genes, like 5S rRNA and $tRNA^{Leu}$ and 7SL genes (Figure 5.9). This interaction is specific and not due to over cross-linking of proteins to DNA, since no signal was detected when primers were used

for the pol II-transcribed ARPP P0 gene. These results demonstrate that the association of E47 with class III genes is specific.

A stable U2OS cell line stably expressing ID3 was also used to detect if E47 was present on pol III-transcribed genes *in vivo*. Chromatin immunoprecipitation assays revealed that E47 was recruited at $tRNA^{Arg}$ and 7SL genes (Figure 5.10). E47 is not associated with the pol II-transcribed gene encoding ARPP P0. Therefore, these results demonstrate the binding of E47 to pol III-transcribed genes. Future experiments could include the use of a U2OS cell line expressing an empty vector control to allow comparison with U2OS cells expressing ID3.

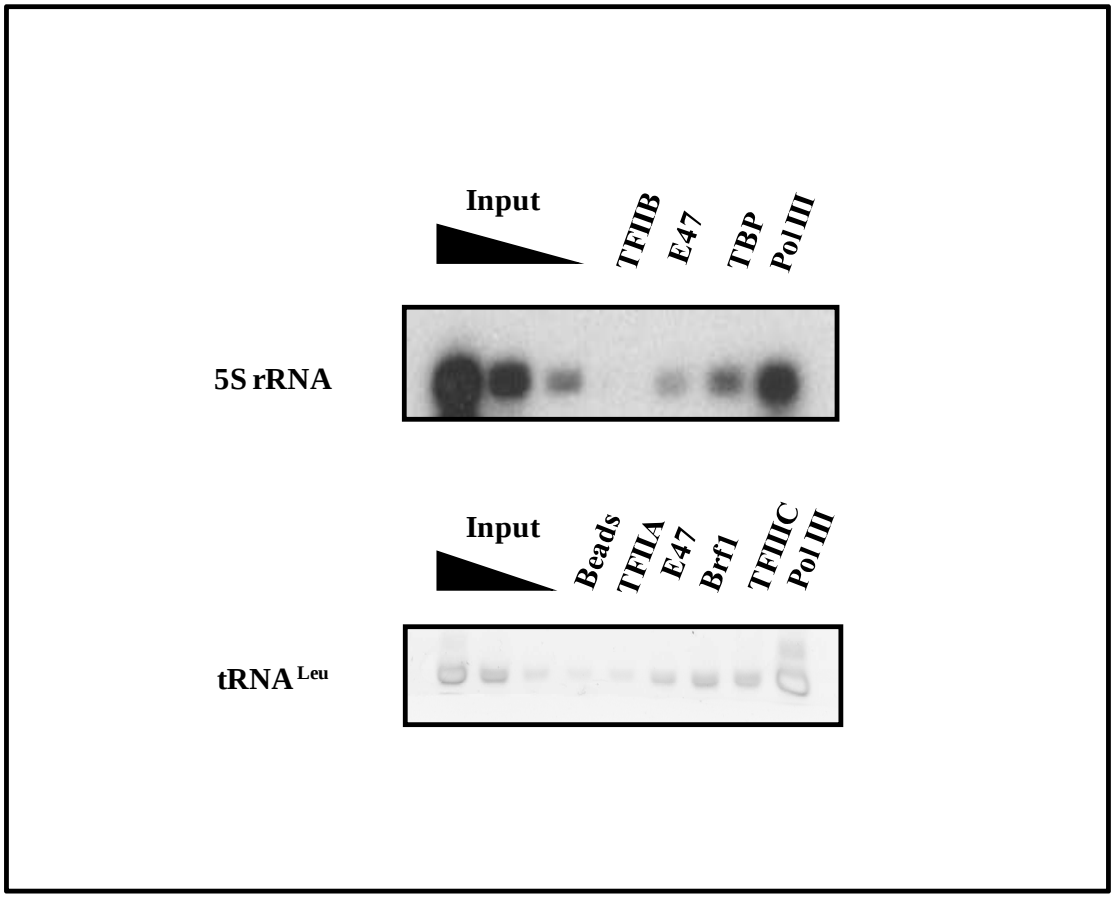

Figure 5.8: Endogenous E47 occupancy on pol III-transcribed genes.

Chromatin immunoprecipitations were performed using HeLa cells. The N-649 antibody was used against E47. FL-109 and C-18 antibodies were used against the TFIIA and TFIIB negative controls respectively. TBP (MTBP-6), Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were used as positive controls for pol III-transcribed genes. The immunoprecipitated DNA samples were analysed by PCR performed on inputs and primers against 5S rRNA and $tRNA^{Leu}$ genes.

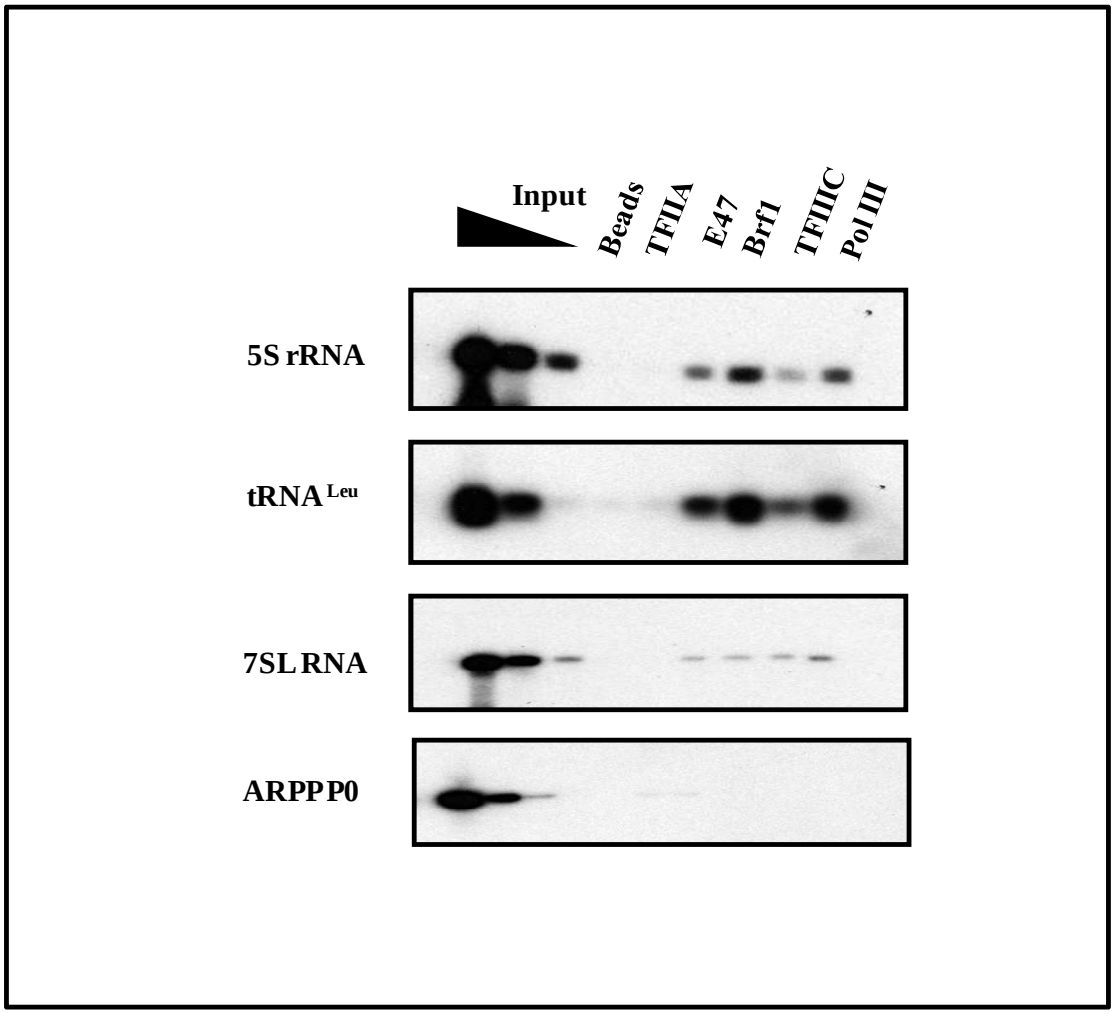

Figure 5.9: E47 is present at pol III-transcribed genes.

Chromatin immunoprecipitations were performed using HEK 293 cells harvested 24 hours after adenoviral expression of E47. The TFIIA (FL-109) antibody and beads were used as negative controls. The N-649 antibody was utilised for E47. The Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were used as positive controls. The samples were analysed by PCR performed on inputs and primers against 5S rRNA, $tRNA^{Leu}$ and 7SL RNA genes.

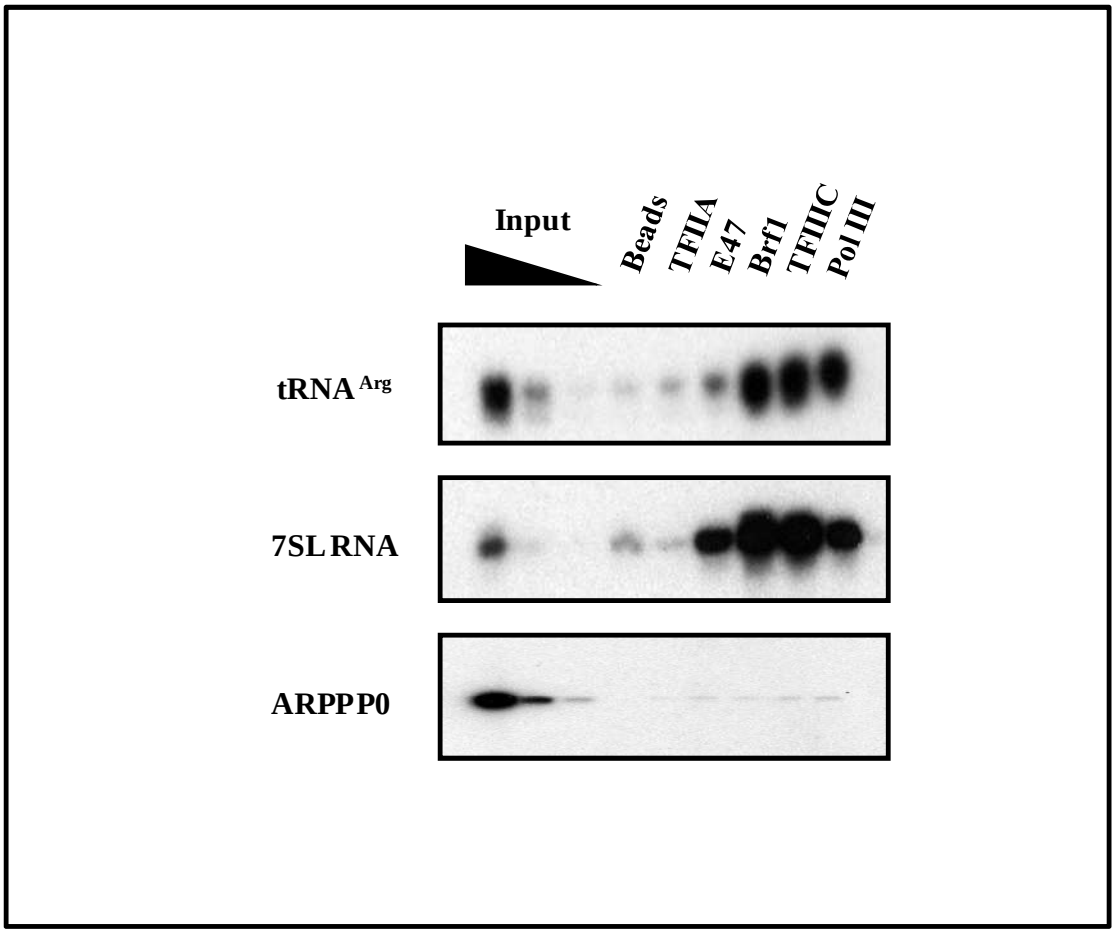

Figure 5.10: E47 is recruited to pol III-transcribed genes.

Chromatin immunoprecipitations were performed using a U2OS cell line stably overexpressing ID3. The TFIIA (FL-109) antibody and beads were used as negative controls. The N-649 antibody was utilised for E47. Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were used as positive controls for pol III-transcribed genes. The samples were analysed by PCR performed on inputs and primers against $tRNA^{Arg}$ and 7SL RNA genes.

5.2.4 Mapping of E47 on the 7SL locus

Chromatin immunoprecipitation experiments have revealed that E47 localises to the promoter of pol III-transcribed genes. To determine if E47 co-localises with the basal transcription factors TFIIIB and TFIIIC on pol III promoters, mapping of the 7SL gene locus was carried out. Five sets of primers were designed, upstream of the gene, at the gene and downstream of the pol III-transcribed 7SL coding region (Figure 5.11, A). HEK 293 cells were infected with an adenovirus expressing E47. Cells were harvested after 24 hours and PCR was used to amplify the DNA extracted from chromatin immunoprecipitation assays. The results revealed that the basal transcription factors TFIIIB and TFIIIC exhibit a different distribution on the 7SL locus (Figure 5.11, B). E47 co-localises with Brf1 and TFIIIC on the second and third position of the pol III template (Figure 5.11, C). Also, a very exciting result, revealed by using this E47 infected cell line, was that ID1 and ID2 are recruited to the 7SL locus. ID1 and ID2 co-localise with Brf1 at the start of the gene on the second position, and decrease at the end of the gene at position three (Figure 5.11, C). However, ID3 does not appear to localise on the 7SL locus (Figure 5.11, B). Therefore, from these results E47 co-localises with Brf1 and TFIIIC. Co-immunoprecipitation experiments are required to test for interactions of E47 with these basal factors that might recruit E47 to pol III-transcribed genes. ID1 and ID2 appear to co-localise with Brf1 rather than TFIIIC, suggesting that ID1 and ID2 are recruited by TFIIIB. For the recruitment of IDs to pol III-transcribed genes, a HEK 293 cell line overexpressing GFP could be compared to the HEK 293 cells overexpressing E47. Also, the effect of overexpression of E47 on the protein levels of Pol III, TFIIIC, ID2, ID1 and Brf1 should be determined by western blotting. The overexpression of E47 could potentially lead to the expression or suppression of basal pol III factors or ID proteins directly or indirectly which could then result in affecting pol III transcription. In the future, western blotting experiments should be done in order to determine whether the levels of ID and E47 proteins change upon overexpression or knockdown of ID and E47 proteins. Results have demonstrated that when the ID2 or E47 proteins are overexpressed the protein levels of each other do not change (Rothschild et al., 2006).

