In vivo studies of repressors of RNA polymerase III transcription

Theodoros Kantidakis

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Institute of Biomedical and Life Sciences

University of Glasgow

Abstract

RNA polymerase III (Pol III) is responsible for transcribing a relatively small but vital set of genes, including 5S rRNA and tRNAs. Pol III transcription has been shown to be upregulated in transformed and cancer cells, suggesting an important role in cell growth and proliferation. Its tight regulation is, therefore, fundamental for cell welfare, and a number of factors have been shown to be implicated in its control. These include the tumour suppressors p53 and Rb, as well as p107 and p130, and the basal transcription factor Dr1. The work in this thesis focused on the role of these repressors in regulating Pol III transcription in human cells.

The Dr1-DRAP1 complex has been identified as a transcriptional repressor of Pol II transcription. Dr1 has also been shown to inhibit Pol III transcription when expressed at high levels, either *in vitro* or in yeast cells. Here it is shown that depletion of endogenous Dr1 by RNAi resulted in upregulation of tRNA expression. This seems to be a direct effect, as the expression of subunits of TFIIIB, TFIIIC and Pol III remained unaltered. ChIP experiments revealed that both Dr1 and DRAP1 are found at Pol III-transcribed genes in human cells, in contrast to previous studies in yeast, and sequential ChIP experiments indicated co-occupancy of Dr1 and Pol III, suggesting the presence of Dr1 at active Pol III templates. Moreover, promoter occupancy by Dr1 is increased under hypoxic conditions, which correlates with negative regulation of Pol III transcription. Dr1 can interact with TFIIIB and TFIIIC subunits, and promoter mapping experiments suggest that TFIIIB might be responsible for its recruitment to Pol III-transcribed genes.

Both Dr1 and DRAP1 can be phosphorylated, but little is known about their regulation. It was found that under stress conditions, such as heat shock and hypoxia, DRAP1, but not Dr1, protein expression is induced. This seems to be a posttranscriptional effect, as the mRNA of both Dr1 and DRAP1 remained stable. Moreover, p53 seems to be involved, as DRAP1 expression was decreased under hypoxic conditions in p53-impaired cells. Furthermore, serum-starved mouse cells presented comparable protein levels of DRAP1 with growing cells, but not of Dr1, which was reduced. These experiments, thus, indicate that both Dr1 and DRAP1 can be posttranscriptionally regulated differentially under stress conditions.

The tumour suppressors p53 and Rb, as well as p107 and p130, have been previously shown to regulate Pol III transcription in mouse cells. An RNAi approach was employed to study the effect of the depletion of their endogenous levels on Pol III transcription in human cells. It was found that they can negatively regulate tRNA expression in U2OS or IMR90 cells, in accordance with the studies in mouse cells.

The tumour suppressor ARF has been shown to suppress rRNA production in a p53-independent manner. Therefore, it was investigated if ARF has a role in the regulation of the Pol III system. It was found that ARF can repress tRNA transcription, but in a p53-dependent manner, as it was unable to inhibit tRNA gene transcription when p53 was ablated.

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List of Abbreviation

^{o}C:	degrees Celsius
µg:	microgram
µl:	microlitre
µM:	micromolar
4E-BP1:	4E-binding protein 1
A:	alanine
A_{260}:	absorbance at 260 nm
A_{280}:	absorbance at 280 nm
AMP:	adenosine monophosphate
AMPK:	AMP-activated protein kinase
ARF:	alternative reading frame
ARPP P0:	acidic ribosomal phosphoprotein P0
ATM:	ataxia telangiectasia mutated
ATP:	adenosine triphosphate
ATR:	ATM- and Rad3- related
Bdp1:	B double prime 1
bp:	base pair
Brf1:	TFIIB-related factor 1
Brf2:	TFIIB-related factor 2
BUR6:	bypass UAS requirement 6

cDNA:	complementary DNA
Cdk:	cyclin-dependent kinase
ChIP:	chromatin immunoprecipitation
Chk:	checkpoint kinase
CHO:	Chinese hamster ovary
CK2:	casein kinase 2
co-IP:	co-immunoprecipitation
CTD:	carboxy-terminal domain
DFX:	deferoxamine mesylate salt
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dNTP:	2' deoxy (nucleotide) triphosphate
Dr1:	down regulator of transcription 1
DRAP1:	Dr1-associated protein 1
DSE:	distal sequence element
DTT:	dithiothreitol
EBER:	Epstein-Barr virus-encoded small RNA
EBV:	Epstein-Barr virus
EDTA:	ethylene diamine tetra acetic acid
eEF2:	eukaryotic elongation factor 2
EMSA:	electrophoretic mobility shift assay
Erk:	extracellular signal-regulated kinase

FBS:	foetal bovine serum
FCS:	foetal calf serum
FKBP12:	FK506-binding protein
g:	relative centrifugal force
GTP:	guanosine triphosphate
h:	hour
HAT:	histone acetyltransferase
HDAC:	histone deacetylase
Hdm2:	human double minute 2
HIF:	hypoxia inducible factor
HPV:	human papilloma virus
HRP:	horseradish peroxidase
Hsp:	heat shock protein
HTLV-1:	human T-cell leukaemia virus 1
IgG:	immunoglobulin G
IP:	immunoprecipitation
IPTG:	β-D-thiogalactopyranoside
JNK:	c-Jun N-terminal kinase
kb:	kilo base
kD:	kilo Dalton
LEU:	leucine
LINE:	long interspersed element
LS-MEB:	low salt microextraction buffer

M:	molar
MAPK:	mitogen-activated protein kinase
Mdm2:	mouse double minute 2
MdmX or Mdm4:	mouse double minute 4
mg:	milligram
min:	minutes
miRNA:	micro RNA
ml:	millilitre
mM:	millimolar
MMS:	methane methylsulfonate
mRNA:	messenger RNA
MRP:	mitochondrial RNA processing
mTOR:	mammalian target of rapamycin
MW:	molecular weight
NC2:	negative cofactor 2
ncRNA:	non-coding RNA
ng:	nanograms
nM:	nanomolar
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PI:	pre-immune
PIC:	preinitiation complex

PKA:	protein kinase A
PKB:	protein kinase B
pmol:	picomole
Pol I:	RNA polymerase I
Pol II:	RNA polymerase II
Pol III:	RNA polymerase III
pre-rRNA:	precursor rRNA
PSE:	proximal sequence element
Q:	glutamine
Rb:	retinoblastoma
RCF:	relative centrifugal force
rDNA:	ribosomal DNA
Rheb:	Ras homolog enriched in brain
RNA:	ribonucleic acid
RNAi:	RNA interference
RNase:	ribonuclease
rRNA:	ribosomal RNA
RT:	room temperature
RT-PCR:	reverse transcriptase-PCR
s:	seconds
S6K:	ribosomal p70 S6 kinase
SDS:	sodium dodecyl sulphate
shRNA:	short hairpin RNA

SINE:	short interspersed nuclear element
siRNA:	small interfering RNA
$SNAP_c$:	snRNA activator protein complex
snoRNA:	small nucleolar RNA
snRNA:	small nuclear RNA
SRP:	signal recognition particle
SUMO-1:	SMT3 suppressor of mif two 3 homolog 1
SV40:	simian virus 40
T:	thymine
TAF:	TBP-associated factor
TBP:	TATA-binding protein
TBS:	tris buffered saline
TE:	tris-EDTA
TFIIA:	transcription factor IIA
TFIIB:	transcription factor IIB
TFIIIA:	transcription factor IIIA
TFIIIB:	transcription factor IIIB
TFIIIC:	transcription factor IIIC
Tris:	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA:	transfer RNA
TSC:	tuberous sclerosis complex
TYR:	tyrosine
U:	units

Ubc13:	ubiquitin-conjugating enzyme 13
UBF:	upstream binding factor
UCE:	upstream control element
UV:	ultraviolet
V:	Volt
v/v:	volume per volume
W:	watt
w/v:	weight per volume
WWP1:	WW domain-containing E3 ubiquitin protein ligase 1

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Author's Declaration

I hereby declare that the work presented in this thesis is my own and has not been submitted for a degree at another institution.

Theodoros Kantidakis

Chapter 1

Introduction

1.1 Transcription and RNA polymerases

Genetic information stored in the genome of prokaryotes and eukaryotes is retrieved by DNA-dependent RNA polymerases, which initiate gene expression programmes by transcribing DNA to RNA. While in prokaryotes and archaeabacteria a single RNA polymerase is responsible for the transcription of all cellular RNA, eukaryotes employ a set of different polymerases to share the task of eukaryotic transcription (White, 2001). More specifically, in the nucleus of eukaryotic cells, three RNA polymerases named RNA polymerase I, II and III, have been found to transcribe different sets of genes (White, 2001). This list has been recently extended by the discovery of a polymerase expressed from an alternative transcript of the mitochondrial RNA polymerase gene in mammalian cells (Kravchenko et al, 2005) and a plant specific polymerase that participates in transcriptional silencing (Herr et al, 2005). Moreover, outside the eukaryotic nucleus, mitochondria and chloroplasts contain their own RNA polymerase systems, which bear similarity to the prokaryotic ones (Falkenberg et al, 2007; Lysenko & Kuznetsov, 2005).

Each nuclear RNA polymerase transcribes specific target genes. RNA polymerase I (Pol I) is responsible for the transcription of one single transcript, the 45S ribosomal RNA precursor (pre-rRNA), which is then processed to the 18S, 5.8S and 28S ribosomal RNAs (rRNAs). These RNAs constitute essential components of the ribosomes and therefore, depending on the cellular demand for protein synthesis, Pol I transcription can contribute 35-60% of the total nuclear transcription (Moss & Stefanovsky, 2002). RNA polymerase II transcribes protein-coding genes to produce messenger RNAs (mRNAs) and many non-coding (nc)RNAs, such as most of the small nuclear RNAs (snRNA), small nucleolar (sno) and micro (mi)RNAs. RNA polymerase III (Pol III) is dedicated to the transcription of several small, untranslated RNAs with a variety of functions and can account for 10-20% of total nuclear transcription (Moss & Stefanovsky, 2002). Finally, the recently discovered RNA polymerase of mitochondrial origin, named spRNAP-IV, seems to transcribe the mRNA of certain protein-coding genes (Kravchenko et al, 2005).

Pol I, II and III all consist of several subunits, forming complex multimeric enzymes (Cramer, 2002). They share five common subunits and also contain subunits homologous to subunits of prokaryotic and archaeal RNA polymerases (Cramer, 2002), indicating functional similarity and common evolutionary origin. It must be noted, however, that the three polymerases not only have distinct target genes, but also different

nuclear localisations, with Pol I residing at the nucleolus, and Pol II and III at separate locations in the nucleoplasm (Pombo et al, 1999).

1.2 RNA polymerase III transcripts

The Pol III products are small, typically less than 400 bp, untranslated RNAs that are involved in a variety of cellular functions (Table 1.1).

Table 1.1. Summary of class III gene products and their functions

Pol III product	Known Function
5S rRNA	Protein synthesis
tRNA	Protein synthesis
U6 snRNA	mRNA splicing
7SL RNA	Intracellular protein transport
7SK RNA	Control of transcriptional elongation by Pol II
H1 RNA	tRNA processing
MRP RNA	rRNA splicing
VA RNA	Adenovirus translational control
EBER RNA	Epstein-Barr virus translational control
SINE	Potential role in cellular stress responses

5S rRNA is the smallest, 121 bp in length in human, of the rRNAs and the one transcribed by Pol III rather than Pol I. As with the other Pol III-transcribed gene products, it is synthesised in the nucleoplasm, but is then transferred to the nucleolus, where it is incorporated into the large ribosomal subunit together with 5.8S and 28S rRNAs and several ribosomal proteins (Lafontaine & Tollervey, 2001). There are 200-300 5S rRNA genes in human cells, most of them in tandem repeat clusters (White, 2002).

tRNA genes are transcribed by Pol III as precursors which are quickly processed to the mature tRNAs between 70-90 bp in length. Human cells contain 171 putative tRNA

pseudogenes and about 450 tRNA genes, which encode up to 274 different tRNA species (Goodenbour & Pan, 2006; GtRNADb, 2004). Different tRNA species can code for the same amino acid (through different anticodons) and several genes can code for the same tRNA anticodon resulting in considerable functional redundancy.

U6 snRNA, together with the Pol II-transcribed U1, U2, U4 and U5 snRNAs, is an essential part of the gigantic ribonucleoprotein assembly called the spliceosome. The spliceosome is responsible for the removal of introns from the pre-mRNAs and the formation of mature mRNA (Valadkhan, 2005). U6 snRNA is highly conserved and about 106 nt in length in humans (Valadkhan, 2005; White, 2002).

7SL is 299 bp in human cells and forms the RNA component of the signal recognition particle (SRP). The SRP recognises the N-terminal signal sequences of nascent polypeptides just after they are translated by the ribosome, and targets the whole SRP-polypeptide-ribosome complex to the endoplasmic reticulum through interactions with the SRP receptor (Lutcke, 1995).

7SK is a 331 nt long Pol III transcript that was found to function as a negative regulator of the transcription elongation factor P-TEFb (Nguyen et al, 2001; Yang et al, 2001). P-TEFb is a heterodimer consisting of the RNA polymerase II carboxy-terminal domain (CTD) kinase Cdk9 and a cyclin subunit (T1, T2 or K), and can promote transcription elongation by increasing the phosphorylation of the RNA polymerase II CTD. 7SK can interact with P-TEFb, inhibiting its CTD kinase activity and its ability to associate with transcription complexes; it can therefore play an important role in the regulation of transcription of Pol II-transcribed genes (Nguyen et al, 2001; Yang et al, 2001).

The H1 and MRP RNAs are transcribed by Pol III and are the RNA components of the RNase P and RNase MRP endoribonucleases respectively. RNase P is involved in the processing of the 5'-termini of pre-tRNAs (Bartkiewicz et al, 1989), while the structurally related MRP (mitochondrial RNA processing) RNase is mainly involved in pre-rRNA processing, but also in DNA replication in the mitochondria (Morrissey & Tollervey, 1995; Schmitt & Clayton, 1993). It is interesting that regulation of Pol III transcription is not only affecting translation directly, through the production of tRNAs and 5S rRNA, but also via their posttranscriptional processing by these endoribonucleases.

Certain viruses can employ the Pol III machinery to synthesise specific viral transcripts. The best characterised example is the adenovirus VAI gene, which is highly transcribed, especially at the late stages of infection, and promotes the efficient translation of the viral mRNAs by the host's translational apparatus (Svensson & Akusjarvi, 1984; Thimmappaya et al, 1982). Similarly, the Epstein-Barr virus (EBV) utilises Pol III to transcribe EBER1 and EBER2 genes (Rosa et al, 1981). EBER 1 and 2 can functionally substitute for VAI during adenovirus infection and are believed to allow the efficient synthesis of viral proteins by the host cell's translational machinery (Bhat & Thimmappaya, 1985). More recently, the EBERs have been implicated in oncogenesis, providing the first example of untranslated RNA with oncogenic functions (Komano et al, 1999; Ruf et al, 2000).

Pol III is also responsible for the transcription of short interspersed nuclear elements (SINEs). SINEs comprise a large family of repetitive elements that includes Alu elements in primates, and B1 and B2 elements in rodents. B1 elements show about 80% homology to Alu and both are considered to have involved from a 7SL gene, while B2 evolved from tRNA genes (Batzer & Deininger, 2002). Alus are present in more than a million copies in human cells and therefore represent about 10% of the whole human genome (Lander et al, 2001), but are highly silenced by DNA methylation (Batzer & Deininger, 2002). Although Alu elements are the most successful transposons in humans and contain their own internal promoters, they lack coding sequences and depend on long interspersed nucleotide elements (LINEs) for their retrotransposition (Dewannieux et al, 2003).

Although Alu, and other repetitive elements, were traditionally considered to be parasites of the genome, 'selfish' or 'junk' DNA (Orgel & Crick, 1980), several recent lines of evidence suggest otherwise. SINEs have been proposed to be involved in cellular stress responses, as heat shock and DNA damaging agents have been found to induce their transcription (Li et al, 1999a; Liu et al, 1995; Rudin & Thompson, 2001). B2 elements have been shown to be induced after heat shock, bind to Pol II and repress its transcription (Allen et al, 2004; Espinoza et al, 2004). Furthermore, Alu elements have been shown to be involved in alternative splicing (Lev-Maor et al, 2003), RNA editing (Athanasiadis et al, 2004) and translational regulation (Hasler & Strub, 2006; Rubin et al, 2002), while it has also been suggested that Alu sequences upstream from gene promoters provide binding motifs for transcription factors (Polak & Domany, 2006). It is slowly emerging that even if

SINEs started as genomic parasites, they might have acquired important cellular functions throughout evolution.

In addition to the above-mentioned Pol III transcripts, the Pol III repertoire includes several other products of less well known or unknown function, such as the vault RNAs (van Zon et al, 2003), Y RNAs (Deutscher et al, 1988) and the neural-specific BC1/BC200 RNAs (Cao et al, 2006). Furthermore, novel Pol III-transcribed non-coding RNAs of different classes have emerged over the last few years and more are expected to be found in intron-transcribed regions (Dieci et al, 2007; Nakaya et al, 2007). Of interest is the finding that in human cells, miRNAs can be transcribed by Pol III via upstream Alu elements that lack a transcription termination signal (Borchert et al, 2006). Since miRNA was so far considered to be exclusively transcribed by Pol II, this discovery extends the potential of Pol III transcription in relation to translational and transcriptional regulation.

1.3 RNA polymerase III promoters

There are three types of Pol III promoters, named type 1, 2 and 3 (Figure 1.1). The 5S rRNA gene promoter is type 1. It is an internal promoter that consists of three elements downstream of the transcription start site; the A-block, an intermediate element and the C-block (Figure 1.1, A) (Pieler et al, 1987). This type of promoter is highly conserved in different species (White, 2002), and mutations in the A- and C-blocks substantially reduce transcription (Pieler et al, 1985a). Mutations in the sequence between the elements does not affect transcription (Pieler et al, 1985a), but alterations of the spacing are not tolerated (Pieler et al, 1987; Pieler et al, 1985b).

Type 2 promoters are also intragenic and the most common promoter type used by Pol III, found in tRNA genes, as well as in SINEs and VA genes (White, 2002). They are well conserved in different species and consist of an A- and a B-block (Figure 1.1, B). The A-block of type 2 is homologous to that of type 1 and, at least in Xenopus, functionally interchangeable (Ciliberto et al, 1983). Similarly to the type 1 promoters, mutations in the type 2 elements result in reduced gene expression (Traboni et al, 1984; Traboni et al, 1983). Although the sequence of the A- and B-blocks is highly conserved, the distance between them can vary, with an optimal 30-60 bp (Baker et al, 1987) that can be stretched

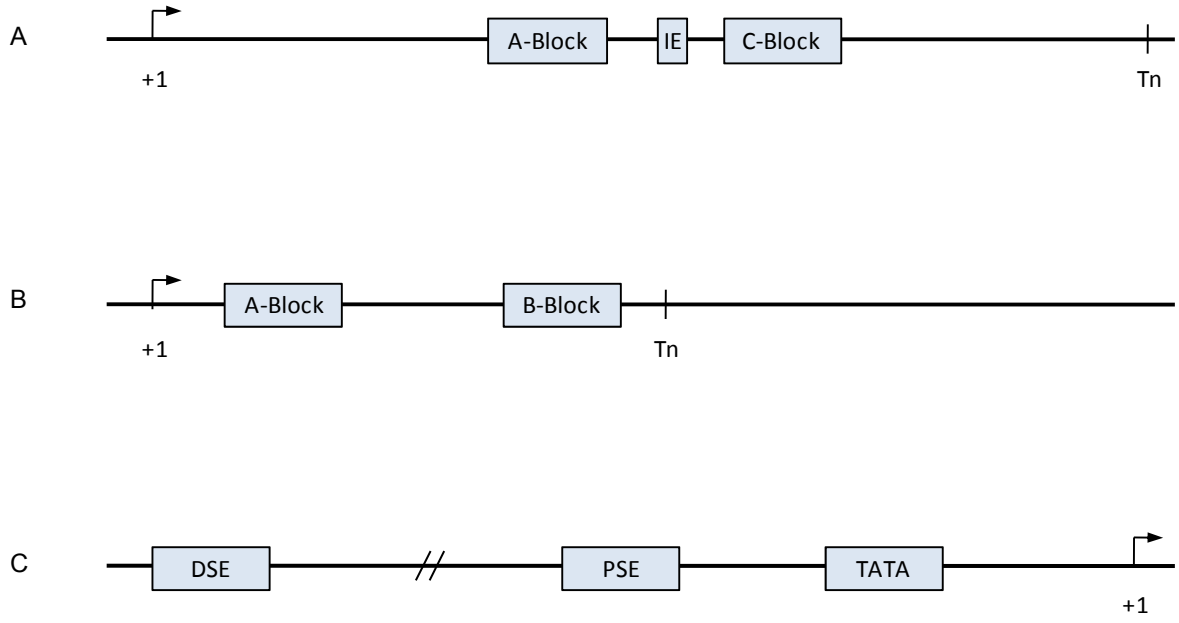


Figure 1.1. Different types of Pol III promoters. A. Type 1 promoter of 5S rRNA genes. **B.** Type 2 promoter of tRNA genes. **C.** Type 3 promoter of U6 snRNA genes. The transcription start site is indicated by +1 and the site of termination by Tn. IE: intermediate element; DSE: distal sequence element; PSE: proximal sequence element. Adapted from White, 2002.

up to 365 bp and maintain functionality (Fabrizio et al, 1987); the naturally occurring differences in the spacing between the two blocks is partly due to introns in some tRNA genes (White, 2002).