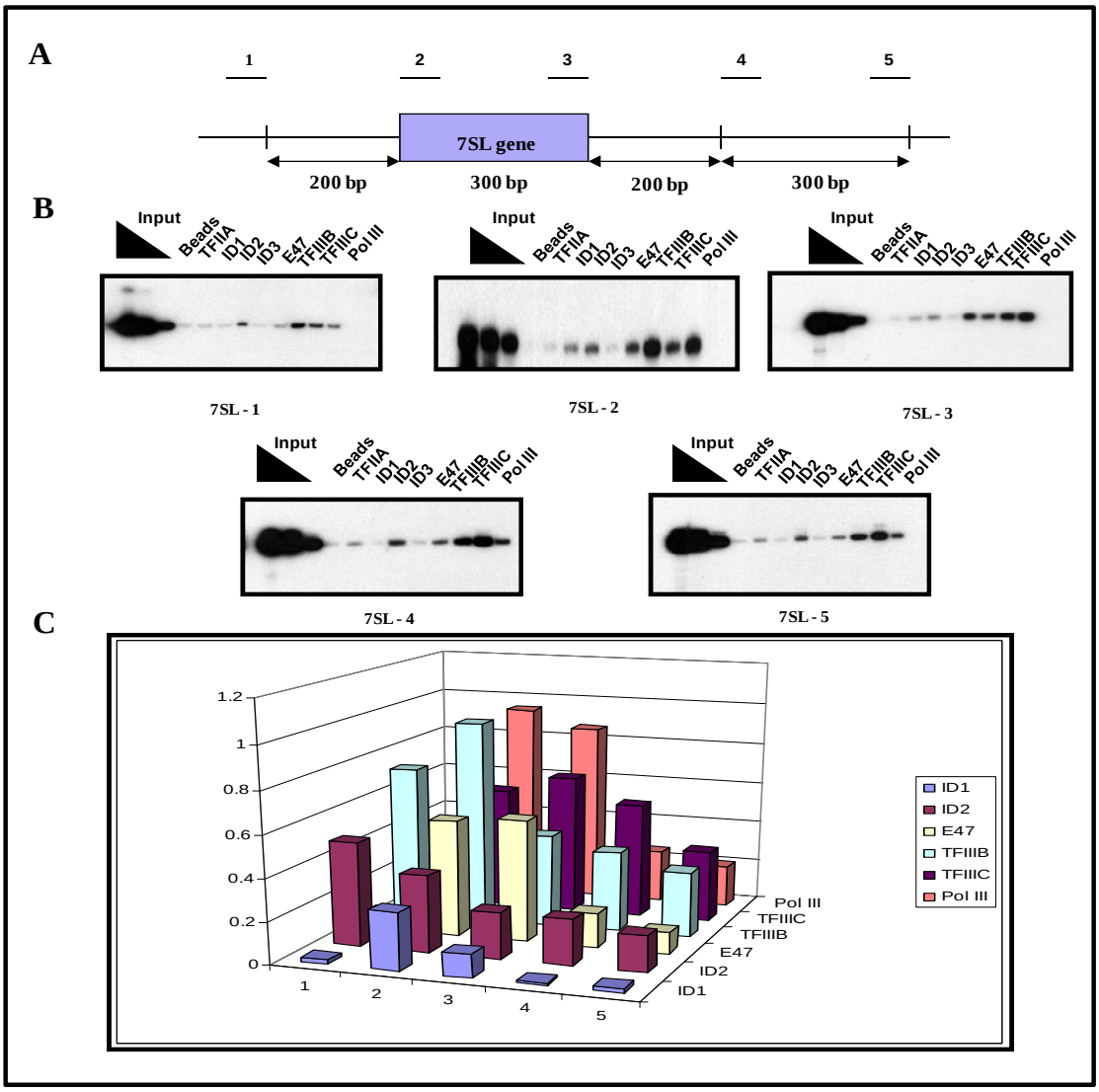

Figure 5.11: Mapping of E47 on the 7SL locus.

Chromatin immunoprecipitations were performed in HEK 293 cells harvested 24 hours after adenoviral expression of E47. **A.** Diagram of the five positions used to design primers on the 7SL locus. **B.** The ID1 (C-20), ID2 (C-20), ID3 (C-20), E47 (N-649), Brf1 (128), TFIIIC (Ab7) and pol III (1900) antibodies were used for ChIP assays. The TFIIA (FL-109) antibody and beads were used as negative controls. **C.** Graphical representation of the ChIP assay. The x-axis shows the five sets of primers. The Y-axis represents the ChIP signals that were normalised against the input and the signals with the TFIIA negative control.

5.2.5 E47 co-immunoprecipitation with Brf1

Chromatin immunoprecipitation assays have revealed that E47 can be recruited to pol III-transcribed genes *in vivo*. Mapping experiments on the 7SL locus indicate that E47 may co-localise together with TFIIIB and TFIIIC. It is therefore possible that E47 could bind directly or indirectly to specific factors such as TFIIIB and TFIIIC. E47 can form homodimers or heterodimers and bind to DNA through E-box sequences on pol II-transcribed genes (Murre *et al.*, 1989). Pol III templates rarely contain E-box DNA sequences which could be targeted by E47. Thus, E47 is more likely to regulate pol III transcription by protein-protein interactions with one or more factors of the pol III apparatus. TFIIIB is a plausible target, since it is a transcription factor that is regulated by a number of different tumour suppressor proteins such as RB, p53 and Maf1 (Sutcliffe *et al.*, 2000; Crighton *et al.*, 2003; Goodfellow *et al.*, 2008). To determine if E47 can interact with endogenous TFIIIB, co-immunoprecipitation assays were performed. Western blot analysis revealed that the Brf1 subunit of TFIIIB was able to co-immunoprecipitate with endogenous E47 in HeLa nuclear extracts (Figure 5.12). This interaction is specific since no signal was detected with the negative control TFIIB.

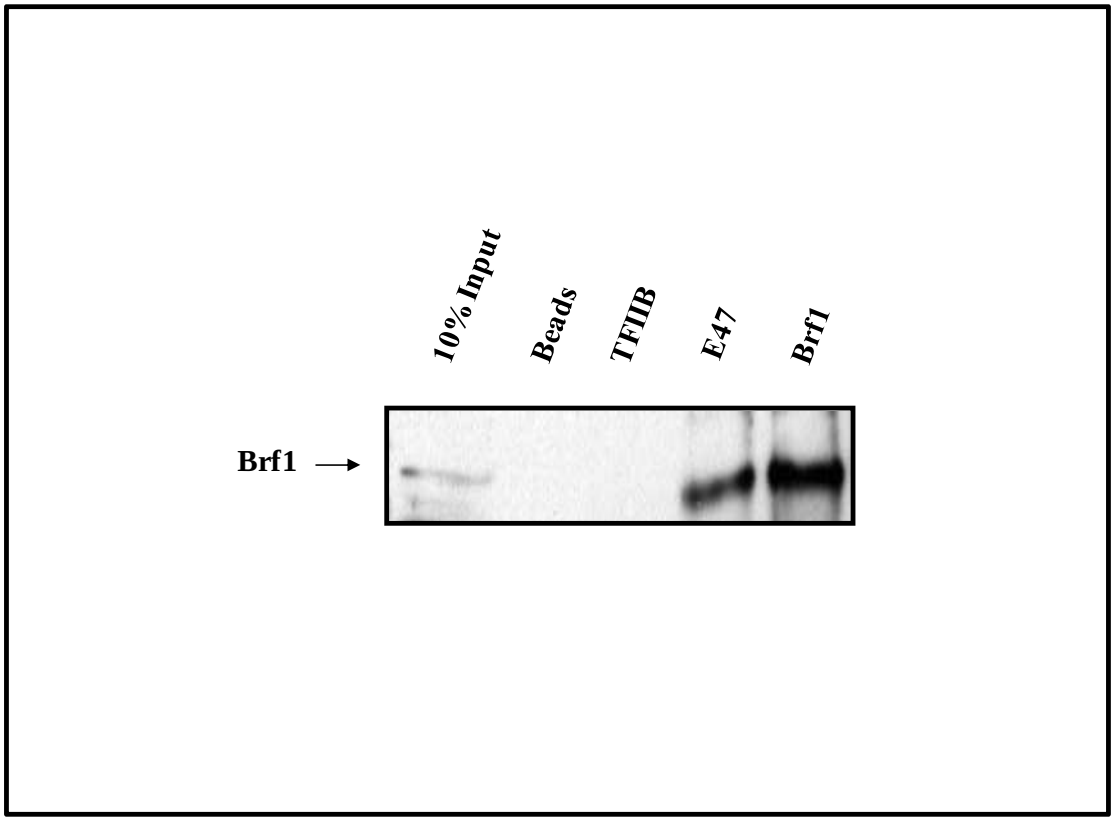

Figure 5.12: Co-immunoprecipitation demonstrates interaction of E47 with Brf1.
HeLa nuclear extracts were used for the co-IP experiments. The TFIIB (C-18) antibody and beads were used as negative controls. The extracts were immunoprecipitated with the E47 (N-649) and Brf1 (128) antibodies. Immunoprecipitated material was resolved on an SDS-7.8% polyacrylamide gel and analysed by western immunoblotting with the 128 antibody against Brf1. 10% of input material was used.

5.2.6 E47 interacts with Brf1 and TFIIIC

Protein co-immunoprecipitation experiments identified the interaction of E47 with Brf1. However, using the above assay it is not possible to distinguish between direct or indirect binding. To determine if E47 can interact with TFIIIB and TFIIIC, *in vitro* transcription and translation assays using ^{35}S-methionine were performed. In order to test this hypothesis the first step involved the expression of GST-fusion proteins, such as GST-E47, and the negative empty vector control GST. The results demonstrate that the overexpression of the GST-E47 fusion protein was successful (Figure 5.13). The GST-E47 protein has a molecular weight of about 103 kDa, however, there are breakdown products. The GST control vector has a molecular weight of about 25 kDa. The Brf1 and TFIIIC110 subunits were *in vitro* translated and labelled with ^{35}S-methionine. Incubating the GST and GST-E47 protein with Brf1 revealed that E47 binds to Brf1 (Figure 5.14, A). Also, E47 binds to the TFIIIC110 subunit (Figure 5.14, B). These interactions are specific, since in the control GST lane there is no signal. Taken together, the above results indicate that E47 can bind to Brf1 and TFIIIC *in vitro*.

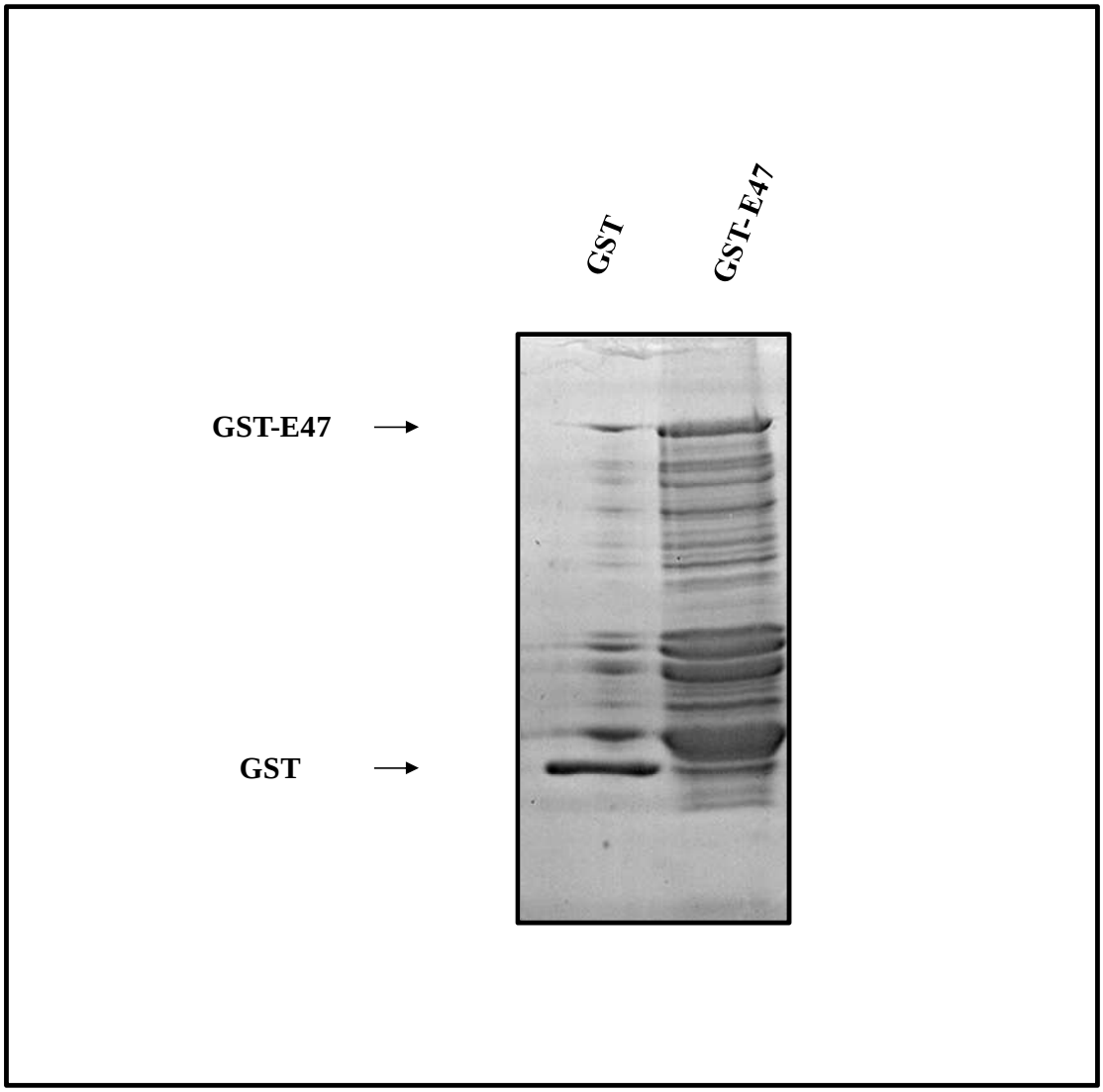

Figure 5.13: Overexpression of GST and GST-E47 fusion protein.

E. coli cells were used to overexpress GST and the GST-E47 fusion protein. Cells were induced with IPTG for 2 hours and harvested. Cell pellets were resuspended and sonicated. The GST and GST-E47 fusion protein were coupled to glutathione-agarose beads. The protein samples were resolved on an SDS-7.8% polyacrylamide gel.

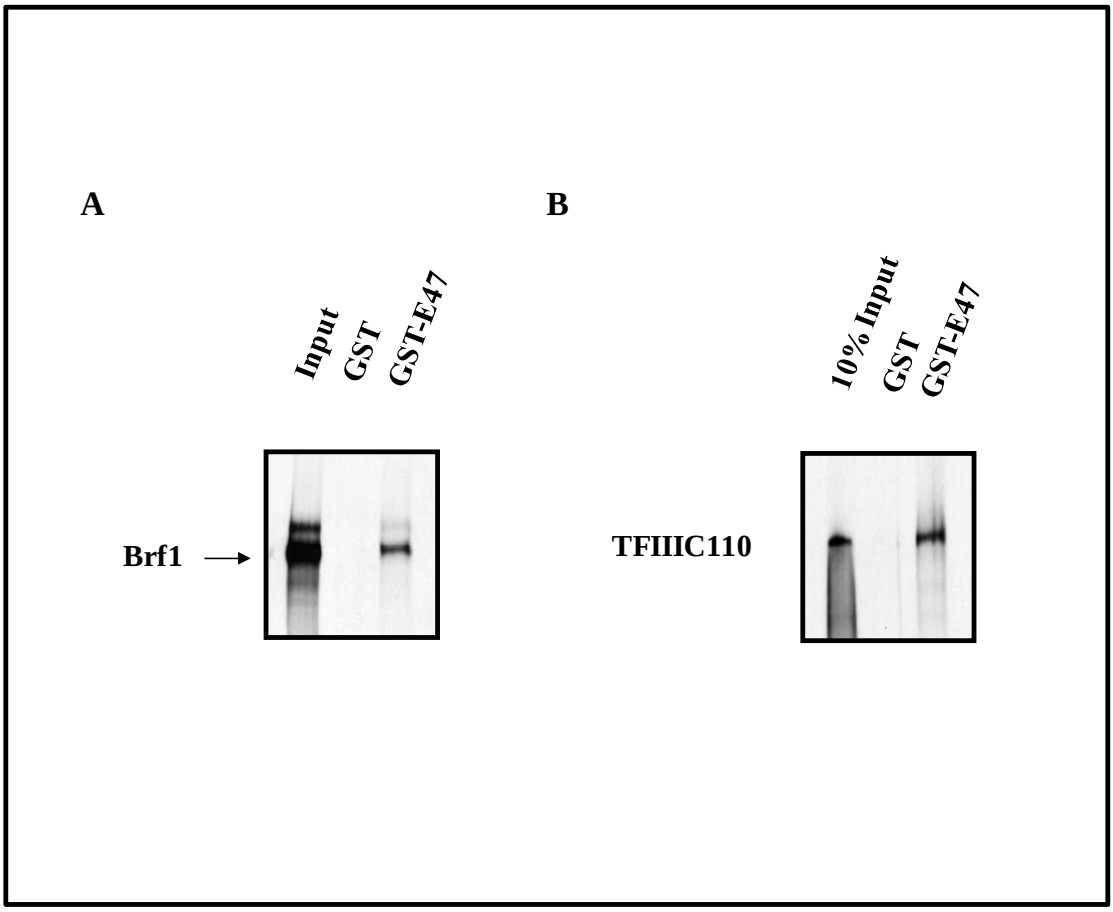

Figure 5.14: *In vitro* transcription/translation using ^{35}S-methionine reveals an interaction of E47 with TFIIIB and TFIIIC subunits.

A. *In vitro* translated Brf1 was labelled with ^{35}S-methionine and incubated with GST used as negative control or GST-E47 at 4°C with agitation. The samples were washed and resolved on an SDS-7.8% polyacrylamide gel and visualised by autoradiography. **B.** *In vitro* translated TFIIIC110 was radiolabelled with ^{35}S-methionine and incubated with GST control or GST-E47 at 4°C with agitation. Proteins retained after washing were resolved on an SDS-7.8% polyacrylamide gel and visualised by autoradiography.

5.2.7 The ID2 protein is recruited to pol III-transcribed genes in an E47-dependent manner

E47 has been shown to regulate the cellular localisation of the ID transcription factor family members. Studies have demonstrated that expression of E47 leads to modulation of ID1 localisation, from cytoplasmic to nuclear (Lingbeck *et al.*, 2005). Also, E47 can stabilise ID1 resulting in an increased half-life of ID1. In another study, co-transfection of E47 and ID3 resulted in translocation of ID3 to the nucleus, whereas E47 was not sequestered into the cytoplasm (Deed *et al.*, 1996). Chromatin immunoprecipitation assays have indicated that in a U2OS cell line stably expressing ID3, E47 was recruited to the promoters of pol III templates (Figure 5.8). Also, in a mapping experiment on the 7SL locus, E47 was overexpressed by adenoviral infection and ID1 and ID2 were found present on the 7SL gene (Chapter 4). To determine whether E47 can recruit ID2 to pol III-transcribed genes *in vivo* or the opposite, HEK 293 cells were infected with an adenovirus expressing ID2 (Ad-ID2), E47 (Ad-E47) or with the control vector (Ad-Vector). Cells were harvested after 30 hours and chromatin immunoprecipitation was performed. The inputs from the three different infections of the control vector, E47 and ID2 were normalised against each other. The results revealed that upon infection of cells with E47 or ID2 this led to an increase in the levels of these proteins on the promoters of 5S rRNA and $tRNA^{Leu}$ genes (Figure 5.15). It is interesting that the expression of E47 appears to recruit ID2, since the ID2 ChIP signal increases in response to Ad-E47. These interactions are specific and not due to over cross-linking of proteins to DNA, since minimal signal was detected using the control TFIIA antibody. The ChIP assay cannot show whether ID2 and E47 are present together at pol III-transcribed genes. However, sequential chip analysis could be performed to determine whether these proteins with opposite functions in cell growth are present together at pol III-transcribed genes. These results will indicate whether recuitment of ID2 by E47 shows presence of these proteins on active or inactive pol III-transcribed genes. PCRs could be done using serial dilution of the DNA templates to confirm that the assay is linear. The effect of overexpression of ID2 and E47 on each other can be determined by western blot analysis to detect the protein levels.

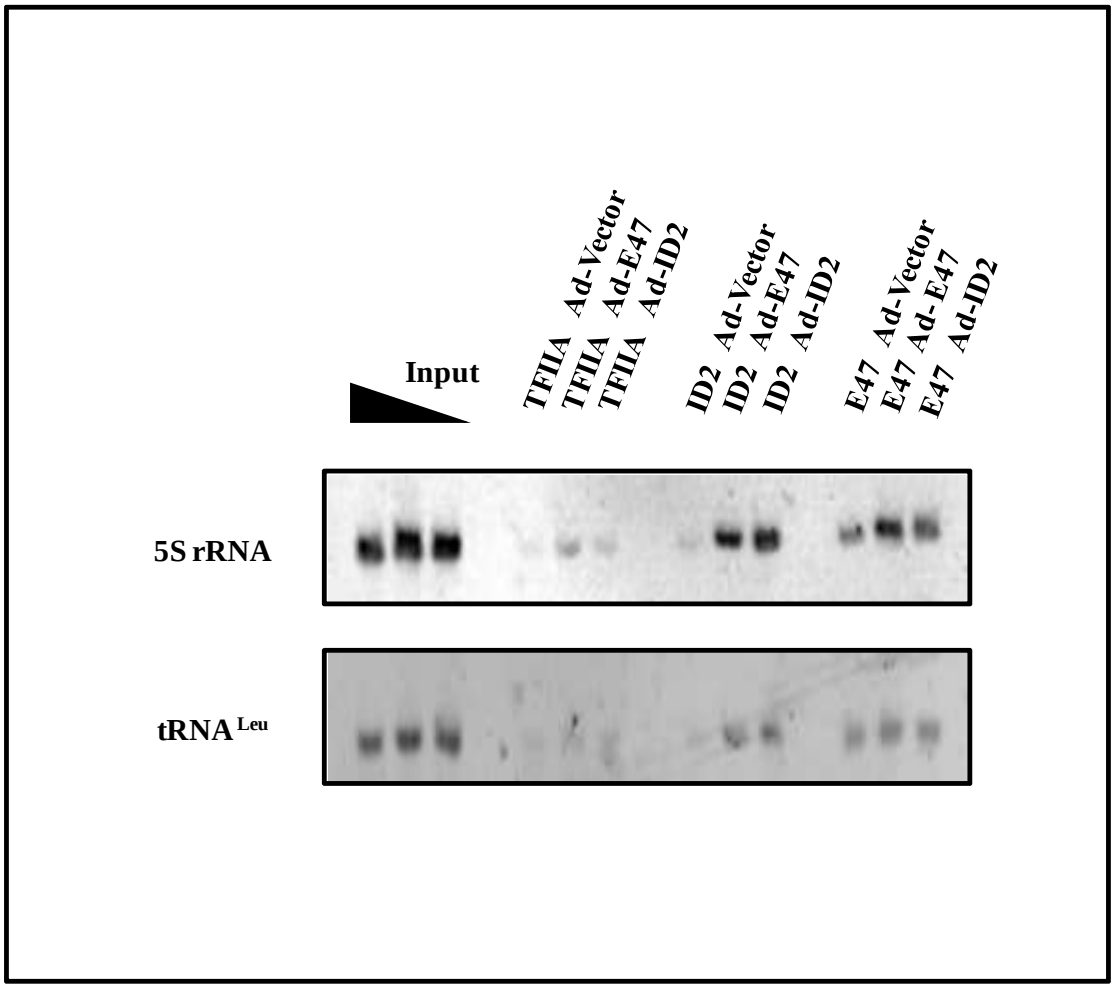

Figure 5.15: ID2 and E47 interact with pol III-transcribed genes *in vivo*.
Chromatin immunoprecipitations were performed using HEK 293 cells harvested 30 hours after infection with the adeno-Vector (Ad-Vector), adeno-E47 (Ad-E47), or adenovirus ID2 (Ad-ID2). The TFIIA (FL-109) antibody was used as negative control. The N-649 and C-20 antibodies were used for E47 and ID2 respectively. The samples were analysed by PCR performed on inputs and primers against 5S rRNA and $tRNA^{Leu}$ genes. ChIP signals were normalised against the inputs.

5.3 Discussion

Studies have identified E47 to be involved in regulation of cell proliferation. Expression of E47 has been demonstrated to suppress cell growth in HeLa, Saos-2 and U2OS cell lines (Pagliuca *et al.*, 2000). It was found that E47 decreased the proliferation rate by promoting cell death associated with the induction of apoptosis. E47 is the strongest transcriptional activator of the CDK inhibitors p15, p16 and p21 compared to the other E-protein family members E12, E2-2 and HEB indicating a mechanism by which E-proteins negatively control cell growth (Zheng *et al.*, 2004; Prabhu *et al.*, 1997; Pagliuca *et al.*, 2000). Another CDK inhibitor targeted by E47 in human neuroblastoma cells is $p57^{kip2}$. Overexpression of E47 by adenoviral infection prevents progression into S phase and induces cell cycle arrest (Rothschild *et al.*, 2006). These studies indicate that E47 is a negative regulator of cell proliferation through mechanisms that involve programmed cell death and enhancement of the expression of CDK inhibitor genes.

In order to investigate if E47 can regulate pol III transcription in human cells *in vivo* an RNAi approach was employed. Transfection of human HeLa cells with siRNA against E2A resulted in significant reduction of protein and mRNA of E47 and E2A respectively compared to the controls (Figure 5.4). Also, the knockdown of E2A by siRNA did not result in overexpression or silencing of the associated ID1, ID2 and ID3 proteins. The next step involved the determination of whether this depletion of E2A would have an effect on pol III transcription. When the protein and mRNA levels of E47 and E2A respectively were reduced, pol III transcription was clearly upregulated. The levels of 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, $tRNA^{Tyr}$, 7SL RNA, 7SK RNA, MRP, and U6 snRNA were significantly increased (Figure 5.5). Also, the E2A knockdown results in an increase in the levels of the pol I transcript pre-rRNA (Figure 5.5). These results indicate that the knockdown of E2A demonstrates a repressing role in pol I and pol III transcription *in vivo*. An adenoviral infection approach was used to determine the effect of expressing E47 in human cells *in vivo*. HEK 293 cells were infected with adenoviruses expressing E47 or the vector control. Infection with the E47 expressing adenovirus resulted in overexpression of E47 protein and E2A mRNA in comparison to

the control (Figure 5.6). The overexpression of E47 caused a reduction in the levels of the pol III transcripts 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 7SK RNA and MRP compared to the control (Figure 5.7).

The use of Oct-1 as a control siRNA has limitations because it is a basal pol III transcription factor. Oct-1 is present on type III promoters and its removal leads to a delay of 30 minutes for the onset of transcription. In addition, Oct-1 is involved in transcription of type III genes, although it is not essential for basal transcription (Hu *et al.*, 2003). The knockdown of E2A using siRNA experiments, should lead to the knockdown of its two known splice variants, the E47 and E12 transcription factors. In the future, siRNAs should be used against either the E47 or the E12 proteins to be able to determine which of them or if both can affect pol III transcription. Western blot experiments could be performed to determine whether the E12 protein levels are also reduced upon treatment of cells with siRNA against E2A, as has been observed for E47 protein levels. Controls other than the Oct-1 control to compare the knockdown of E2A and the effects on pol III transcripts such as mock or non-silencing controls could be employed. The use of a non-silencing control could also be used since it does not target a protein and should avoid non-specific targeting but leads to the activation of the siRNA response pathway. Furthermore, a mock control using only lipofectamine 2000 without adding siRNA should be used to determine whether there is an effect mediated by the transfection reagent on the cells. In addition, the implementation of other techniques for determining the effects of the knockdown of E47 proteins could be employed. Since the RT-PCR technique is semi-quantitative the more sensitive Q-PCR technique could be used. In the future western blot experiments could be performed to detect the expression levels of the other E-protein family members such as HEB and E2-2 and also of the associated ID1, ID2 and ID3 proteins upon overexpression of E47. Studies have shown that when the E47 or ID2 proteins are overexpressed the protein levels of each other do not change (Rothschild *et al.*, 2006). Also, immunofluorescence techniques could be employed in order to investigate where E47 and ID proteins are localised in the cell and whether they co-localise.