In contrast to the intragenic type 1 and 2 promoters, type 3 promoters are gene-external (Figure 1.1, C). They consist of a proximal sequence element (PSE), a TATA box at a fixed distance downstream the PSE, and a distal sequence element (DSE) upstream of the PSE (Schramm & Hernandez, 2002). Pol III-transcribed genes with this kind of promoter include the U6 snRNA (Krol et al, 1987), 7SK RNA (Murphy et al, 1986), H1 RNA (Baer et al, 1990) and MRP RNA genes (Topper & Clayton, 1990). Interestingly, the yeast U6 snRNA promoter contains A- and B-blocks, which, together with the TATA box, are required for efficient transcription (Brow & Guthrie, 1990; Eschenlauer et al, 1993), suggesting that the extragenic promoter type 3 genes have evolved relatively recently within the Pol III system (Paule & White, 2000).

A number of Pol III-transcribed genes present promoters that cannot be characterised as type 1-3. For example, the EBER2 gene of EBV contains A- and B-blocks

typical of type 2, but also a TATA box and upstream elements, all of which are required for efficient transcription (Howe & Shu, 1989). The 7SL RNA gene also falls in the category of Pol III promoters that rely on both internal and upstream sequences, as it requires the first 37 nt upstream of the transcription start site, in conjunction with the internal promoter elements (Ullu & Weiner, 1985).

1.3 Transcription complex assembly at Pol III promoters

Pol III promoters are recognised by Pol III-specific transcription factors that form a transcription preinitiation complex (PIC), which is completed by the recruitment of Pol III. Depending on the type of the promoter, different transcription factors are required in order to form a functional PIC.

1.3.1 Transcription complex assembly at type 2 promoters

In type 2 promoters (Figure 1.2), the A- and B-block sequences are recognised by transcription factor IIIC (TFIIIC) (Lassar et al, 1983). This large transcription factor can recognise and bind simultaneously the two promoter elements, even though their separation can vary between different genes. Human TFIIIC has been shown to consist of five subunits, known as TFIIIC220, 110, 102, 90 and 63, with a total mass approaching 600 kD (Kovelman & Roeder, 1992; Yoshinaga et al, 1989). The TFIIIC220 and 110 subunits interact with the promoter DNA, with 220 specifically binding to the B-block (Shen et al, 1996; Yoshinaga et al, 1987; Yoshinaga et al, 1989). TFIIIC63 is also thought to contribute to the binding of TFIIIC to the DNA, by interactions with the A-block (Hsieh et al, 1999b). TFIIIC90 binds to TFIIIC220, 110 and 63 subunits and appears to bridge the two subdomains (220/110 and 102/63) and extend over the entire gene (Hsieh et al, 1999a). Interestingly, two or three (110, 90 and probably 220) subunits of the TFIIIC complex exhibit histone acetyltransferase (HAT) activity, which might have an important role in the chromatin remodelling of Pol III genes, before the formation of a functional PIC (Hsieh et al, 1999a; Kundu et al, 1999).

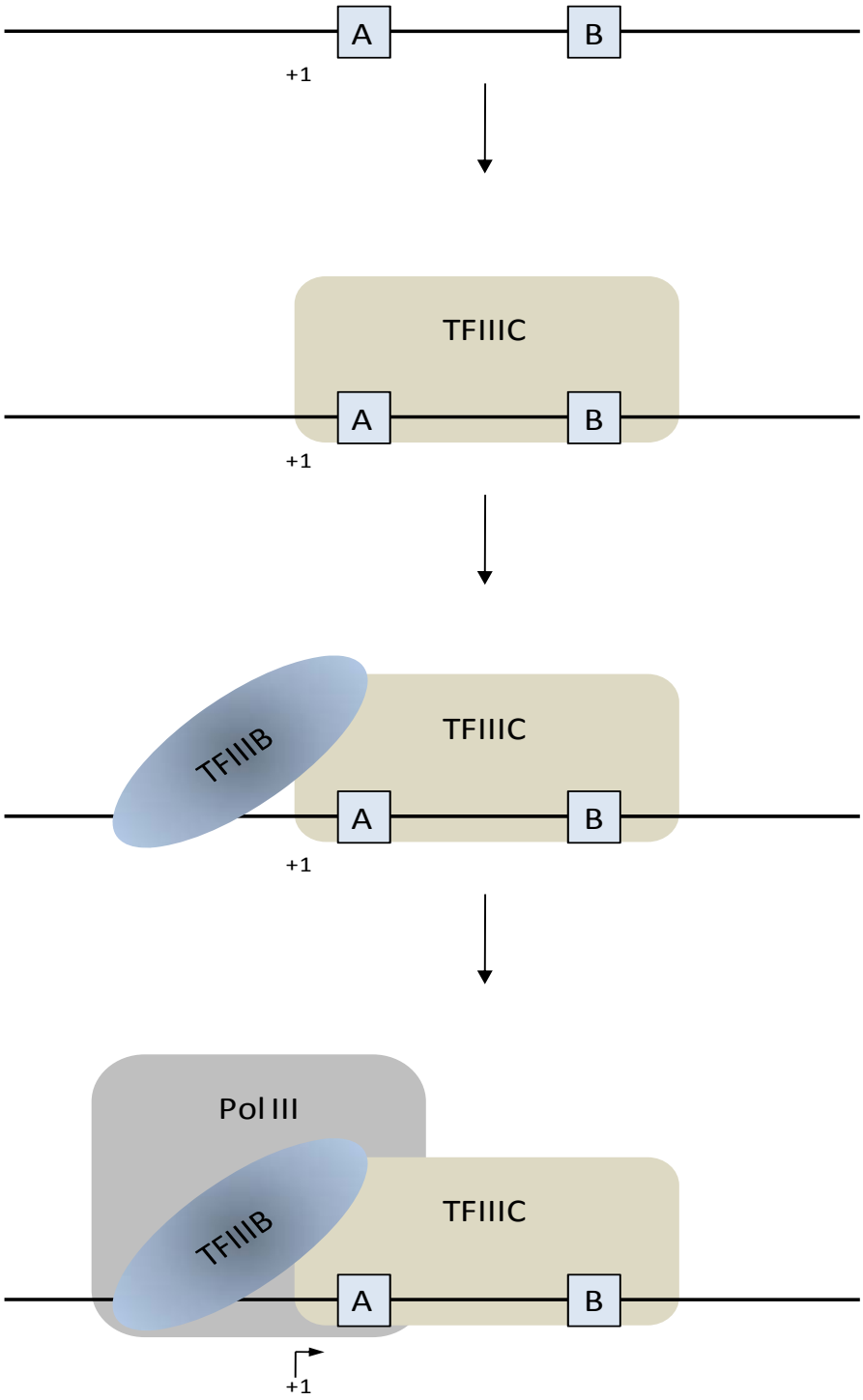


Figure 1.2. Transcriptional complex assembly on a type 2 promoter. TFIIIC binds to A- and B-blocks on DNA and recruits TFIIIB via protein-protein interactions. Once bound, TFIIIB recruits Pol III and transcription is initiated. The transcription site is indicated by +1.

The main role of TFIIIC is to recruit transcription factor IIIB (TFIIIB) onto the Pol III promoters. TFIIIB consists of three subunits: TBP (TATA-binding protein), Brf1 (TFIIB-related factor) and Bdp1 (B double prime 1). TBP and Brf1 strongly interact with each other (Khoo et al, 1994; Wang & Roeder, 1995), while Bdp1 binds weakly with the complex (Kassavetis et al, 1995). The recruitment of TFIIIB by TFIIIC is achieved by protein-protein interaction between the subunits of the two factors. More specifically, the TFIIIC102 subunit is thought to initially contact Brf1 and several subsequent interactions between the other subunits contribute to the formation of a stable complex (Schramm & Hernandez, 2002). These interactions include association of TFIIIC90 with Brf1, and of TFIIIC102 and 63 with both Brf1 and TBP (Hsieh et al, 1999a; Hsieh et al, 1999b).

Once TFIIIB has formed a stable complex with TFIIIC, Pol III is recruited. TBP and Brf1 have been shown to interact with Pol III subunits and these interactions are considered to be important for Pol III recruitment (Brun et al, 1997; Wang & Roeder, 1997; Werner et al, 1993). Furthermore TFIIIC102, 90 and 63 also interact with Pol III subunits and, therefore, TFIIIC might not only recruit TFIIIB, but also facilitate the recruitment of the polymerase (Hsieh et al, 1999a; Hsieh et al, 1999b), although TFIIIB is sufficient to recruit Pol III *in vitro* and initiate multiple rounds of transcription (Kassavetis et al, 1990).

1.3.2 Transcription complex assembly at type 1 promoters

Recruitment of TFIIIC onto 5S rRNA genes requires transcription factor IIIA (TFIIIA) (Figure 1.3). TFIIIA is a single polypeptide of approximately 40 kD and has nine zinc-finger domains. These domains bind to the A-block, the intermediate element and mainly the C-block of the type 1 promoters, with the latter contributing about 95% of the binding affinity of TFIIIA (Clemens et al, 1992; Nolte et al, 1998). As TFIIIC has little affinity for type 1 promoters, which lack a B-block, TFIIIA serves as an adaptor, allowing TFIIIC to be recruited onto 5S rRNA genes (Paule & White, 2000). It is not clear how TFIIIA recruits TFIIIC, but both factors must form a complex at the promoter prior to recruitment of TFIIIB, which in turn will recruit Pol III (Bieker et al, 1985; Carey et al, 1986).