Overexpression of E-protein family members (E47, E12, E2-2 and HEB) using HeLa, U2OS and SaOS-2 cell lines results in suppression of cell colony formation (Pagliuca *et al.*, 2000). Also, it has been demonstrated that the p15, p16 and p21 promoter sequences, which contain E-box sequences, can be activated by E-protein members. In contrast to previous experiments where overexpression of E47 lead to inhibition of cell growth arrest due to block of G1 progression (Peverali *et al.*, 1994), later work showed that the mechanism responsible for this effect does not involve arrest of G1 progression (Pagliuca *et al.*, 2000). After ectopic expression of E-protein family members, the cell cycle profiles of cells were determined using FACS analysis. Flow cytometry demonstrated that cells transfected with E-proteins showed an increase in the number of cells in S phase and an increase of cells with subdiploid DNA content (Pagliuca *et al*., 2000). In addition, trypan blue dye exclusion experiments demonstrated an increase in cell death after ectopic expression of E-proteins. TUNEL assays showed a significant induction of apoptosis levels following transfection of cells with E-proteins. Furthermore, BrdU incorporation assays showed that a significant fraction of cells were in S phase (Pagliuca *et al*., 2000). These results demonstrate that overexpression of E-proteins promotes cell death associated with apoptosis.

Other studies have revealed that the E47 protein is involved in promoting cell cycle progression (Zhao *et al.*, 2001). A tissue culture based system was employed where E47 protein levels were regulated in an inducible manner, using an E47 oestrogen fusion construct. Also, a dominant negative E47 oestrogen construct was used. These constructs were expressed in several cell lines such as in the pre-B-cell line 697, an NIH 3T3 cell line and the myeloid progenitor cell line K562. Suppression of E47 led to inhibition of cell cycle progression and FACS analysis demonstrated a decrease in the fraction of cells being in S phase, suggesting an inhibition of G1 cell cycle progression (Zhao *et al.*, 2001). BrdU incorporation experiments also confirmed that suppression of E47 led to decrease in cell cycle progression. Furthermore, the overexpression of E47 in NIH 3T3 cells promoted entry into S phase, which was monitored by FACS analysis (Zhao *et al.*, 2001). Further experiments could be performed on pol III transcription to investigate the biological response on cell growth and apoptosis after knockdown or overexpression of E47 proteins. FACS analysis could be used to determine the

biological consequences such as cell cycle progression and cell viability mediated by the knockdown or ectopic expression of E47. Furthermore, TUNEL assays could be employed to measure the percentage of apoptotic cells upon knockdown or overexpression of E47 proteins. Because the E47 protein appears to be involved in cell growth and apoptosis the effect of overexpressing or knocking down E47 should be further investigated to determine the possible biological effects mediated by expressing or depleting E47. A potential explanation for the effects on pol III transcription seen here by knocking down or overexpressing E47 could be attributed to indirect effects. These indirect effects could result in affecting cell proliferation, which in turn could affect pol III transcription. The slower growth of cells could result in the reduction of pol III transcription. However, chromatin immunoprecipitation and co-immunoprecipitation experiments argue that the effects demonstrated by altering E47 levels could be due to direct effects of E47 on pol III activity. Thus, a possible explanation for the role of E47 on pol III transcription could be that E47 is capable of regulating cell proliferation and cell viability by directly affecting pol III transcription, since pol III transcription is involved in cell growth. In order to address indirect effects caused by the overexpression or knockdown of E47, cell lines containing functional RB and p53 that represent a more physiological condition should be employed instead of HeLa cells. In addition, the siRNA used to target the E47 protein should be designed to be specific for E47 and also reduce the possibility of off-target effects in order to avoid indirect effects. Also, *in vitro* transcription assays should be performed to demonstrate whether E47 can affect pol III transcription *in vitro*, because the results obtained from these experiments might be less expected to be affected by indirect consequences.

In conclusion, the results shown here demonstrate that in both cases, knockdown or overexpression, E47 is involved in the repression of pol III transcription, suggesting a mechanism by which E47 regulates cell growth.

Studies have demonstrated that E47 is found at the promoters of CDK inhibitors and their activation leads to cell cycle arrest (Prabhu *et al.*, 1997). The E47 transcription factor is thought to activate or repress transcription by interacting with the E-box promoter sequences of pol II-transcribed genes (Zheng *et al.*, 2004; Perez-Moreno *et al.*, 2001). Since the results have shown that E47 can repress pol III transcription it would be

interesting to investigate if E47 could be interacting directly with the pol III machinery to repress pol III activity. Chromatin immunoprecipitation assays were used to demonstrate whether E47 localises at pol III-transcribed genes *in vivo*. In the HeLa cell line used, endogenous E47 was found at pol III-transcribed genes like 5S rRNA and $tRNA^{Leu}$ (Figure 5.8). In another cell line, (HEK 293) cells were infected with an adenovirus expressing E47. ChIP assays revealed that this protein showed a high enrichment and at 5S rRNA and $tRNA^{Leu}$ and 7SL genes (Figure 5.9). Also, a stable cell line expressing ID3 was used to identify if E47 localises at pol III-transcribed genes. The results demonstrate that E47 is present at the $tRNA^{Arg}$ and 7SL genes indicating a possible recruitment by ID3 (Figure 5.10). When E47 was expressed in HEK 293 cells this protein was found at the 7SL gene. This led to investigate, by mapping experiments on the 7SL locus, the association of E47 with potential factors that could recruit E47 to pol III promoters. The results revealed that E47 co-localises with Brf1 and TFIIIC at different positions on the 7SL gene (Figure 5.11). Therefore, a possibility is that *in vivo* Brf1 and/or TFIIIC could recruit E47 to pol III templates. Interestingly, there is recruitment of ID1 and ID2 which are present at the start of the 7SL gene but not at the end of the gene displaying a similar pattern with Brf1 (Figure 5.11). These data indicate that ID1 and ID2 co-localise with Brf1 and not TFIIIC or pol III. This suggests that Brf1 could be a factor recruiting ID1 and ID2 to the promoters of pol III-transcribed genes. Overall, these results demonstrate that E47 is present at pol III-transcribed genes in several cell lines. This suggests that E47 may regulate pol III activity through a direct interaction with one or more components of the pol III apparatus.

Given that E47 was demonstrated to localise on pol III-transcribed genes, possible mechanisms involved in this process were investigated. One possibility could be that E47 recognises a specific DNA sequence in pol III templates. However, the pol III genes rarely contain the E-box sequences that E47 could target and bind to. Therefore, protein-protein interactions of E47 with factors of the pol III apparatus were investigated. A possible target is TFIIIB, which has been demonstrated to be regulated by a number of oncoproteins, like c-Myc and ERK (Gomez-Roman *et al.*, 2003; Felton-Edkins *et al.*, 2003a,b) and tumour suppressor proteins such as RB, p53 and Maf1 (Sutcliffe *et al.*, 2000; Crighton *et al.*, 2003; Goodfellow *et al.*, 2008). To investigate if

E47 can bind to endogenous TFIIIB, co-immunoprecipitations assays were performed. The results revealed that the Brf1 subunit of TFIIIB co-immunoprecipitates *in vivo* with E47 and this effect is specific, since no signal is detected in the negative control (Figure 5.12). However, using this assay it cannot be distinguished whether an interaction is direct or indirect. Therefore, it cannot be excluded that TFIIIB could be present in a complex with TFIIIC. To investigate if E47 binds to TFIIIB or TFIIIC, *in vitro* transcription and translation assays using ^{35}S-methionine were performed. The first step involved the expression of the GST and GST-E47 fusion proteins. These proteins were overexpressed and purified, although in the GST-E47 protein there were breakdown products (Figure 5.13). The results demonstrate that E47 binds to the Brf1 subunit of TFIIIB and the TFIIIC110 subunit of TFIIIC (Figure 5.14). Thus, E47 is associated with Brf1 and TFIIIC110, and these interactions could be responsible for recruitment of E47 to the promoters of pol III-transcribed genes.

The correct subcellular localisation is important for the function of numerous proteins. E47 can act as a nuclear chaperone for ID proteins. When ID2 was co-transfected in SMCs with a vector containing full length E47, the wild type ID2 was localised in the nucleus (Matsumura *et al.*, 2002). Studies have investigated if the nucleo-cytoplasmic shuttling of ID2 is involved in the regulation of E-box mediated transcription. It was demonstrated that decreased nuclear export of ID2 enhances repression of E47-mediated transcription (Kurooka & Yokota, 2005). These results suggest that the nuclear export of ID proteins has an inhibitory role in their suppressive ability against E47. The bHLH protein E47 has also been demonstrated to regulate the cellular localisation of ID1 and ID3 proteins from cytoplasm to nucleus when co-transfected with these proteins (Deed *et al.*, 1996; Lingbeck *et al.*, 2005). Also, when E47 was co-expressed with ID proteins, E47 remained mainly nuclear. Therefore, E47 can regulate the available pool of its binding inhibitory partners, by modulating the nuclear localisation of ID proteins. To investigate whether E47 and ID2 can recruit one another to the promoters of pol III-transcribed genes *in vivo*, ChIP assays were performed using HEK 293 cells infected with adenovirus expressing E47, ID2 and the control vector. After infection with E47 or ID2, these proteins demonstrated a higher occupancy on the 5S rRNA and tRNA^{Leu} gene promoters (Figure 5.15). Also, ID2 appears to be present on pol III templates in an E47

sensitive manner, and demonstrates a higher enrichment on pol III-transcribed genes compared to ID2 recruiting E47. In addition, it would be interesting to investigate if E47 and ID2 could increase DNA binding of pol III-specific factors and this association is important for downregulation or upregulation of pol III activity. In the future, ChIP on ChIP assays of IDs and E47 will be required to dissect recruitment issues and whether IDs and E47 proteins are present together at pol III templates. Also, sequential ChIPs of various mutants lacking interaction domains of IDs and E47 could be employed to investigate further their presence at pol III promoters and whether these proteins can be found together with pol III at active or inactive pol III-transcribed genes. By overexpressing ID or E47 mutants it could be possible to determine whether E47 is capable of recruiting ID proteins using the HLH domain of E47. Mutants of E47 lacking the HLH domain or containing point mutations in this domain, which is required for the interaction with ID proteins, may not be able to recruit ID proteins at the promoters of pol III genes.

In conclusion, the data in this chapter suggest a new function for E47, in repressing pol III transcription. Furthermore, it is possible that other members of the E-protein family could regulate pol III activity since they show similar functional properties.

Chapter 6

Discussion and Conclusions

6.1 RNA polymerase III transcription, cell growth and cancer

High rates of protein synthesis are needed for increased growth, since 80-90% of a cell's dry mass is mainly constituted of protein (Zetterberg & Killander, 1965). Therefore, the growth rate is proportional to the rate of protein accumulation (Baxter & Stanners, 1978). Moreover, cells can withdraw from the cell cycle and become quiescent if there is a 50% decrease in the translational output (Brooks, 1977; Rønning *et al.*, 1981). Since 5S rRNA and tRNA availability is essential for protein synthesis, increased pol III transcription is required to maintain rapid cell growth. These observations may explain a requirement for stimulation of pol III transcription by tumour cells.

Pol III transcription has been frequently found to be abnormally elevated in transformed cells and tumours. Early studies demonstrated that pol III is deregulated in mice with myelomas, even though pol II activity remains normal (Schwartz *et al.*, 1974). Several types of transformed cells have been shown to overexpress products of pol III, including cell lines transformed by RNA tumour viruses (e.g., human T cell leukaemia virus 1), DNA tumour viruses (e.g., simian virus 40, hepatitis B virus) and chemical carcinogens (e.g., methylcholanthrene) (Gottesfeld *et al.*, 1996; Liebhaber *et al.*, 1978; Larminie *et al.*, 1999; Wang *et al.*, 1995; Scott *et al.*, 1983; White *et al.*, 1990; Singh *et al.*, 1985). These studies have focused on cell lines, however, their relevance has also been explored in tumours *in situ*. In particular, increased levels of 5S rRNA and $tRNA^{Tyr}$ have been found in ovarian cancers (Winter *et al.*, 2000). In another study, analysis of 80 tumours revealed raised levels of 7SL RNA in each of the tumour samples representing 19 types of cancer (Chen *et al.*, 1997b). Moreover, carcinomas of the lung, breast and tongue demonstrated elevated levels of pol III transcripts in neoplastic cells in comparison to healthy control tissue (Chen *et al.*, 1999b). Therefore, increased pol III activity appears to be associated with the transformed state.

The deregulation of pol III transcription in cancer can be attributed to and categorised into three general mechanisms. The first mechanism involves the activation of pol III transcription by oncoproteins such as c-Myc, Ras and CK2 (Figure 6.1)

(Gomez-Roman *et al.*, 2003; Wang *et al.*, 1997; Johnston *et al.*, 2002). Pol III transcription can also be activated through loss of tumour suppressors such as RB, p53 and PTEN and this constitutes the second mechanism (Figure 6.1) (White *et al.*, 1996; Crighton *et al.*, 2003; Woiwode *et al.*, 2008). Finally, the third mechanism involved in the deregulation of pol III transcription is mediated by the overexpression of a specific pol III factor such as TFIIIB or TFIIIC (Felton-Edkins & White, 2002; Larminie *et al.*, 1999). Increased levels of the Brf1 subunit of TFIIIB have been found in patients with cervical carcinomas (Daly *et al.*, 2005). Also, other cases where Brf1 is elevated in prostate and bladder cancers can be found in microarray databases (http://www.oncomine.org). This last mechanism has been recently re-examined revealing very interesting findings.

Specifically, studies have focused on the effects of Brf1 overexpression on mammalian systems. To determine whether Brf1 is limiting for pol III transcription, Brf1 levels were raised in Brf1-inducible MEF cells and an increase in the levels of pol III transcripts was observed (Marshall *et al.*, 2008). These effects were specific since there was no change in the levels of pol I and pol II transcripts tested. In addition, increased occupancy of Brf1 and pol III was observed at the promoters of 5S rRNA and tRNA genes when Brf1 was induced. Increased proliferation was observed in MEF cells containing the inducible Brf1 construct and in MEFs infected with a Brf1 expressing adenovirus (Marshall *et al.*, 2008). Since activation of pol III transcription is observed in transformed and tumour cells, the role of Brf1 in stimulating transformation was investigated. Brf1 induction in MEF and CHO cells resulted in oncogenic transformation of these cells. The cells displayed morphological changes, focus formation and grew in an anchorage-independent manner. Moreover, mice injected with clones overexpressing Brf1 developed tumours (Marshall *et al.*, 2008). One of the targets of Brf1 is the gene encoding the $tRNA_i^{Met}$. Overexpression of $tRNA_i^{Met}$ displayed similar effects to the induction of Brf1 such as increased protein synthesis and formation of tumours in mice. Therefore, the effects demonstrated by expression of Brf1 may be attributed to raised expression of $tRNA_i^{Met}$. Interestingly, the levels of Myc and cyclin D1 were elevated in response to overexpression of Brf1 and $tRNA_i^{Met}$. Therefore, it is likely that the capacity of Brf1 and $tRNA_i^{Met}$ to regulate oncoproteins like Myc may be responsible for their

transforming ability. These observations suggest a possible requirement for the activation of pol III transcription in cancer progression.