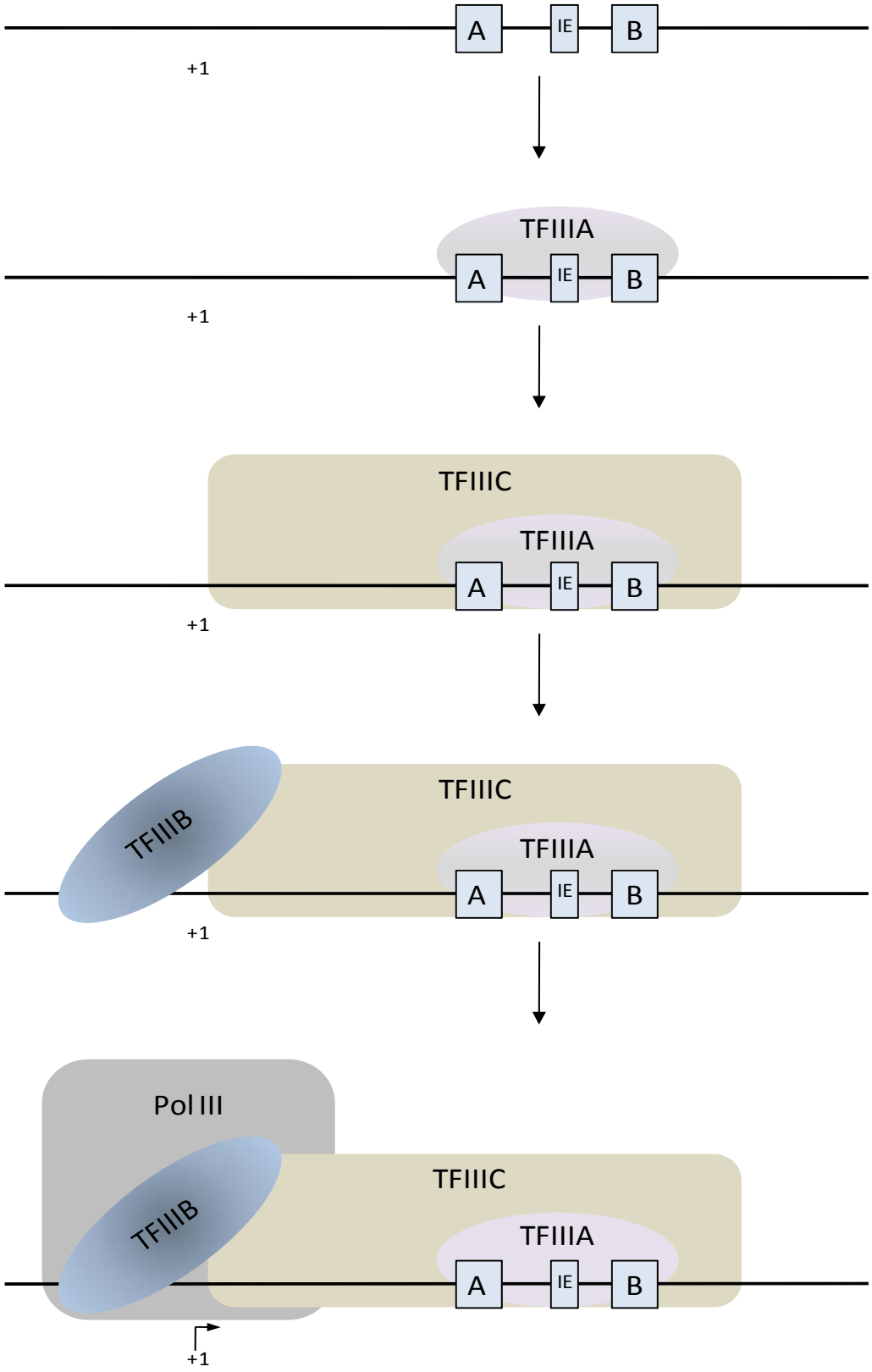


Figure 1.3. Transcriptional complex assembly on a type 1 promoter. TFIIIA binds to the promoter elements on DNA and recruits TFIIIC, which results in consequent recruitment of TFIIIB and TFIIIC and transcription initiation. The transcription site is indicated by +1.

1.3.3 Transcription complex assembly at type 3 promoters

In contrast to the intragenic type 1 and 2, type 3 promoters are gene-external and contain different promoter elements, namely the TATA box, PSE and DSE (Figure 1.1, C). Therefore, they do not require TFIIIC or TFIIIA, but employ a different set of transcription factors (Figure 1.4) (Schramm & Hernandez, 2002).

The PSE is recognised by $SNAP_c$ (snRNA activator protein complex), a five subunit transcription factor (Schramm & Hernandez, 2002) that is involved in the transcription of snRNA genes by both Pol II and Pol III (Henry et al, 1998). The TATA box is recognised by TBP, which is a subunit of TFIIIB. In the case of type 3 promoters, Brf1 is substituted in the TFIIIB complex by the related factor Brf2 (Schramm et al, 2000), which is specifically required for this type of promoter and contributes to TBP-promoter binding (Ma & Hernandez, 2002). Both $SNAP_c$ and TBP can bind weakly to DNA on their own, but cooperative binding interactions between them result in their efficient recruitment to the DNA (Mittal & Hernandez, 1997). The interaction of $SNAP_c$ and TFIIIB with the promoter DNA is further enhanced by Oct-1, a transcription factor that binds to the DSE and stabilises $SNAP_c$ and TFIIIB by protein-protein interactions (Mittal et al, 1996; Murphy et al, 1992). However, although Oct-1 contributes to a stable $SNAP_c$-TFIIIB complex on the promoter, it is not essential for basal transcription (Hu et al, 2003). Assembly of the $SNAP_c$-TFIIIB complex at type III promoters facilitates Pol III recruitment and, subsequently, transcription initiation.

1.4 RNA polymerase III

As discussed previously, TFIIIA, TFIIIC and $SNAP_c$ are considered recruitment factors whose main role is to recruit TFIIIB to Pol III promoters, which will then allow the recruitment of Pol III (Schramm & Hernandez, 2002).

Pol III consists of 17 subunits in yeast and human and 16 of them have been shown to be essential in yeast (Geiduschek & Kassavetis, 2001). Of the 17 subunits, five are common to Pol I, II and III, two common to Pol I and III and ten unique to Pol III (Schramm & Hernandez, 2002). It is thought that the Pol III-specific subunits contribute to

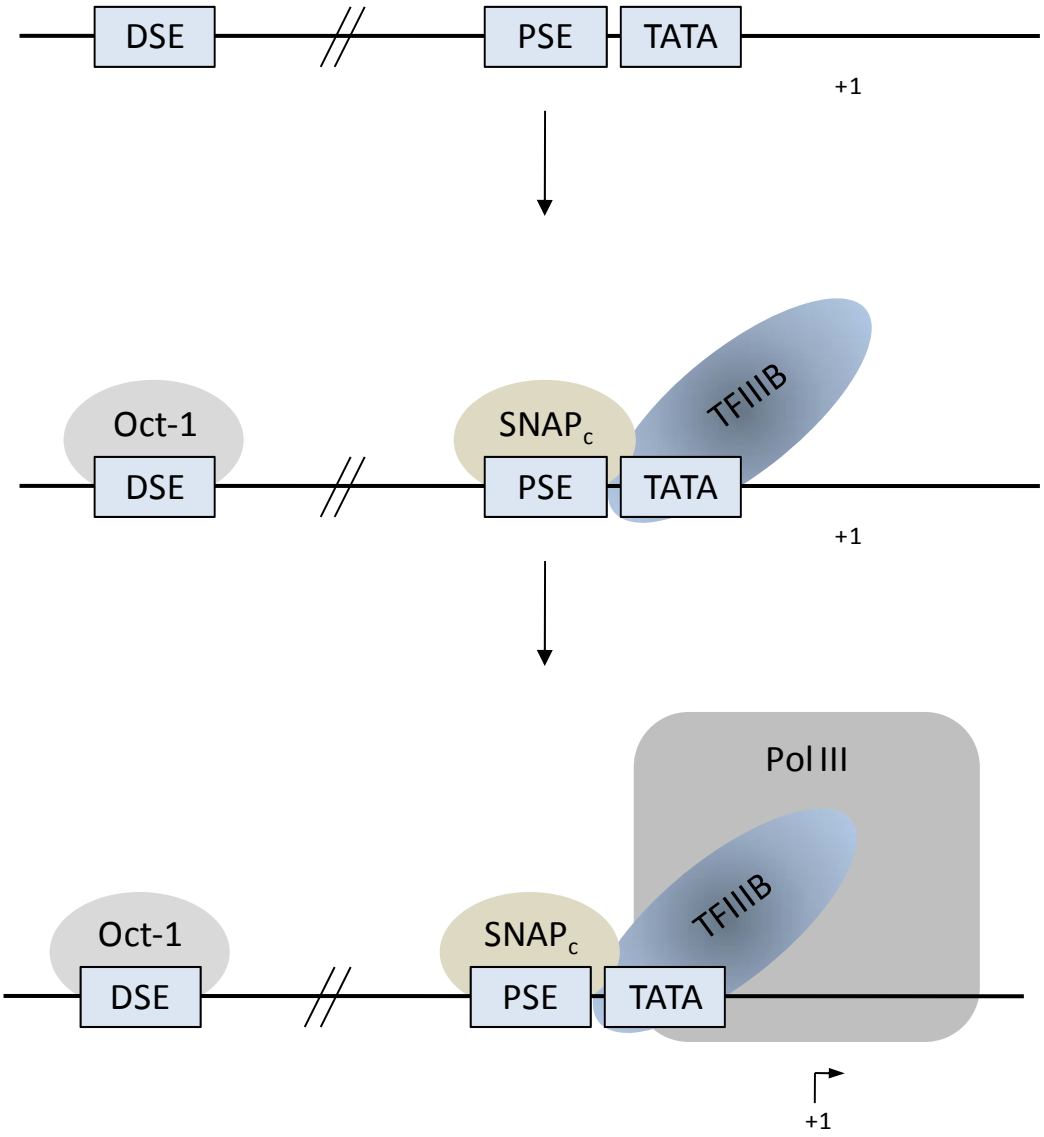


Figure 1.4. Transcriptional complex assembly on a type 3 promoter. TFIIIB and SNAP$_C$ bind cooperatively to the TATA box and PSE respectively, and recruit Pol III to initiate transcription. Binding of Oct-1 to the DSE enhances the recruitment of SNAP$_C$/TFIIIB. The transcription site is indicated by +1.

the Pol III-specific properties, like its nuclear localisation, the interactions with the promoter factors and RNA-related enzymes and its elongation and termination properties (Geiduschek & Kassavetis, 2001).

Experiments regarding the formation of the preinitiation complex at Pol III promoters have been executed *in vitro*, allowing for the possibility that *in vivo* many of the factors that mediate Pol III promoter recognition and Pol III itself are recruited to the promoter as a holoenzyme. Indeed, it has been reported that subunits of TFIIIB and TFIIIC can be immunoprecipitated with Pol III as an active holoenzyme in human cells and that about 10% of the Pol III molecules in the immunoprecipitate were associated with TFIIIB and/or TFIIIC components (Wang et al, 1997b). A similar approach in yeast also showed association of Pol III with TFIIIB and TFIIIC subunits, but in this case the holoenzyme was not functional and required supplementation with all three TFIIIB subunits for transcriptional activity (Chedin et al, 1998). Although the presence of functional Pol III holoenzymes *in vivo*, at least in yeast, is not clear, it is believed that it might be a valid possibility (Geiduschek & Kassavetis, 2001).

1.5 Transcription initiation, elongation and termination by RNA polymerase III

Pol III recruitment onto the promoter DNA results in the formation of a complete preinitiation complex. Following that, the DNA strands are separated without requiring ATP hydrolysis and a transcription bubble, is formed (Kassavetis et al, 1992). The TFIIIB subunits Brf1 and Bdp1 have an important role in promoter opening; impairment of specific domains on them can result in failure to form the transcription bubble and initiate transcription, although Pol III is properly recruited (Kassavetis et al, 1998; Kassavetis et al, 1999).

Once the DNA strands have been separated, RNA synthesis can be initiated. Pol III dissociates from promoter-bound TFIIIB and progresses along the DNA. It is unclear how Pol III manages to read through the DNA when factors like TFIIIC and TFIIIA (in the case of 5S rDNA) are stably bound to it and the assembled transcription complexes are not removed even after multiple rounds of transcription (Bogenhagen et al, 1982; Wolffe et al,

1986). However, one possibility might be that the factor is transiently displaced by Pol III during transcription, but remains associated with TFIIIB, which interacts with DNA upstream of the start site, due to protein–protein contacts.

In contrast to other polymerases, Pol III has the ability to recognise transcription termination sites independently of other factors (Cozzarelli et al, 1983); clusters of four or more T residues are sufficient to signal the accurate and efficient termination of transcription (Paule & White, 2000). Mutations or deletion of these termination signals resulted in the production of run-off transcripts, but also diminished the efficiency of single- and multiple-round transcription, suggesting that the termination signal might contribute to the efficiency of initiation and re-initiation (Schramm & Hernandez, 2002; Wang et al, 2000; Wang & Roeder, 1996).

After termination, Pol III can either disassociate or undergo a termination-coupled, re-initiation event and go through a new round of transcription, much more rapidly than the initial one (Dieci & Sentenac, 1996). This is because Pol III can re-initiate transcription on the same template, without being released from it and, thus, avoid the slow step of polymerase recruitment (Dieci & Sentenac, 1996). It is thought that bending of the DNA by TFIIIA, TFIIIB and TFIIIC may facilitate re-initiation by bringing the two ends of Pol III-transcribed genes into close proximity (Paule & White, 2000). Indeed, it has been shown that TFIIIB and TFIIIC are involved in the efficient polymerase recapture and transcription re-initiation (Ferrari & Dieci, 2008; Ferrari et al, 2004).

The La autoantigen and NF1 polypeptides have been implicated in the efficient termination and re-initiation of Pol III transcription in human cells. La binds to nascent Pol III transcripts and promotes their processing to mature forms (Maraia, 2001), but is also involved in transcription; addition of purified La to immobilised DNA templates increased the release of RNA and the overall level of transcription (Maraia et al, 1994), while presence of La resulted in more efficient transcription re-initiation (Maraia, 1996). NF1 polypeptides were implicated in transcription termination due to their binding sites downstream of VAI terminators and were reported to increase VAI transcription in crude extracts, but not in a purified system, suggesting that NF1 might counteract negative factors not present in the latter (Wang et al, 2000). However, although a recent *in vivo* study showed La to associate with several Pol III-transcribed genes, it failed to detect NF1 at Pol III templates (Fairley et al, 2005).

1.6 RNA polymerase III transcription regulation

By being responsible for the transcription of 5S rRNA and tRNAs, Pol III is directly associated with translation and protein synthesis. Protein synthesis is essential for cell growth, since growth is defined as increase in cell mass and 80-90% of the dry mass of a cell is protein (Zetterberg & Killander, 1965). Therefore, Pol III transcription is tightly associated with cell growth. Because adequate cell growth is a requirement for cell cycle progression and proliferation (Neufeld & Edgar, 1998), one would expect that conditions that deregulate cell growth would also affect transcription by Pol III.

Indeed, a wide variety of transformed cell types, including lines transformed by DNA tumour viruses, RNA tumour viruses, or chemical carcinogens, have been found to express abnormally high levels of Pol III products (White, 2004b). Furthermore, conclusions inferred from studies in cell culture have been validated in tumour cells. For example, 7SL was found to be elevated in each of 80 tumour samples tested, representing 19 different types of cancer, when compared to healthy tissue (Chen et al, 1997a). Moreover, 5S rRNA and tRNA, as well as 7SL, were consistently overproduced in ovarian cancers (Winter et al, 2000), while breast, tongue and lung carcinomas also revealed increased levels of Pol III transcripts in cancer cells (Chen et al, 1997b). These experiments indicate that the deregulation of growth and proliferation in cancer cells is associated with deregulation of Pol III transcription.

One way to increase Pol III output would be to raise the levels of transcription factors which are required for Pol III transcription and are found at limiting concentrations. To this end, it has been shown that adenoviral infection (Hoeffler et al, 1988) and simian virus 40 (SV40) (White et al, 1990), polyomavirus (Felton-Edkins & White, 2002) or EBV transformation (Felton-Edkins et al, 2006) can result in TFIIIC overexpression. Furthermore, TFIIIC was also overexpressed in ovarian carcinomas, confirming that upregulation of TFIIC can be observed in tumours and suggesting clinical significance (Winter et al, 2000). Subunits of TFIIIB can also be found at elevated levels in transformed cells. TBP can be induced after activation of Ras signalling by hepatitis B virus (Wang et al, 1997a) and is also elevated in colon carcinomas (Johnson et al, 2003). Bdp1 was also found to be increased in cell lines transformed by EBV, SV40 or polyoma virus (Felton-Edkins et al, 2006; Felton-Edkins & White, 2002), as well as in some cervical tumours (Daly et al, 2005), while Brf1 mRNA was found to be induced in biopsies positive for the

oncogenic HPV16 strain, but not for the lower risk strains or HPV-negative biopsies (Daly et al, 2005).

Pol III transcription has also been found to be upregulated by direct activation of TFIIIB. The Tax oncoprotein of the human T cell leukaemia virus-1 (HTLV-1) was shown to activate Pol III transcription by targeting TFIIIB and accelerating the rate and/or extent of transcription initiation complex assembly (Gottesfeld et al, 1996). Moreover, c-Myc, one of the most frequently activated oncogenes that is estimated to be involved in 20% of all human cancers (Dang et al, 2006), interacts with TFIIIB and potently upregulates Pol III transcription (Gomez-Roman et al, 2003). c-Myc can be found at 5S rRNA and tRNA genes, and its depletion by RNA interference or genetic knock-out results in decreased tRNA expression (Felton-Edkins et al, 2003b; Gomez-Roman et al, 2003).

Kinases have also been found to bind to TFIIIB and activate transcription by Pol III. CK2 can directly interact with and phosphorylate Brf1, resulting in the upregulation of Pol III transcription by facilitating recruitment of TFIIIB to TFIIIC (Johnston et al, 2002) and it has been shown to be oncogenic in transgenic mice (Seldin & Leder, 1995) and hyperactive in some human cancers (Faust et al, 1996; Munstermann et al, 1990; Notterman et al, 2001). The MAP (mitogen-activated protein) kinase Erk (extra-cellular signal regulated kinase) can also activate Pol III transcription by binding and phosphorylating Brf1 and stimulating the assembly of Pol III transcription complexes (Felton-Edkins et al, 2003a). Erk, which is downstream of the Ras-Raf-Mek signalling pathway, is found to be abnormally elevated in about 30% of cancers, most frequently because of mutational activation of the upstream Ras (Downward, 2003). It must be noted, however, that phosphorylation of Brf1 (TFIIIB) does not always result in upregulation of Pol III transcription. Cyclin-dependent kinases (Cdks) from mitotic frog extracts inhibit expression of Pol III templates (Gottesfeld et al, 1994; Hartl et al, 1993), with cdc2–cyclin B kinase being sufficient to repress expression of a *Xenopus* 5S rRNA gene (Gottesfeld et al, 1994). Work in human cells showed that hyperphosphorylation of Brf1 at mitosis, with kinase(s) other than cdc2–cyclin B, can result in compromised Pol III recruitment and gene expression, due to release of Bdp1 from chromosomal templates (Fairley et al, 2003).

A third way to stimulate Pol III transcription is by derepression of TFIIIB. TFIIIB has been shown to be bound by tumour supressors and transcription repressors that bind to TFIIIB and inhibit the formation of a functional preinitiation complex, resulting in repression of Pol III transcription (White, 2004a). The tumour suppressors p53 (Cairns & White, 1998; Crighton et al, 2003) and Rb (Retinoblastoma) (Larminie et al, 1997; White

et al, 1996), as well as the Rb-family proteins p107 and p130 (Sutcliffe et al, 1999), have been shown *in vitro* and *in vivo* to repress Pol III transcription in mouse cells. The tumour suppressor ARF also has the ability to repress Pol III transcription in human cells (Jen Morton, personal communication). Furthermore, Maf1 can repress Pol III transcription in yeast and human cells (Johnson et al, 2007; Reina et al, 2006; Upadhya et al, 2002), while the general transcriptional repressor Dr1 has been shown to repress Pol III transcription in yeast (Kim et al, 1997) and in human cell extracts (White et al, 1994).