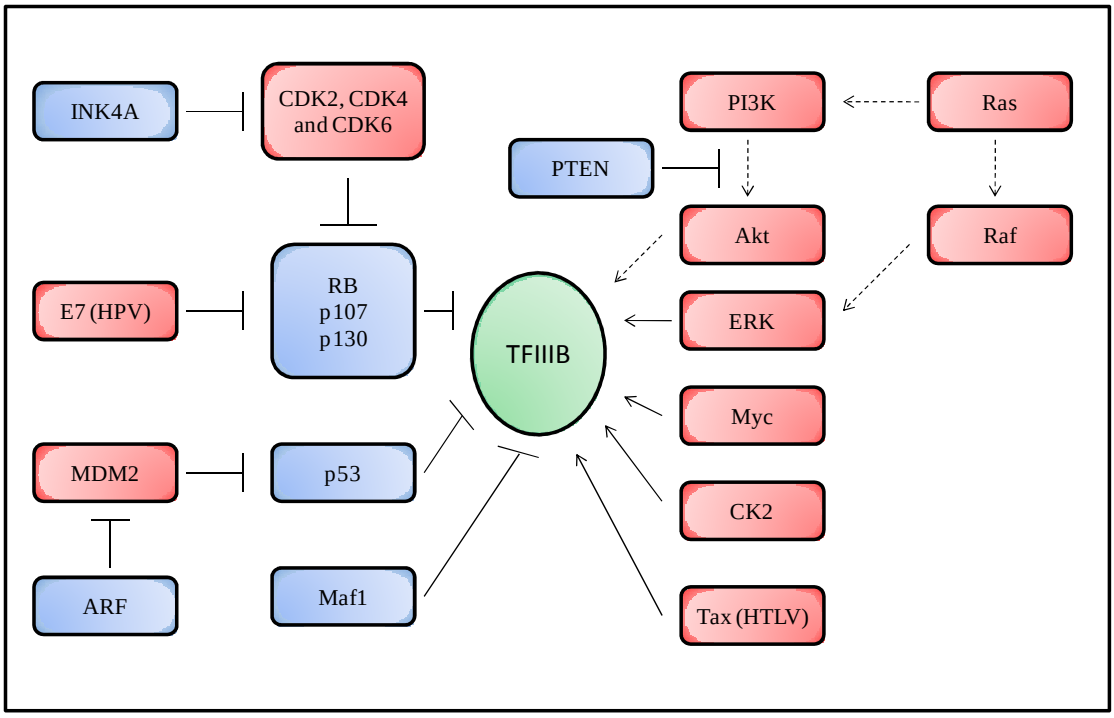

Figure 6.1: The transcription factor TFIIIB is regulated by several activators and repressors.

The blue boxes indicate proteins that have been demonstrated to repress pol III transcription through TFIIIB, and those in red boxes have been shown to function as activators. Dotted lines represent indirect interactions. The growth repressors p53, RB, p107, p130 and Maf1 can repress TFIIIB by direct interactions. Their function can be blocked by viral proteins such as HPV E7 and the cellular oncoprotein mouse double minute 2 (MDM2). Also, the MDM2 oncoprotein causes p53 degradation, thereby increasing TFIIIB activity. RB, p107 and p130 phosphorylation by CDK2, CDK4 and CDK6 leads to release of TFIIIB from suppression. The tumour suppressor INK4A inhibits the phosphorylation mediated by CDK4 and CDK6, thus leading to TFIIIB repression by RB, p107 and p130. The ARF tumour suppressor inhibits p53 degradation

by MDM2 and therefore causes TFIIIB suppression. In contrast, a variety of activators have been shown to bind and activate TFIIIB directly, including CK2, Myc, ERK and HTLV1. Ras and Raf signalling activate ERK, and Ras also stimulates phosphatidylinositol 3-kinase - Akt signalling. PTEN can antagonise the PI3K-Akt pathway (adapted from Marshall & White, 2008).

6.2 Induction of pol III transcription by ID1, ID2 and ID3

The findings of this thesis have shown that the ID1, ID2 and ID3 oncoproteins can regulate pol III transcription. The ID transcription factor proteins were considered because they are known to be targets of the Myc oncogene (Lasorella *et al.*, 2000), which has been shown to induce pol III transcription (Gomez-Roman *et al.*, 2003). Studies have shown that ID2 expression is upregulated in neuroblastoma cell lines (Lasorella *et al.*, 2002). These cell lines show amplification of neuronal Myc (N-Myc), and overexpression of ID2 is possibly due to direct control by N-Myc expression. Other studies have identified that ID2 is also a target of c-Myc, and that the transcription of the ID1, ID2 and ID3 genes is activated by Myc via E-boxes within their promoters (Swarbrick *et al.*, 2005; Lasorella *et al.*, 2000; Light *et al.*, 2005). Also, the ID2 family member has been demonstrated to bind and inhibit the function of the hypophosphorylated form of RB, which is known to repress pol III transcription (White *et al.*, 1996; Larminie *et al.*, 1997). Therefore, the ID2 protein directly and the ID1 and ID3 proteins indirectly may have oncogenic activity by interfering with the RB tumour suppressor pathway. In addition, p53 has been shown to be involved in the regulation of ID proteins (Qian & Chen, 2008; Yan *et al.*, 2008; Wilson *et al.*, 2001; Hernandez-Vargas *et al.*, 2006). The expression levels of the ID1, ID2 and ID3 proteins correlate with mitotic index and with p53 expression levels in human colorectal adenocarcinomas (Wilson *et al.*, 2001). ID protein levels are increased in p53 knockout mice and knockdown of ID proteins in colorectal adenocarcinomas resulted in growth arrest (Wilson *et al.*, 2001). It has been demonstrated that the ID1 protein becomes down regulated in HCT116 and U2OS cells treated with chemotherapeutic drugs in a p53

dependent manner (Qian & Chen, 2008). Also, ID1 has been found to be regulated by DEC1, which is a bHLH transcription factor that is targeted by p53. ChIP assays showed that the DEC1 protein is present at the promoter of the ID1 gene and leads to the inhibition of ID1 expression (Qian & Chen, 2008).

Previous studies have demonstrated that ID proteins play a crucial role in the regulation of cell growth (Barone *et al.*, 1994). Experiments have shown that the silencing of ID proteins reduces cell proliferation (Hara *et al.*, 1994; Peverali *et al.*, 1994). Furthermore, the depletion of IDs by silencing results in the induction of expression of representative genes involved in proliferation such as the cyclin-dependent kinase inhibitors p16 and p21 (Zheng *et al.*, 2004; Asirvatham *et al.*, 2007). In chapter 3 it was investigated whether the partial depletion of endogenous ID1, ID2 and ID3 proteins by RNAi would affect pol III transcription *in vivo*. It was demonstrated that the knock-down of ID proteins by RNAi resulted in downregulation of the expression of the 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, 7SL RNA, 7SK RNA, MRP RNA and U6 snRNA pol III transcripts in human cells. Furthermore, it was shown that the ID2 and ID3 but not ID1 proteins may be involved in the regulation of pol I transcription, since knocking-down these proteins reduced the levels of the pol I transcript pre-rRNA. The difference in the regulation of the expression of pre-rRNA between ID proteins could be attributed to differences in the sequences of these members. The four ID family members share a 69-78% similarity in their HLH domain, however the rest of their sequences are unrelated (Deed *et al.*, 1994; Norton *et al.*, 1998). Even a few amino acid changes can play a crucial role in protein-protein interactions, an example is the association of ID2 with the RB protein. ID2 binds directly to RB and the related p107 and p130 pocket proteins through the HLH domain of ID2 (Lasorella *et al.*, 1996). However, ID1 and ID3 cannot bind to RB and its related proteins, which suggests that a few amino acid changes even in the homologous HLH domain can be important for the specificity of these proteins for RB and possibly pol I factors. In the future it would be interesting to further investigate these preliminary findings on the regulation of pol I transcription by ID proteins, in order to understand in more detail the role that these proteins exert on cell proliferation. Immunoprecipitation experiments could be carried out to investigate whether ID proteins intetact with pol I basal transcripion factors.

Several cancer types and tumour cell lines overexpress the ID1 protein. In particular, ID1 is overexpressed in squamous cell carcinoma and small cell lung cancer cell lines (Langlands *et al.*, 2000; Kamalian *et al.*, 2008). ID1 has also been found to be overexpressed in human tumours such as cervical cancer and pancreatic carcinomas (Schindl *et al.*, 2001; Maruyama *et al.*, 1999). Evidence for the oncogenic role of ID2 has been demonstrated in transgenic mice where expression of ID2 resulted in the development of lymphomas (Morrow *et al.*, 1999). Overexpression of ID2 by adenovirus infection can upregulate the expression of the $tRNA^{Leu}$, 5S rRNA, $tRNA^{Tyr}$, MRP RNA, $tRNA^{Leu}$ and U6 snRNA pol III transcripts *in vivo* in human cells. Studies have linked ID3 to tumourigenesis as well. ID3 is expressed at high levels in hepatocellular carcinomas and prostate tumours (Damdinsuren *et al.*, 2005; Li *et al.*, 2004).

In order to ensure that the depletion of ID1, ID2 or ID3 by RNAi was specific for each of these proteins, western blotting was performed to determine the expression levels of these proteins. It was shown that upon depletion of each ID specifically by RNAi, the expression levels of their related proteins remained unaltered. These results are in agreement with studies where ID1, ID2 or ID3 silencing did not result in overexpression or suppression of other ID family members, and other work establishing that the knockdown of ID1 protein levels had no effect on the levels of the related ID proteins (Asirvatham *et al.*, 2007; Zheng *et al.*, 2004). Furthermore, it has been demonstrated that overexpression of ID2 by adenovirus infection does not result in repression or overexpression of the other ID family members and the E47 protein (Rothschild *et al.*, 2006).

To further confirm the *in vivo* effects of knocking-down ID proteins by RNAi on pol III transcription, *in vitro* transcription was employed. *In vitro* experiments revealed a reduction in the levels of pol III transcription when ID2 was depleted. The results in this thesis suggest that ID proteins can positively affect pol III transcription, and therefore play a crucial role in the regulation of cell growth.

6.3 Interactions of ID1, ID2 and ID3 with the pol III transcription machinery

ID proteins are known to function by sequestering bHLH proteins, inhibiting their DNA binding ability, with ID proteins being unable to bind DNA (Benezra *et al.*, 1990). In chapter 4, ChIP experiments demonstrated that endogenous ID2 is present at the promoters of the pol III-transcribed 5S rRNA and $tRNA^{Leu}$ genes. Furthermore, overexpression of ID2 by adenovirus infection resulted in strong enrichment of the ID2 protein at other pol III templates, such as 5S rRNA, $tRNA^{Leu}$, 7SL RNA and U6 snRNA. Interestingly, E47 was present at the same pol III-transcribed genes as ID2, suggesting a possible recruitment of E47 by ID2. In addition, ID3 was found to localise at the 5S rRNA, $tRNA^{Leu}$, $tRNA^{Tyr}$ and 7SL RNA pol III-transcribed genes using a U2OS cell line stably expressing this protein. In the same cell line, E47 was also recruited to the promoters of pol III templates demonstrated in chapters 4 and 5. A possible explanation for the presence of endogenous ID2 and not ID1 and ID3 on pol III genes could be that in the cell lines tested the antibodies used, which were not ChIP grade, were unable to detect ID1 and ID3 convincingly, even though they are expressed. From these results, it can be concluded that ID proteins are present at pol III-transcribed genes of promoter types I, II and III *in vivo* in human cells (Figure 6.2).

Furthermore, scanning ChIP assays indicated that ID2 co-localises with TFIIIB at the promoters of pol III templates. Studies have demonstrated that another oncoprotein, c-Myc, is able to co-localise with TFIIIB using promoter mapping experiments (Kenneth *et al.*, 2007). The results showed that ID2 and the TFIIIB factor were present at the start of the gene, rather than the end of the gene where TFIIIC and pol III localise. Therefore, these data suggest that TFIIIB and more specifically Brf1 may be involved in the recruitment of ID2 to pol III-transcribed genes. Moreover, in the HEK 293 cell line where ID2 was overexpressed, E47 was also recruited to the promoter of the pol III gene and E47 localises with ID2 at the beginning of the gene. These findings indicate that ID2 might recruit E47 and that an ID2-E47 complex may exist, which localises with TFIIIB at pol III promoters. These data are very interesting since

the HLH ID proteins are shown to localise at DNA and, as mentioned earlier, ID proteins lack a DNA binding domain and the pol III factors examined have no HLH domain.

Since ID proteins are localised at the promoters of pol III-transcribed genes they may be recruited by a specific mechanism. Experiments employing co-IPs revealed that ID1, ID2 and ID3 can interact with TFIIIB *in vivo*, results which are in agreement with the findings from ChIP data. Furthermore, *in vitro* transcription and translation experiments showed that GST-ID1, GST-ID2, GST-ID3, GST-ID2 HLH and GST-ID2 δHLH fusion proteins interacted with TFIIIB and in particular Brf1. These results suggest that ID2 containing the HLH (ID2-HLH) and ID2 lacking the HLH domain (ID2-δHLH) might both have binding sites for Brf1, therefore ID2 may have more than two binding domains available for the interaction with the Brf1 subunit of TFIIIB. The ID proteins can interact with their target proteins (e.g. E47, MyoD and RB) through their HLH domain (Murre, 2005; Hasskarl & Münger, 2002). If the HLH domain of ID2 is at some point masked by the binding of another protein, such as E47, the amino or carboxyl domain may be able to interact with Brf1. Therefore, if ID2 and E47 are localised as a complex together at pol III templates, this may be a mechanism by which ID2 and possibly other IDs can still exert their function by utilising another domain other than the HLH.