Since most of the work on the negative regulators of Pol III transcription has been done in yeast or mouse cells, the work in this study focused on human cells. The regulation of Pol III transcription by Dr1 and its interacting protein DRAP1, in human cells, is investigated in chapters 3 and 4 respectively, while the effects of different stress conditions on their regulation, as well as on Pol III transcription are examined in chapter 5. The effect of RNAi knock-down of Rb, p107 and p130 on Pol III transcription in human cells is explored in chapter 6, while similar experiments are reported in chapter 7, regarding the role of p53 in the repression of Pol III transcription, by itself or in response to ARF.

CHAPTER 2

Materials and Methods

2.1 Cell culture

Cell culture was performed in a class II hood, using aseptic techniques and sterile equipment and reagents. All plasticware used was supplied by Corning.

2.1.1 Cell lines and maintenance

HeLa, U2OS and A31 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). NARF2 cells were maintained as the above mentioned cells lines, but with the addition of 150 µg/ml hygromycin (Duchefa Biochemie) and 300 µg/ml G418 sulfate (Sigma). The E6-expressing NARF2-E6 cells were cultured the same way as NARF2, but with puromycin (Sigma) at a concentration of 1.5 µg/ml. IMR90 cells were maintained in McCoy's medium (Cambrex) supplemented with 20% FBS (Sigma), 2 mM L-Glutamine (Sigma), 50 U/ml penicillin and 100 U/ml streptomycin. The CHO-Brf1 cell line was maintained in αMEM (Cambrex), supplemented with 10% doxycycline-free FCS (Clontech), 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), 100 µg/ml G418 sulfate, 100 µg/ml hygromycin (Duchefa Biochemie) and 2 µg/ml doxycycline (Melford Laboratories).

Cells were maintained at 37 °C in humified atmosphere containing 5% CO_2 and were passaged when 80-90% confluent. For cell passaging, the media were 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 75 cm^2 flask or adjusted properly) and then aspirated immediately. A further 2 ml were added and left for approximately 2 minutes at 37 °C. Following trypsinisation, fresh medium was immediately added to the dissociated cells in order to neutralise the trypsin and cell suspensions were appropriately transferred to new flasks.

2.1.2 Cryo-freezing and recovery

Cryo-freezing was used for storage of all cell lines. Cells were trypsinised as described, pelleted by centrifugation at 1000 *g* for 5 min, and resuspended in 80% DMEM, 10% FBS and 10% dimethyl sulphoxide (DMSO, Sigma). Cells from a sub-confluent 75 cm^2 flask were aliquotted into 2 ml in cryo-tubes and frozen in two steps, initially by being placed at -80 ^oC overnight and subsequently by being transferred to liquid nitrogen storage. For cell recovery after liquid nitrogen storage, the content of the cryo-tubes was rapidly thawed at 37 ^oC and mixed with fresh medium. The cells were pelleted by centrifugation and the supernatant was aspirated off to ensure removal of DMSO, prior to resuspension in maintenance medium in 25 cm^2 flasks.

2.1.3 Protein induction in inducible cell lines

Induction of ARF in NARF2 cells was achieved by adding 1 mM IPTG in the medium and harvesting the cells 24 hours later, as previously described (Stott et al, 1998).

Brf1 induction in the CHO-Brf1 cell line was achieved by washing the cells twice with pre-warmed PBS and re-culture with doxycycline-free medium for 48 hours (Marshall et al, 2008).

2.1.4 Application of stress conditions and chemical treatments of the cell lines

HeLa and U2OS cells were subjected to hypoxic stress by incubation for 24 hours in a hypoxic incubator (Wolf Laboratories, UK) at 37 ^oC, with a gas mixture containing 5% CO_2 and 1% O_2, balanced with nitrogen. Cells under normoxic conditions were treated the same way, but using a gas mix of 5% CO_2 and 20% O_2.

HeLa and U2OS cells were subjected to heat shock by incubating at 45 ^oC for 30 minutes, as described previously (Liu et al, 1995), but in a cell culture incubator. Cells were then either harvested immediately (0h) or left to recover at 37 ^oC for 2, 4 or 8 hours before harvesting. Control cells were not subjected to heat shock.

HeLa and U2OS cells were subjected to chemically-induced anoxia by addition of the iron chelator deferoxamine mesylate salt (DFX, Sigma). Cells were treated with 150 µM DFX for 24 hours before harvesting. Control cells were cultured the same way, but with the addition of an equal volume of H_2O rather than DFX.

HeLa cells were treated with the mTOR inhibitor rapamycin (Calbiochem) for 4 hours at a concentration of 100 nM, before harvesting. Control cells were treated with the equal volume of DMSO.

Serum starvation experiments took place by washing A31 cells twice with PBS, before subjecting them in DMEM supplemented with 0.5% FBS (Sigma), 2 mM L-Glutamine (Sigma), 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma), for 24 hours. Control cells were treated equally, but maintained in 10% FBS.

2.2 Preparation of whole cell extracts

Cells were cultured in either 10 cm culture dishes or 6-well plates to about 80% confluency before harvesting. Preparation was performed on ice, as rapidly as possible, and all solutions and plasticware were kept ice-cold to maintain cell activity. The maintenance medium was aspirated and the cells were washed twice with ice-cold PBS. They were then scraped into cell lysis buffer (20 mM HEPES (pH 7.8), 150 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, 0.5 µg/ml leupeptin, 1.0 µg/ml trypsin inhibitor, 0.5 µg/ml aprotinin and 40 µg/ml bestatin) and transferred to sterile microfuge tubes. 100 or 500 µl of buffer was used per well or per 10 cm plate, respectively. The cell lysates were then passed through a 26G needle five times and centrifuged at 16000 *g* for 10 minutes at 4 ºC. The supernatants were aliquoted and snap-frozen on dry ice, before being stored at -80 ºC.

2.3 Determination of protein concentrations

The protein concentrations of whole cell extracts were determined using Bradford's reagent (BioRad) diluted 1 in 5 with distilled H_2O. For each experiment, a standard curve was constructed by measuring the absorbance at 595 nm of 1, 2, 4, 6, 8, 10 and 12 μg of BSA in 1ml of Bradford's reagent. The absorbance readings of the whole cell extracts were performed in triplicate at 595 nm, and the protein concentration of each sample was determined from the standard curve.

2.4 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cell extracts containing 50 μg of protein per lane were resolved by denaturing SDS-PAGE typically on 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). Prior to loading, samples were boiled for 2 minutes in 1x protein sample buffer (62.5 mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue). Electrophoresis was performed in 1x SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris pH 8.3) at 200 V, until the bromophenol dye had moved to the bottom of the gel.

2.5 Western blot analysis

After separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes (BioRad) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system (BioRad). The transfer was carried out in 1x transfer buffer (76.8 mM glycine, 10mM Tris pH 8.3, 20% methanol) at 100 V for an hour at room temperature or at 50 V, overnight at 4 ºC and the proteins immobilised on the nitrocellulose were then visualised by staining with 1x Ponceau S solution (Sigma) to ensure their efficient transfer. Subsequently, the membranes were washed with PBS and then blocked in milk buffer

(32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20, 5% skimmed milk powder (Marvel)) for 1 hour at room temperature. The membranes were then incubated in the presence of the appropriate primary antibody diluted in milk buffer for 2 hours at room temperature or at 4 ºC overnight. The primary antibodies used and their appropriate concentrations are listed in table 2.1. After the primary antibody incubation, the membranes were washed three times for 5 minutes each in PBS containing 0.5% Tween-20, in order to remove excess primary antibody. Following the washes, the membranes were incubated in the presence of the appropriate secondary antibody (Dako) at a dilution of 1:1000 in milk buffer. The excess of secondary antibodies on the membranes was washed off by sequential washes with western wash buffer (32.5 mM Tris, 150 mM NaCl, 0.2% Tween-20); three washes of 5 minutes each, followed by two washes of 15 minutes and a final wash of 5 minutes with 1x TBS (2.5mM Tris-HCl pH 7.6, 15mM NaCl). The bound antibodies were then detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham). The obtained signals were quantified by densitometry (ImageJ v1.37), normalised to the respective actin signals and represented in graphs as the average fold increase or decrease, along with the standard deviations.

2.6 Co-immunoprecipitation

25 µl of packed protein G sepharose beads (Sigma) were used per immunoprecipitation. The beads were washed three times with 1 ml 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 phenylmethylsulfonyl fluoride (PMSF), 0.2 mM EDTA, 40 µg/ml bestatin, 1 µg/ml trypsin inhibitor, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin) prior to incubation with 5 µl anti-Dr1 (1162) and the respective pre-immune sera or anti-Brf1 (128) antibodies and rabbit IgGs (sc-2027, Santa Cruz Biotechnology); the total volume was made up to 50 µl with LS-MEB and the antibodies were incubated with the beads rotating for 2 hours at 4 ºC. Following that, the beads were washed three times with 1x LS-MEB to remove excess antibody. 300 µg of HeLa protein extract (Computer Cell Culture Belgium) were added to the beads and incubated rotating for 1 hour at 4 ºC. The beads were then washed five times with 1x TBS,

Table 2.1. Primary antibodies used for western blot analysis

Protein	Antibody	Dilution	Source
Dr1	1162	1:1000	In house
Dr1	1163	1:1000	In house
DRAP1	V-18	1:1000	Santa Cruz Biotechnology
DRAP1	D9390-01	1:1000	US Biological
TBP	58C9	1:1000	Santa Cruz Biotechnology
TBP	MTBP-6	1:200	In house
Brf1	128	1:1000	In house
Bdp1	2663	1:1000	In house
TFIIIC90	1898	1:1000	In house
TFIIIC110	3208	1:1000	In house
TFIIIC220	Ab7	1:1000	In house
Pol III (155)	1900	1:1000	In house
Actin	C11	1:5000	Santa Cruz Biotechnology
TFIIB	C18	1:1000	Santa Cruz Biotechnology
HIF1α	NB100-105	1:500	Novus Biologicals
p-S6K	9202	1:1000	Cell signalling technologies
Oct-1	12F11	1:1000	Santa Cruz Biotechnology
p53	554293	1:1000	BD Pharmingen
Hsp70	4E7	1:10000	Santa Cruz Biotechnology
Rb	G3-245	1:1000	BD Pharmingen
p107	SD9	1:1000	Santa Cruz Biotechnology
p130	C-20	1:1000	Santa Cruz Biotechnology
ARF	FL-132	1:200	Santa Cruz Biotechnology

before adding 50 µl of 2x protein sample buffer. The samples were then boiled for two minutes and either stored at -20 ºC for future use or directly subjected to western blotting.