Previous studies have demonstrated that the overexpression of Brf1 stimulates the occupancy of Brf1 and pol III at class III templates, inducing the transcription of 5S rRNA and tRNA genes (Marshall *et al.*, 2008). Other studies have shown that induction of Myc resulted in enhanced recruitment of Brf1 and pol III at 5S rRNA genes (Kenneth *et al.*, 2007). Furthermore, when wild type and Myc null rat fibroblast cell lines were compared, the occupancy of pol III at a tRNA template was reduced in the cell line in which the Myc oncogene was knocked-out (Kenneth *et al.*, 2007; Ernens *et al.*, 2006). Also, stimulation of the tumour suppressor p53 led to a decrease of Brf1 recruitment to a pol III template (Crighton *et al.*, 2003). The results have shown that in p53 knockout MEF cell lines the pol III occupancy on pol III templates was higher than in wild type cells (Crighton *et al.*, 2003). Interestingly, knockout of ID2 in MEF cells results in a decrease in the promoter occupancy of Brf1, E47 and pol III. This suggests that ID2 may

be involved in the recruitment of Brf1, which then leads to recruitment of pol III and stimulation of pol III transcription. These findings also reveal a possible role for the recruitment of E47 by ID2 and the existence of an ID-E47 complex that might be involved in the regulation of pol III-transcribed genes.

Future experiments will be required to determine whether increased pol III transcription mediated by ID proteins plays a role in oncogenesis. The pol III 7SK RNA has been implicated in cell transformation and tumourigenicity by regulating the expression of the c-Myc oncoprotein (Luo *et al.,* 1997). Other studies suggest that the induction of pol III transcription might play a role in the transforming effect of c-Myc (Johnson *et al.*, 2008). When RNAi was used to deplete Brf1 and to inhibit c-Myc stimulating pol III transcription, this compromised the ability of c-Myc to drive tumour growth in mice (Johnson *et al.*, 2008). From these results it might be concluded that the control by c-Myc and possibly ID proteins of pol III genes may present a positive feedback towards their own expression, in addition to affecting cell growth and cancer progression. In the future, Q-PCR experiments could be done in order to precisely calculate the differences in the levels of pol III transcripts upon altering the levels of ID proteins. FACS analysis could be performed to determine the cell viability and to measure the percentage of apoptotic cells upon overexpression or knockdown of ID proteins. Also, mouse knockouts of ID1, ID2 and ID3 could be created to study pol III target gene expression in various mouse tissues. In addition, tumour samples from patients showing ID upregulation can be used in order to study pol III target gene expression.

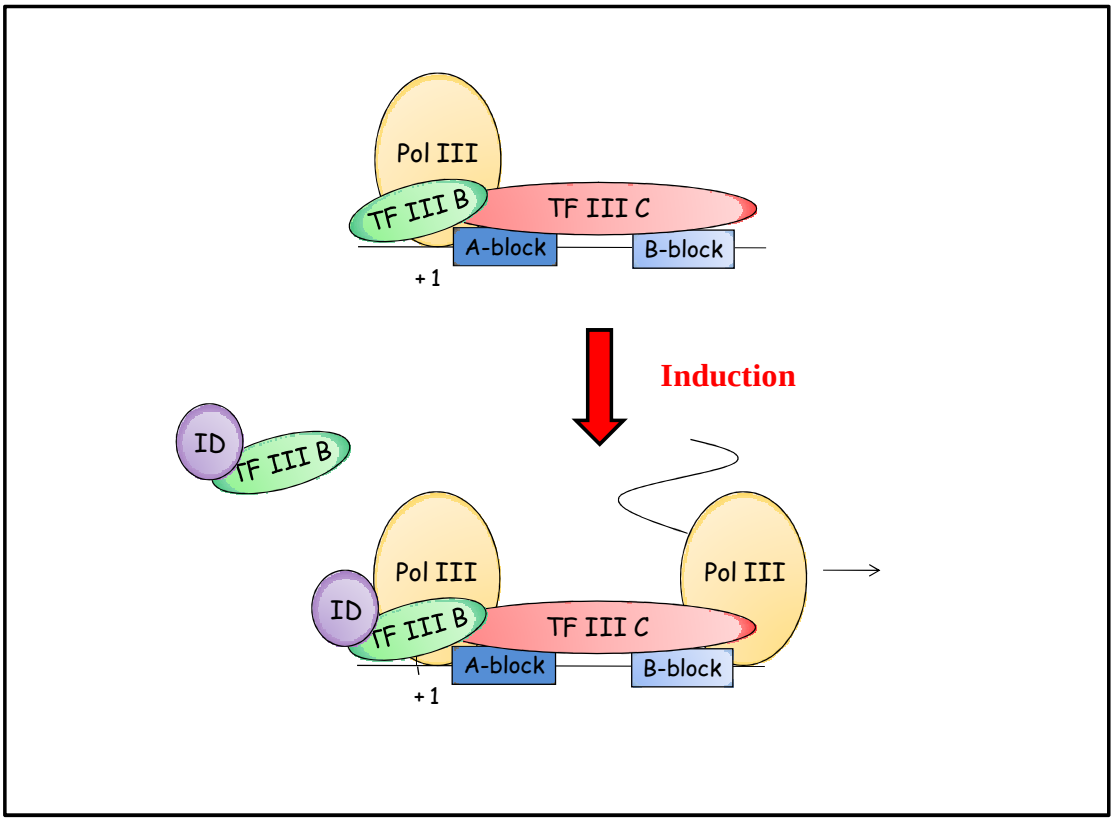

Figure 6.2: Possible model for the induction of pol III transcription by the ID proteins.

Schematic representation of changes to the pol III transcription machinery which might be occurring in transformed cells due to ID protein expression. Most pol III genes have internal promoters within the transcribed region. TFIIIC (red) binds to the promoter DNA sequences of tRNA genes. In turn, TFIIIC recruits TFIIIB (green) by protein-protein interactions. TFIIIB recruits pol III (gold) at the transcription initiation site. Considerable levels of pol III transcription are maintained in all cells, such as untransformed cells. In transformed cells, increased levels of ID proteins (violet), might stimulate recruitment of TFIIIB followed by recruitment of pol III and activation of pol III transcription.

6.4 Suppression of pol III transcription by E47

The E47 protein has been shown to be involved in processes such as cellular growth and apoptosis. Increased or decreased levels of E47 activity can lead to cell death, suggesting that lymphocytes can only survive in an optimum range of E47 activity (Kim *et al.*, 1999; Engel & Murre, 1999; Park *et al.*, 1999). Studies using mouse models have demonstrated that E2A null mice develop malignant T cell lymphomas (Bain *et al.*, 1997; Yan *et al.*, 1997). Restoring the E47 or E12 activity in cells obtained from E2A-deficient lymphomas led to apoptosis (Engel & Murre, 1999). Furthermore, when E47 was ectopically expressed in human T cell acute lymphoblastic leukaemia cell lines cell cycle progression was inhibited and programmed cell death was activated (Park *et al.*, 1999; Quong *et al.*, 2002). Therefore, it is thought that E47 functions as a tumour suppressor and its inactivation is a crucial step in the development of human leukaemias (Engel & Murre, 1999; Park *et al.*, 1999). In chapter 5, it was determined whether the partial depletion of endogenous E47 would affect pol III transcription. The knockdown of E47 by RNAi resulted in upregulation of the expression of the 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, $tRNA^{Tyr}$, 7SL RNA, 7SK RNA, MRP RNA, and U6 snRNA pol III transcripts in human cells *in vivo* (Figure 5.5). Moreover, it was demonstrated that the E47 protein might be involved in the regulation of pol I transcription, since the depletion of E47 induced the expression levels of the pre-rRNA transcript (Figure 5.5). These data suggest that E2A might target type I, type II and type III promoters and negatively coordinate the regulation of both pol I and pol III transcription. Since components synthesised by pol I and pol III are constituents of the translational apparatus, it might be a beneficial strategy for the cellular economy to utilise the same protein to regulate expression of ribosomal proteins. Overexpression of E47 by adenovirus infection results in downregulation of the expression of the 5S rRNA, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Sec}$, 7SK RNA and MRP RNA pol III transcripts *in vivo* in human cells. These results demonstrate that overexpression of E47 reduces pol III transcription, suggesting a mechanism by which E47 is involved in the regulation of cell growth.

6.5 Possible mechanism of repression of pol III transcription by E47

E47 proteins have a dual function since they can activate or repress gene expression (Ikawa *et al.*, 2004; Murre, 2005). The AD1 and AD2 domains mediate the transactivation and repression activities of E-proteins. In eukaryotic cells, DNA can be condensed into nucleosomes which are composed of histones. The modification of histones through acetylation by HATs and deacetylation by HDACs, can have a positive or negative effect, respectively, on transcription. The modulation of acetylation levels by activators or repressors which are involved in the recruitment of HATs or HDACs, respectively, can influence transcriptional activity. The AD1 domain of E-proteins also has the capability to repress transcription by recruiting a family of corepressors known as ETO (Zhang *et al.*, 2004). There are three known ETO members, ETO, ETO-2 and MTGR1 (myeloid transforming gene-related protein 1), which are highly related and are found in the mammalian genome. The ETO family members have been demonstrated to interact with the nuclear corepressors N-CoR (nuclear receptor corepressor) and Sin3A. Furthermore, the ETO members have the ability to recruit the HDAC1 and HDAC3 complexes in order to modulate chromatin accessibility (Zhang *et al.*, 2004).

In chapter 5 the repression of pol III transcription by E47 was shown to be possibly mediated by recruitment of this protein to gene promoters by specific pol III factors. The mechanism of this suppression, however, remains to be addressed. Since the expression of a number of E47 target genes is modulated by histone deacetylation, this could be a potential mechanism for the regulation of pol III transcription. Studies have revealed that chromatin remodelling can affect pol III activity (Tse *et al.*, 1998; Sutcliffe *et al.*, 2000; Kenneth *et al.*, 2007). Therefore, E47 might be involved in the recruitment of HDACs to pol III-transcribed genes. It has been demonstrated that the AML1-ETO fusion protein interacts with the AD1 domain of E-proteins leading to a silencing mechanism of E-protein target genes (Zhang *et al.*, 2004). This results in recruitment of HDACs to target promoters. Other studies have shown that TAL1/SCL interferes with E47 target genes which are important for cell differentiation and survival. The repression

is regulated by the recruitment of the mSin3A/HDAC1 corepressor complex to E47 target promoters (O'Neil *et al.*, 2004). Evidence suggests that ETO and ID family members have the ability to regulate the activity of E-proteins and this occurs in different ways. ID proteins inhibit the activity of E-proteins, interfering with both positive and negative regulation by sequestering them. However, the ETO members could be recruited to a particular set of genes to modulate transcriptional repression. Whether E-proteins can recruit HDAC members to their target pol III genes is an issue that remains to be addressed.

E47 has been reported to occupy several pol II genes in mammalian cells (Zheng *et al.*, 2004; Prabhu *et al.*, 1997; Perez-Moreno *et al.*, 2001). In chapter 5, ChIP assays demonstrated that endogenous E47 was present at the 5S rRNA and $tRNA^{Leu}$ pol III-transcribed genes *in vivo* in human cells. In addition, the overexpression of E47 revealed that E47 was strongly enriched at the promoters of 5S rRNA, $tRNA^{Leu}$ and 7SL genes. Furthermore, the use of the U2OS cell line stably expressing ID3 showed that E47 was recruited to the $tRNA^{Arg}$ and 7SL pol III templates. This result represented an indication that recruitment between E47 and ID proteins might be occurring at pol III-transcribed genes. These results demonstrate that the E47 protein is present at several pol III templates of promoter types I and II and in a number of cell lines of human origin.

Moreover, mapping ChIP assays suggested that E47 localises with TFIIIB and TFIIIC *in vivo* at the pol III promoter examined. These results indicate that TFIIIB and/or TFIIIC might be involved in the recruitment of E47 to the promoter of pol III templates. Furthermore, using the HEK 293 cell line in which E47 was overexpressed, ID1 and ID2 were also recruited; however, they co-localised with TFIIIB at the start of the gene. These findings indicate that E47 might be involved in the recruitment of ID1, ID2, and possibly other ID family members which are known to interact with the E47 protein, to the promoters of pol III-transcribed genes.

In chapter 5, experiments were employed to identify possible factors that might be involved in the recruitment of E47 to the promoters of pol III-transcribed genes. The results from co-IPs demonstrated that E47 interacts with TFIIIB *in vivo*, an observation which is consistent with ChIP data. In addition, *in vitro* transcription and translation assays revealed that the GST-E47 fusion protein binds to the Brf1 and TFIIIC110

subunits. However, the above technique cannot distinguish between direct or indirect interactions and thus it is possible that the TFIIIC subunit may be in a complex together with another pol III factor, representing an indirect interaction with the E47 protein. Therefore, these results suggest that E47 is able to repress pol III transcription through a direct mechanism, since E47 is present at pol III templates and is demonstrated to be recruited to pol III-transcribed genes, possibly through protein-protein interactions (Figure 6.3). Future experiment could include Q-PCR, which could be performed in order to precisely calculate the differences in the levels of pol III transcripts upon altering the levels of E47 proteins. Furthermore, FACS analysis could be done in order to determine the cell viability and to measure the percentage of apoptotic cells upon overexpression or knockdown of E47 proteins. An E47 mouse knockout model could be created to study pol III target gene expression in various mouse tissues. In addition, tumour samples from patients showing chromosomal translocations or deletions affecting E47 can be used in order to study pol III target gene expression.

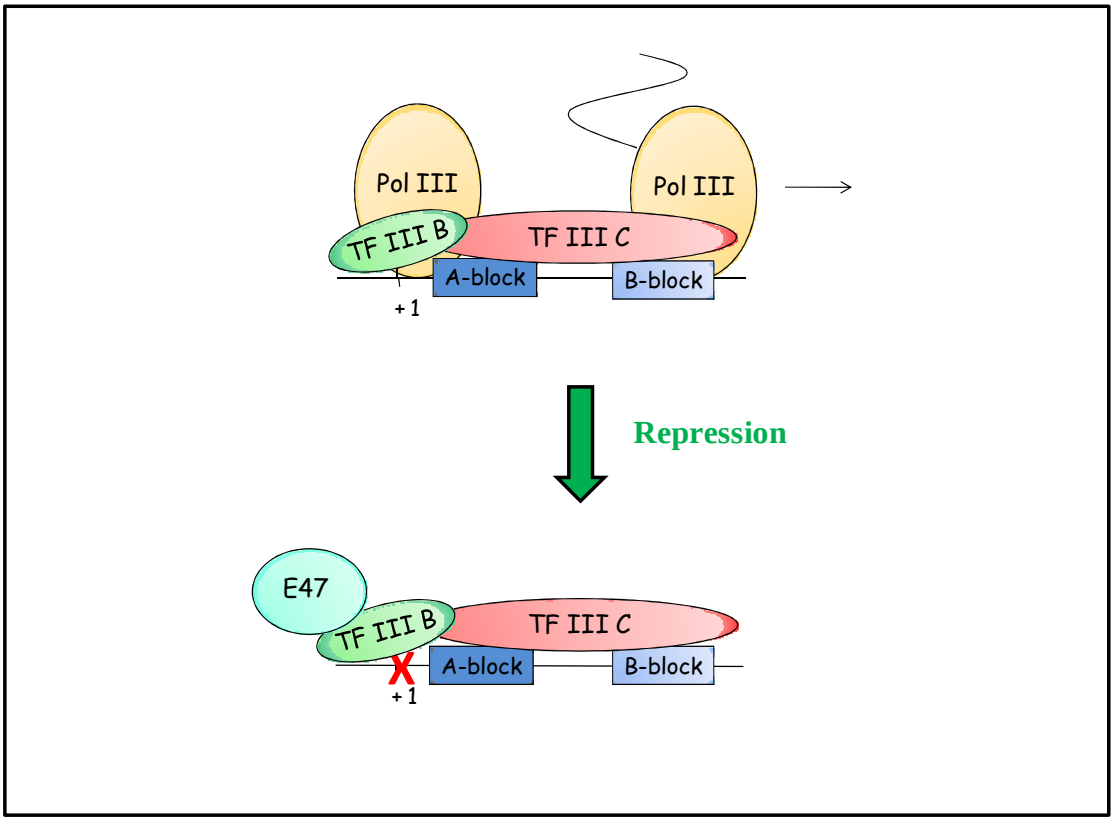

Figure 6.3: Possible model for the repression of pol III transcription by E47.
Schematic illustration of effects on the pol III transcription machinery that might be occurring under growth repression conditions due to E47 protein expression. Most pol III genes have internal promoters within the transcribed region, which are recognised by TFIIIC (red). TFIIIC binds to the promoter region of tRNA genes. TFIIIC recruits TFIIIB (green) by protein-protein interactions. TFIIIB then recruits pol III (gold) at the transcription start site. Transformation is associated with increased levels of pol III transcription, with several oncoproteins stimulating pol III activity. The restraint on cell growth may involve the interaction of E47 (aquamarine) with TFIIIB, preventing the recruitment of pol III and therefore leading to suppression of pol III transcription.

6.6 Proposed models of regulation of pol III transcription by the ID and E47 proteins

The findings in this thesis demonstrate that the ID proteins are involved in the induction of pol III transcription, whereas, E47 represses pol III output. E47 has been shown to modulate the cellular localisation and degradation of ID proteins. Expression of E47 results in nuclear localisation of ID1, ID2 and ID3 proteins (Lingbeck *et al.*, 2005; Matsumura *et al.*, 2002; Deed *et al.*, 1996). Also, the expression of E47 stabilises ID members leading to an increased half-life of ID proteins (Lingbeck *et al.*, 2005; Deed *et al.*, 1996). In chapter 5, it is shown that when E47 or ID2 are overexpressed, this results in increased enrichment of either protein at the promoters of pol III-transcribed genes. The expression of E47 was shown to result in recruitment of ID2, since the ID2 signal observed in the ChIP experiments increases in Ad-E47 infected cells. Furthermore, the knockout of ID2 in MEF cells results in reduced promoter occupancy of E47, as shown in chapter 4. In addition, scanning ChIP assays showed that upon expression of E47, the ID1 and ID2 proteins were recruited to the 7SL promoter and co-localised with TFIIIB. Also, overexpression of ID2 resulted in recruitment of E47 to the above promoter, and both proteins localised with TFIIIB at the start of the gene examined. These findings suggest that the ID and E47 proteins might exert their function on pol III activity by a regulatory cross talk between these transcription factors. Future experiments will be needed to determine the mechanism by which ID and E47 proteins regulate the expression of pol III-transcribed genes. In proposed models of pol II transcription, the ID proteins act by binding and sequestering E47 proteins, the ratio of ID proteins to E47 proteins ultimately determining the level of ID protein activity. Studies have shown that the overall effect of ID proteins on proliferation can be independent of E-proteins (Asirvatham *et al.*, 2007). However, in some cases the E-proteins can determine the magnitude of the effect or reverse the ID-mediated target expression (Asirvatham *et al.*, 2007).

In this thesis, it is demonstrated that both ID and E47 proteins are present at the pol III templates, however, several possible models for the induction or repression of pol III transcription are proposed. ID proteins are shown to bind to TFIIIB in the absence of DNA by co-immunoprecipitation and *in vitro* transcription and translation experiments (Figure 4.6; Figure 4.8). This might suggest that there is an initial interaction in the cytoplasm preceding DNA binding, which may lead to the activation of TFIIIB that increases its affinity for TFIIIC. ID proteins might activate TFIIIB by precluding a TFIIIB/suppressor complex interaction, by competing for binding sites with pol III suppressor proteins RB and p53 (Sutcliffe *et al.*, 2000; Crighton *et al.*, 2003). In addition, the ID members might induce a conformational change to the TFIIIB complex by binding or through a post-translational modification by an ID-associated factor (such as a HAT complex). Moreover, ID proteins might function through sequestration of their target proteins such as RB, E47 and the ETS family proteins, leading to the induction of pol III transcription (Figure 6.4). Furthermore, the E47 transcription factor may promote the translocation of ID proteins to the nucleus by forming an E47-ID complex, which interacts with TFIIIB leading to the repression of pol III transcription. However, it is not known whether an E47-ID complex is present at pol III templates, or if these proteins are present separately at different sites of the pol III transcription complex. Another model might also involve the E47 protein acting as a molecular switch repressing pol III transcription (Figure 6.5). The models proposed here serve as hypotheses of what might be occurring for the regulation of pol III transcription by the ID and E47 proteins, however, different mechanisms may apply. Sequential ChIPs will be required to determine whether IDs and E47 proteins are present together at pol III templates. Sequential ChIPs can also be used to investigate if these proteins can be found together with pol III at active pol III-transcribed genes. Knockout E47 cell lines would be helpful to address the possible recruitment of IDs by E47 on pol III promoters.

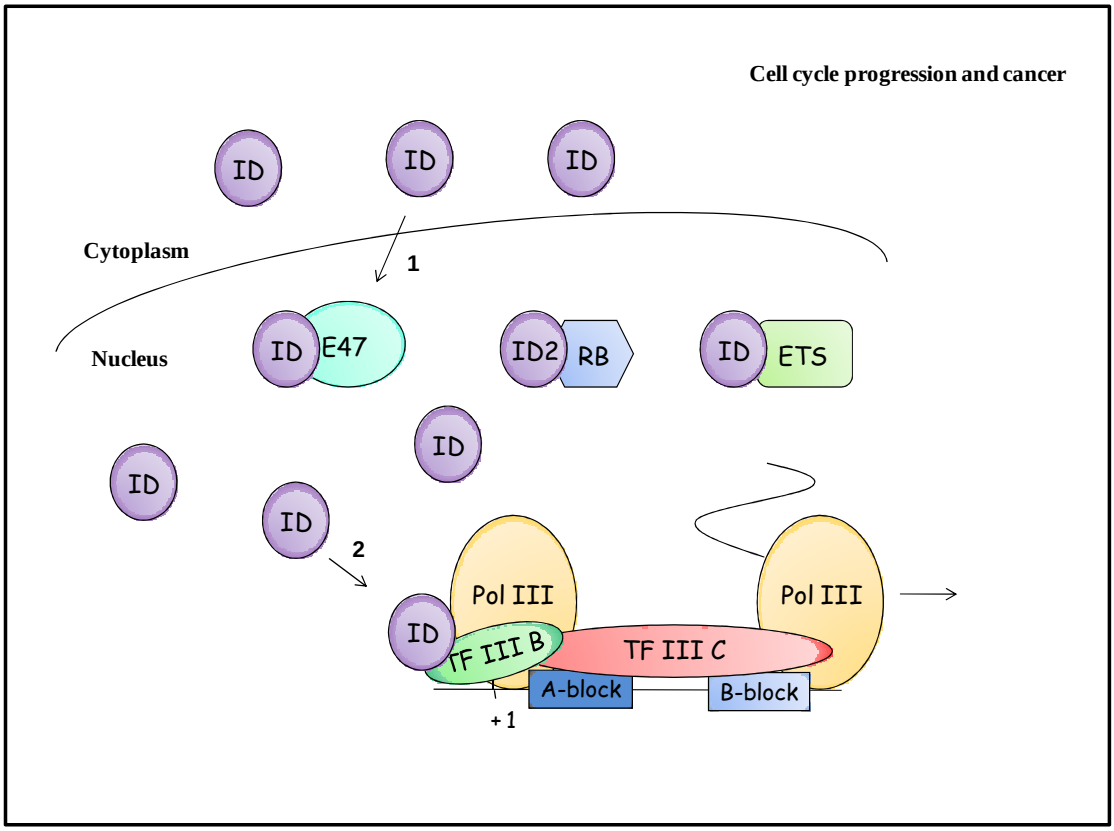

Figure 6.4: Possible model of activation of pol III transcription by ID proteins.
Activation of pol III transcription by ID proteins through sequestration of their target proteins. In undifferentiated cells large amounts of ID proteins translocate from the cytoplasm to the nucleus where they directly bind to and inactivate their targets, the E47, RB and ETS transcription factors (1). ID proteins also bind directly to TFIIIB (2) which promotes the recruitment of pol III, stimulating transcription.

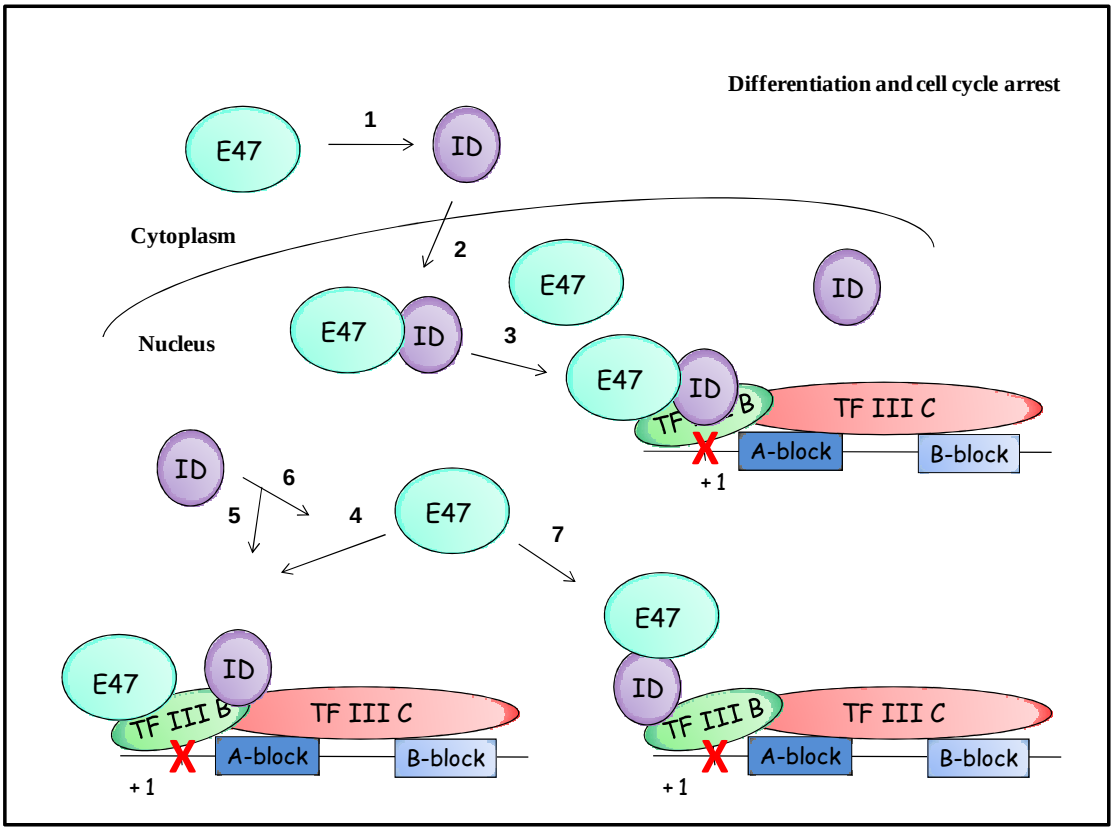

Figure 6.5: Possible models of repression of pol III transcription through interaction with the pol III machinery.

Following the activation of differentiation programs, the expression of E47 increases. E47 binds (1) and translocates ID proteins to the nucleus (2). The E47-ID complex binds to TFIIIB (3) and inhibits the activation of pol III transcription. E47 (4) and ID (5) proteins are present individually at different sites at TFIIIB interfering with the pol III machinery, leading to repression of pol III activity. ID proteins bind to TFIIIB (6), followed by E47 binding to IDs (7). Here, E47 acts as a molecular switch suppressing pol III transcription.

6.7 Possible role of ID and E47 proteins in the recruitment of HATs and HDACs to pol III-transcribed genes

In the 1960s, it was discovered that histone acetylation levels correlate with gene activity (Allfrey *et al.*, 1964). Histone acetylation and deacetylation is controlled by the interplay between HATs and HDACs, which modify chromatin structure and regulate transcription. It is thought that HAT activity results in increased gene transcription through the opening of the chromatin architecture by the addition of acetyl groups (Yang & Seto, 2007; Norton *et al.*, 1990; Lee *et al.*, 1993; Hebbes *et al.*, 1988). In contrast, HDACs remove acetyl groups and their activity is linked to the repression of gene expression through chromatin condensation (Ekwall, 2005; Taunton *et al.*, 1996; Rundlett *et al.*, 1996). It has been demonstrated that HATs and HDACs associate with several transcriptional activator and repressor proteins, such as c-Myc and MeCP2 (methyl CpG binding protein 2), which leads to the recruitment of HATs and HDACs and results in the regulation of gene expression (Vervoorts *et al.*, 2003; Jones *et al.*, 1998). c-Myc interacts with HATs such as CBP- and GCN5-containing complexes, which leads to the recruitment of these complexes to gene promoters (Vervoorts *et al.*, 2003; McMahon *et al.*, 2000). c-Myc has been shown to recruit GCN5 and the cofactor TRRAP to pol III-transcribed genes, which leads to the hyperacetylation of histone H3, followed by recruitment of pol III and activation of transcription (Kenneth *et al.*, 2007). The ID1 protein has been demonstrated to induce the activity of the hypoxia-inducible factor-1α (HIF-1α) in the breast cancer cell line MCF-7 (Kim *et al.*, 2007). This enhancement of the transcriptional activity of HIF-1α by overexpressing ID1 is due to the recruitment of CBP. However, previous experiments that have been performed in order to investigate whether ID proteins interact with HAT complexes have not shown an interaction between these proteins (Lasorella *et al.*, 1996). A possible mechanism by which ID proteins may activate pol III transcription could involve the recruitment of HAT complexes to pol III-transcribed genes (Figure 6.6). In this model, overexpression of ID proteins might lead to binding to TFIIIB to promote the formation of the TFIIIB-TFIIIC interaction. ID proteins could recruit HAT complexes including p300/CBP and

GCN5 to pol III-transcribed genes. The recruitment of the HAT complexes could then result in histone acetylation, which may result in recruitment of pol III and induction of pol III transcription (Figure 6.6).