For co-immunoprecipitation experiments using radiolabelled proteins, the *in vitro* synthesised protein and HeLa nuclear extract were pre-cleared to minimise non-specific interactions between the proteins and the beads. For this, 40 µl of the labelled protein together with 400 µg HeLa nuclear extract and 50 µl packed protein A sepharose beads (Sigma) made up to a final volume of 400 µl with LS-MEB, were incubated rotating for 1 hour at 4 ºC. 100 µl of the pre-cleared supernatant were then aliquoted to new tubes, 5 µl of Dr1 (1162) antibody or the 1162 pre-immune control were added and the volume was made up to 500 µl with LS-MEB. The incubation with the antibodies took place for 2 hours rotating at 4 ºC and then 25 µl of protein A sepharose beads were added and the incubation continued for another hour. Finally, the beads were washed five times with TBS, before being mixed with the appropriate volume of loading dye, boiled for 10 min and run in SDS-PAGE. The gel was then incubated with fixing solution (10% (v/v) acetic acid, 30% (v/v) methanol) for 30 minutes at room temperature and washed with dH_2O for 15 min. Subsequently, it was incubated with Autofluor (National Diagnostics) for 2 hours at room temperature and then vacuum dried for 2 hours at 80 ºC. The co-immunoprecipitated proteins were visualised by autoradiography.

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

2.7.1 RNA Extraction

Total cellular RNA was extracted from cells by using the TRI reagent (Sigma). The maintenance medium was aspirated and the cells were washed three times with ice-cold PBS. 1 ml or 500 µl of TRI were used per 10 cm dish or per well of a six-well plate, respectively, to harvest the cells. The samples were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes, before the addition of 0.2 ml (per ml of TRI used) of chloroform to each. The samples were then thoroughly mixed by vortexing for 15 seconds and were left incubating for 5 minutes at room temperature, before being centrifuged at 16000 *g* for 15 minutes at 4 ºC. This resulted in the separation of the samples into three phases: a lower red organic phase

containing protein, a middle white interphase containing DNA and an upper colourless aqueous phase containing RNA. The aqueous phase was carefully transferred into new tubes and 0.5 ml of isopropanol was added to precipitate the RNA. The samples were mixed by repeated inverting, incubated for 10 minutes at room temperature and centrifuged at 16000 *g* for 10 minutes at 4 ºC. The resulting RNA pellet was washed with 1 ml of 75% ethanol, vortexed briefly and centrifuged at 8000 *g* for 5 minutes at 4 ºC. Subsequently, the supernatant was aspirated off, and the RNA pellets were left to air dry for approximately 10 minutes before being resuspended in 20-50 µl of RNase-free water. Samples were heated to 50 °C to facilitate resuspension. Following resuspension, the RNA concentration was calculated by spectrophotometry at 260 nm, considering that OD of 1 at 260 nm corresponds to 40 µg/ml of RNA and following the formula: RNA concentration (µg/ml) = absorbance at 260 nm x 40 x dilution factor. All RNA samples were stored at -80 ºC.

2.7.2 cDNA production

1 µg of RNA was added to 2 µl of 1x hexanucleotide mix (Roche) and made up, with RNase-free water, to a final volume of 25 µl. Primer annealing was carried out at 80 °C for 10 minutes before transferring to ice. 8 µl of 5x First Strand Buffer (Invitrogen Life Technologies), 4 µl of 0.1 M dithiothreitol (DTT) (Invitrogen Life Technologies), 2 µl of 10 mM dNTP mix (Promega) and 1 µl (200U) of Superscript II Reverse Transcriptase (Invitrogen Life Technologies) were added to initiate reverse transcription. The reaction took place for 1 hour at 42 °C before being terminated by heating at 70 °C for 15 minutes. The cDNA was stored at -20 ºC.

2.7.3 Polymerase Chain Reaction (PCR)

Each PCR reaction had a total volume of 20 µl and contained 1 µl of cDNA, 20 pmol of the appropriate primers, 0.5 U of *Taq* DNA polymerase (Promega), 1x *Taq* DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each non-radioactive dNTP, and 1.8 µCi of [α-^{32}P] dCTP (Amersham). The cycles used for the PCR amplification of the cDNA ranged from 18-30 depending on the amplified target molecule. The sequences of the primers used and the cycling parameters are listed in table 2.2. The PCR products were

Table 2.2. RT-PCR primers and cycling parameters

Transcript	Primers 5'-3': Forward Reverse	PCR Conditions (denaturing; cycling; final elongation)
5S rRNA	GGCCATACCACCCTGAACGC CAGCACCCGGTATTCCCAGG	95ºC-3min; 95ºC-30s; 58ºC-30s; 72ºC-1min; 72ºC-5min
ARPPP0	GCACTGGAAGTCCAACTACTTC TGAGGTCCTCCTTGGTGAACAC	95ºC-2min; 95ºC-1min; 58ºC-30s; 72ºC-1min; 72ºC-5min
$tRNA^{LEU}$	GAGGACAACGGGGACAGTAA TCCACCAGAAAAACTCCAGC	95ºC-3min; 95ºC-30s; 68ºC-30s; 72ºC-30s; 72ºC-5min
$tRNA^{TYR}$	AGGACTTGGCTTCCTCCATT GACCTAAGGATGTCCGCAAA	95°C-3min; 95°C-1min; 65°C-30s; 72°C-15s; 72°C-5min
U6 snRNA	GCTCGCTTCGGCAGCACATATAC TATCGAACGCTTCACGAATTTGCG	95°C-3min; 95°C-1min; 60°C-30s; 72°C-1min; 72°C-5min
7SK	CGATCTGGTTGCGACATCTG CGTTCTCCTACAAATGGAC	95°C-3min; 95°C-30s; 57°C-30s; 72°C-30s; 72°C-10min
Brf1	CACCCAGAATGCATGACTTCCG AAATTCTGTGAGCCTCTTCCGTAGCG	95°C-3min; 95°C-1min; 61°C-30s; 72°C-1min; 72°C-5min
TBP	GCTGCAGCCG TTCAGCAGTC GCGGTACAATCCCAGAACTC	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min
Bdp1	GCTGATAGAGATACTCCTC CCAGAGACAAGAATCTTCTC	95°C-3min; 95°C-1min; 56°C-1min; 72°C-1min; 72°C-5min
TFIIIC90	AAACAGAAGTTGCTGAGTGC ATGGTCAGGCGATTGTCC	95°C-3min; 95°C-1min; 55°C-30s; 72°C-1min; 72°C-10min
TFIIIC110	CCAGAAGGGGTCTCAAAAGTCC CTTTCTTCAGAGATGTCAAAGG	95°C-3min; 95°C-1min; 62°C-30s; 72°C-30s; 72°C-10min
TFIIIC220	TCCAGCGAGACCTTCACACC GGATTGAGTGTTGCTGGGCT	95°C-3min; 95°C-30s; 62°C-30s; 72°C-30s; 72°C-10min
RPC155 (Pol III)	GCACAGAGCATTGGTGAG CGTCATCATCCTTGTCTAG	95°C-3min; 95°C-30s; 60°C-30s; 72°C-30s; 72°C-5min
TFIIB	GCAGACAGAATCAATCTAC CAGTTGTAATCAAATCCACAC	95°C-3min; 95°C-30s; 55°C-30s; 72°C-30s; 72°C-5min
Dr1	AGAGCTGGTGGTGAACTGCT CCAAGGTTTTCCAAACGAGA	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min
DRAP1	GGAACGAAAAGCAAGGACAA CGTCCTCTTCATCAGGTGCT	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min

Hsp70	CCGAGCATTCTCTGAATCCAT CACTTTCGGCTGTCTCCTTC	95°C-3min; 95°C-1min; 62°C-30s; 72°C-1min; 72°C-5min
p53	GTTCCGAGAGCTGAATGAGG TCTGAGTCAGGCCCTTCTGT	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min
p21	CTAGGCGGTTGAATGAGAGG CAGGTCTGAGTGTCCAGGAA	95°C-3min; 95°C-30s; 60°C-30s; 72°C-30s; 72°C-5min
Rb	GGAAGCAACCCTCCTAAACC TTTCTGCTTTTGCATTCGTG	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min
p107	AGAATGCCTCCTGGACCTTT GGGGTGTCACGAGTGAACTT	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min
p130	ATTTGGCATGGAAACCAGAG GTCACCCTTCTGGGAGTCAA	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min

diluted with equal volume of formamide loading buffer (98% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 5mM EDTA) and resolved on 7% polyacrylamide (National Diagnostics) sequencing gels containing 7% urea and 0.5x 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 0.5x TBE. The samples were heated at 95 ºC for 2 minutes and 1.5 µl of each sample was loaded per gel lane. Electrophoresis was carried out for 1 hour at 40 W and the gels were vacuum dried for 1 hour at 80 ºC, prior to being exposed to autoradiography film overnight at -80 ºC. The obtained signals were quantified by densitometry (ImageJ v1.37), normalised to the respective ARPP P0 or TFIIB signals and represented in graphs as the average fold increase or decrease, along with the standard deviations.

2.8 In vitro transcription and translation

Proteins were in vitro transcribed and translated using the TNT reticulocyte lysate kit (Promega), according to the manufacturer's instructions. The newly synthesised proteins were labelled with ^{35}S-methionine. After the reactions took place, 2 µl of the sample were analysed by SDS-PAGE. The gel was incubated with fixing solution (10% (v/v) acetic acid, 30% (v/v) methanol) for 30 minutes at room temperature and washed

with dH_2O for 15 minutes. Subsequently, it was incubated with Autofluor (National Diagnostics) for 2 hours at room temperature and then vacuum dried for 2 hours at 80 ºC. The radiolabelled proteins were visualised by autoradiography.