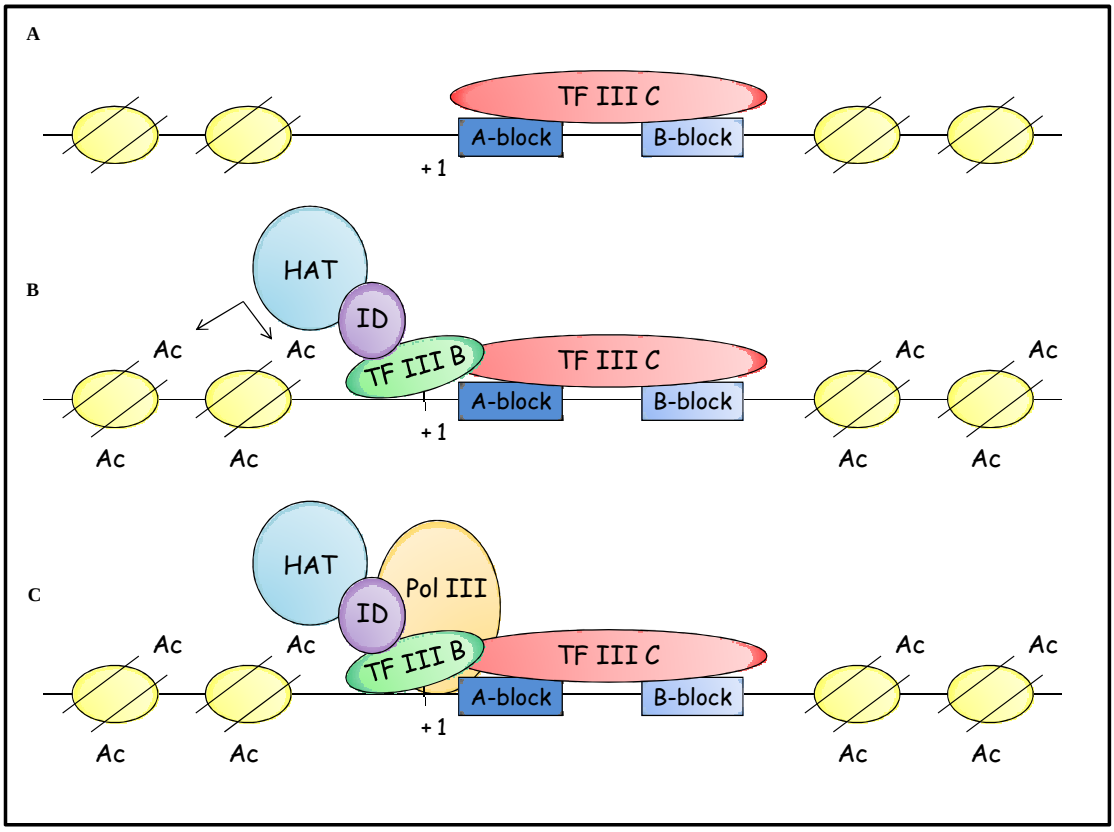

Figure 6.6: Possible model for the activation of pol III transcription by ID proteins, mediated by the recruitment of histone acetylases.

A. tRNA gene with TFIIIC bound in an inactive state. **B.** Activation of ID proteins leads to the recruitment of an ID-TFIIIB-HAT complex to the tRNA promoter, which in turn leads to the acetylation of histones. **C.** This is then followed by the recruitment of the polymerase and the subsequent activation of pol III transcription.

Future experiments are required to determine whether ID1, ID2 and ID3 proteins can associate with HAT complexes.

The tumour suppressor RB and p53 proteins have been demonstrated to repress pol II transcription by recruiting HDAC complexes to promoters (Brehm & Kouzarides, 1999; Luo *et al.*, 1998; Murphy *et al.*, 1999; Luo *et al.*, 2000). It has been shown that HDAC function contributes to regulation of pol III gene expression (Sutcliffe *et al.*, 2000). However, it has been demonstrated that HDAC activity is not required for RB and p53 to repress pol III transcription (Sutcliffe *et al.*, 2000; Crighton *et al.*, 2003). It is suggested that RB and p53 use an HDAC-independent mechanism to regulate pol III transcription *in vivo*. This may be explained by the interaction of RB and p53 with TFIIIB, which results in inhibiting promoter occupancy by TFIIIB (Sutcliffe *et al.*, 2000; Crighton *et al.*, 2003). Interestingly, E-proteins can function as transcriptional repressors by recruiting co-repressors such as ETO and mSin3A (Zhang *et al.*, 2004; Kee, 2009). Also, it has been shown that the E-protein HEB recruits HDAC1 and HDAC3 to its AD1 domain, this AD1 domain is conserved with other E-protein family members, including the E47 protein, suggesting that E47 may also interact with HDAC1 and HDAC3 (Zhang *et al.*, 2004). Experiments in this thesis have shown that E47 represses pol III transcription (Figure 5.5; Figure 5.7) and that E47 localises at pol III-transcribed genes (Figure 5.8; Figure 5.9; Figure 5.10; Figure 5.11), and this repression may be due to the recruitment of HDAC complexes to pol III-transcribed genes. In a potential mechanism the levels of ID proteins decrease during differentiation, which then allows E47 to interact with TFIIIB, since it is not sequestered by ID proteins. E47 may recruit HDAC complexes leading to the repression of pol III transcription (Figure 6.7). Therefore, IDs and E47 proteins might recruit HATs and HDACs respectively, leading to increases or decreases in localised histone acetylation surrounding pol III-transcribed genes. The mechanisms proposed here provide hypotheses for the regulation of pol III transcription; however, different mechanisms may apply as well or instead.

One mechanism by which HAT and HDAC complexes could be responsible for the patterns of acetylation and regulation of transcription would be to remain in the vicinity during replication and reassociate with the newly synthesised chromatin following the passage of the replication fork.

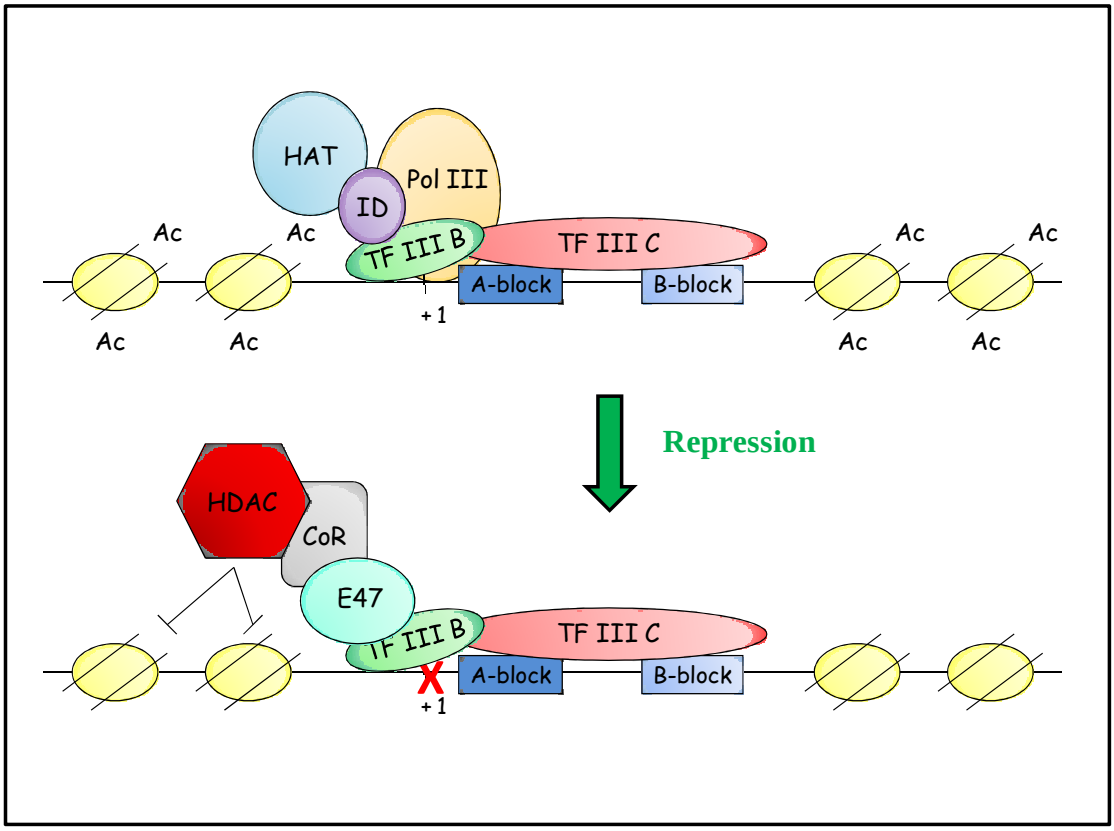

Figure 6.7: Possible model for the repression of pol III transcription by E47, mediated by the recruitment of histone deacetylase complexes.

The levels of ID protein members decrease during differentiation, which correlates with a reduction of pol III transcription. E47 is not under the control of ID proteins which results in binding of E47 to TFIIIB. E47 may then recruit a co-repressor-HDAC complex, leading to the suppression of pol III transcription.

Modification of histones, such as acetytation of the histone tails, can alter the chromatin structure by weakening the histone-histone and histone-DNA contacts leading to the opening of chromatin to assist the interaction of transcription complexes with DNA (Wolffe & Hayes, 1999; Hansen *et al.*, 1998). HDACs could be recruited to methylated DNA through their association with methyl-DNA-binding proteins, like MeCP2 and MBD2 (methyl-CpG binding domain protein 2), and also with the DNA methyl transferase itself (Fuks *et al.*, 2000; Ng & Bird, 2000). Furthermore, acetylated histone tails form interaction domains for the binding of molecules containing bromodomains (brm [brahma]-like domains). Bromodomains are present in HAT and chromatin remodeling complexes and associate with acetylated lysines (Zeng & Zhou, 2002). Sequential recruitment of bromodomain-containing factors and complexes to promoter regions is important for the regulation of gene expression (Agalioti *et al.*, 2002; Hassan *et al.*, 2002). Acetylation of histones surrounding the promoters of pol III-transcribed genes might form interaction domains on the chromatin for bromodomain-containing complexes to serve as co-activators (Fischle *et al.*, 2003; Turner, 2000). It has been shown that the Brg1 ATPase, which is a subunit of SWI/SNF (switch/sucrose nonfermentable) complex and contains a bromodomain, associates with pol III genes and it may be that histone acetylation can recruit ATP-dependent chromatin remodelling proteins (Sif, 2004).

In addition to regulating gene transcription by histone modification, HATs and HDACs can control the function of a wide range of proteins, including transcription factors (Lee & Workman, 2007). CBP acts as a co-activator for the transcription factor p53, and acetylates p53 on a lysine residue adjacent to the DNA-binding domain (Gu & Roeder, 1997). This modification enhances DNA-binding and increases transcriptional activity of p53 (Luo *et al.*, 2004). p53 has also been shown to interact with HDAC1, by forming a complex with the co-repressor mSin3a, leading to the deacetylation of lysine residues of p53 by HDAC1 (Murphy *et al.*, 1999; Luo *et al.*, 2000). As a result, the free lysine residues are targeted for ubiquitination, leading to the degradation of p53 (Ito *et al.*, 2002; Rodriguez *et al.*, 2000). The pol II basal transcription factors TFIIB, TFIIF and TFIIEβ have been shown to be acetylated, with deacetylation also occurring suggesting that this is a reversible catalytic process, in order to control their functions

(Choi *et al.*, 2003; Imhof *et al.*, 1997). The ID and E47 proteins might form a complex with TFIIIB, preceding DNA localisation, recruiting HATs or HDACs to acetylate or deacetylate this complex to regulate TFIIIB activity. Future experiments are required to investigate this possible mechanism of activation or inhibition of TFIIIB by ID or E47 proteins, respectively. Another possibility may be that the recruitment of ID-HAT or E47-HDAC complexes could lead to acetylation or deacetylation of other factors of the pol III machinery, resulting in activation or repression of transcription.

6.8 Conclusions

Products of pol III transcription include the 5S rRNA and tRNAs which play an important role in the biosynthetic capacity of cells and subsequently in growth. Work in this present study demonstrates that the ID1, ID2 and ID3 family members, which have been implicated in the development and progression of tumour growth, are involved in the upregulation of pol III transcripts. Furthermore, the results described in this thesis showed that the E47 protein, a transcription factor thought to play a critical role in the regulation of cell growth and survival, participates in the repression of pol III transcription. The ID family members appear to target the TFIIIB factor and specifically Brf1 in order to induce pol III activity. Also, E47 is demonstrated to interact with the Brf1 subunit to downregulate pol III transcription. Both IDs and E47 members are thought to interact directly with Brf1 and appear to localise at pol III genes. The pol III transcriptional machinery has been demonstrated to be the target of several oncogenes and tumour suppressors. Recently, induction of Brf1 has been shown to stimulate cell proliferation and lead to oncogenic transformation. Therefore, components of the pol III machinery could be explored as potential therapeutic targets. It will be interesting to investigate whether the E47 protein functions by altering the histone code and the methylation status of pol III genes, and whether ID proteins can stimulate transcription after pol III genes become accessible through recruitment of chromatin-modifying factors. Therefore, the ID and E47 protein functions are branching out, adding to their abilities the control of expression of pol III-transcribed genes. Deregulated ID and E47 proteins may utilise a mechanism involving pol III transcription to affect cancer progression. Finally, the knowledge concerning pol III transcription should be aimed to provide therapy to benefit patients.

Chapter 7

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