2.9 Propagation, preparation and analysis of plasmid DNA

2.9.1 Transformation of competent cells

E. coli XL-1 blue competent cells (Stratagene) were used for the bacterial transformations. The cells, stored at -80 °C, were thawed on ice prior to the addition of 20 ng of plasmid DNA per 50 µl of competent cells and gentle mixing. The mixture was then left to incubate on ice for 30 minutes. Following that, the cells were heat-shocked at 42 ºC for 45 seconds and immediately transferred to ice for a further 2 minutes. 500 µl of SOC medium (LB Broth, 0.05% glucose, 10 mM $MgSO_4$, 10 mM $MgCl_2$), pre-heated at 37 ºC, were then added to the cells, followed by a 1 hour incubation at 37 ºC on an orbital shaker (250 rpm). 150 µl of the transformation mixture were then plated onto LB-agar plates (LB-broth, 2% agar, 100 µg/ml ampicillin) and incubated at 37 ºC overnight to allow colony formation.

2.9.2 Preparation of plasmid DNA

A single bacterial colony was selected to inoculate 5 ml of LB (Luria-Bertani) medium containing 100 µg/ml ampicillin as the selection antibiotic. The incubation took place for about 6 hours at 37 °C on an orbital shaker (300 rpm). 500 µl of this culture were then used to inoculate a larger culture of 500 ml of LB, which was incubated overnight at 37 °C on an orbital shaker (300 rpm). Bacteria from the smaller or larger culture were then harvested by centrifugation and their plasmid DNA was isolated using the Qiagen Plasmid Mini or Maxi kit (Qiagen) respectively, according to the manufacturer's instructions. The plasmid DNA was retrieved in 30 µl (Mini) or 50 µl (Maxi) of sterile water and stored at -20 °C. The DNA concentration was determined by measuring absorbance at 260 nm,

according to the formula: DNA concentration (μg/ml) = absorbance at 260 nm x 50 x dilution factor.

2.9.3 Restriction endonuclease analysis

The restriction endonucleases *XhoI* and *EcoRI* (Promega) were used to digest the plasmid DNA isolated from the SUPER RNAi library (Cancer Research UK) and ensure the presence of the shRNA insert. The reactions were performed in a total volume of 30 μl, containing 1 μl of each restriction enzyme, 1 μg of plasmid DNA and 3 μl of the MULTI-CORE buffer (Promega). The DNA was digested for 1 hour at 37 ºC. Subsequently, the digest 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) and analysed by 1% (w/v) agarose gel electrophoreses. The electrophoreses were performed in 1x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) buffer containing 0.1 μg/ml ethidium bromide (Sigma) and visualised under UV.

The plasmids containing the correct size Dr1 shRNA insert were then sent for sequencing (Cogenics) to verify and identify the Dr1 target sequence, using the sequencing primer 5'-GCTGACGTCATCAACCCGCT-3'.

2.10 RNAi

2.10.1 shRNA

The shRNA employed to knock-down the expression of Dr1 gene was retrieved from a human whole genome RNAi library (SUPER RNAi, Cancer Research UK and http:/www.screeninc.nl) (Brummelkamp et al, 2002). The library contains mammalian expression vectors in *E. coli*, with an insert that encodes one to three hairpin transcripts, each of which is later, in the cells, processed into a 21 nucleotide siRNA, targeting the mRNA of a specific gene. The Dr1 vector employed encoded an insert with the sequence: 5'-GAAGAAAGGCCAGTTCTCG-3' targeting the mRNA of the second exon of the Dr1 gene. The p53 (insert sequence: 5'-GACUCCAGUGGUAAUCUAC-3') and DRAP1 (not

sequenced) targeting vectors were also used to knock-down p53 and DRAP1 expression respectively; an empty vector, containing no insert, was used as control. The plasmid containing the specific shRNA insert was isolated, identified by restriction enzyme digests and/or sequencing, propagated and transfected in HeLa cells.

2.10.2 siRNA

Commercially available siRNAs targeting the first (AM16704-44899, Ambion) and third (AM16704-114186, Ambion) exons of Dr1 were also used to knock-down Dr1 expression, while a validated, non-targeting siRNA (AM4390844, Ambion) was used as control. siRNAs targeting DRAP1 (sc-38091, Santa Cruz Biotechnology), Oct-1 (sc-36119, Santa Cruz Biotechnology), Rb (sc-29468, Santa Cruz Biotechnology), p107 (sc-29423, Santa Cruz Biotechnology) and p130 (sc-29425, Santa Cruz Biotechnology) were also purchased and employed to reduce the expression of their respective targets.

2.11 Transient transfections

2.11.1 Transient transfections using Nucleofector

The Nucleofector (Amaxa Biosystems) transfection system is an electroporation-based method. Cells were grown to about 80% confluency, trypsinised and harvested by centrifugation at 1000 *g* for 5 minutes. They were then washed twice with, pre-warmed at 37 ºC, 1x PBS and $1x10^6$ cells were resuspended to 100 µl of Nucleofector solution R per transfection reaction. 1 µg of plasmid DNA containing a shRNA insert or 100 nM siRNA were added to the above suspension and the cells were electroporated with the appropriate, depending on the cell line, programme using the Amaxa Nucleofector apparatus (Amaxa Biosystems). 500 µl of the culture medium were added immediately after the electroporation and the suspension was first transferred to 2 ml of medium and then to six-well plates containing 2.5 ml of growing medium. The medium was replaced with fresh the day after and the cells were left to grow for 48 hours, unless otherwise stated, before harvesting.

2.11.2 Transient transfections using Lipofectamine 2000

Transfections using Lipofectamine 2000 (Invitrogen) were performed according to the manufacturer's instructions. Briefly, the siRNA used per reaction was added to Optimem (Gibco) to a final volume of 100 µl while 2.5 µl Lipofectamine 2000 were added to 97.5 µl Optimem. The mixtures were left incubating for 10 minutes at room temperature and were then mixed together and left for an extra 10 minute incubation at room temperature. During this time, cells of about 70% confluency, grown on 6-well plates, were washed twice with pre-warmed Optimem and finally covered with 800 µl Optimem. 200 µl of the siRNA/Lipofectamine 2000/Optimem mixture were added per well. Fresh medium was added to the cells the next day and they were harvested 48 hours after transfection, unless otherwise stated.

2.12 Chromatin immunoprecipitation (ChIP) assay

Cells were grown in 10 cm plates to about 80% confluency and one 10cm dish of cells was used per IP. Formaldehyde, to a final concentration of 1%, was added to the cell maintenance medium to cross-link the protein-DNA complexes for 10 minutes at 37 °C. The cross-linking was stopped by the addition of glycine at a final concentration of 0.125 M and the plates were transferred to ice for harvesting. The cells were harvested in the culture medium/formaldehyde/glycine mix and pelleted by centrifugation at 500 *g* for 5 minutes at 4 °C. The cell pellets were resuspended/washed in ice cold PBS, centrifuged at 500 *g* for 5 minutes at 4 °C, resuspended/washed for a second time with ice cold PBS/0.5% NP-40, and centrifuged at 500 *g* for 5 minutes at 4 °C. The cells were then resuspended in 40 ml of high salt buffer (0.5% NP-40, PBS, 1 M NaCl) and incubated on ice for 30 minutes. Following that step, the cells were pelleted by centrifugation at 1500 rpm for 5 minutes at 4 °C, washed with 40 ml PBS/1% NP-40 and resuspended in 40 ml of low salt buffer (0.5% NP-40, 10 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl) for a 30 minute incubation on ice. Following this incubation, the cells were centrifuged as previously, resuspended in 1 ml of low salt buffer and passed through a 26G needle five times. 300 µl of 20% sarcosyl were then added to the cell suspension and the final volume was made up to 3 ml by low salt buffer. This volume was then transferred to a sucrose cushion and

centrifuged at 4000 *g* for 10 minutes at 4 °C. The supernatant was then discarded, the pellet resuspended in 3 ml TE, transferred to a second sucrose cushion and centrifuged at 4000 *g* for 10 minutes at 4 °C. The pelleted material, containing genomic DNA and cross-linked proteins, was then resuspended in 2 ml TE (10 mM Tris pH 8.0, 1 mM EDTA) and the DNA was sheared into smaller fragments, of about 0.5 kb, by sonication (Branson sonifier 250, 10x for 10 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 material, which was then centrifuged at 13000 *g* for 5 minutes. The supernatant was then aliquoted evenly in microfuge tubes, while 10% of the aliquoted volume was retained for use as input control. 25 µl of the custom-made or 5 µg of commercial antibodies were added per aliquot (Table 2.3), and the tubes were incubated rotating overnight at 4 °C.

The next day, 50 µl of protein G sepharose beads (Sigma) (25 µl packed beads, washed three times and made up to 50 µl with 1x NET buffer) were added to each tube and left to incubate rotating for 2 hours at 4 °C. The beads were then recovered on polypropylene columns (Pierce), washed twice with 10 ml ice-cold RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice with 10 ml ice-cold LiCl buffer (10 mM Tris HCl, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0) and finally twice with ice-cold TE. The beads were then incubated for 10 minutes with 400 µl TE/1% SDS at room temperature. The eluted material was then incubated overnight at 42 °C in the presence of 0.125 mg/ml proteinase K.

The DNA was extracted twice using 400 µl phenol/chloroform/isoamylalcohol (25:24:1) and once using 400 µl of chloroform alone. For the DNA precipitation, 1 ml of ethanol and 40 µl of 3 M sodium acetate were added to the tubes, which were then mixed by inversion and left at -20 ºC overnight. The next day, the samples were centrifuged at 16000 *g* for 20 minutes to pellet the DNA, which was washed with 1 ml of 70% ethanol, prior to being air-dried and resuspended in 50 µl of TE. The samples was then kept at 4 °C and analysed by PCR, as previously described. The primer sequences and conditions used for the ChIP assays' PCR analyses are displayed in table 2.4.

For sequential ChIP experiments the beads, after the final TE wash, were incubated with 150 µl TE/1% SDS, rather than 400 µl, and the eluted material was diluted up to 1.5 ml with TE. This was then aliquted to new tubes, while 10% of the aliquot volume was

retained as secondary input control. The secondary antibodies were then added as appropriate and the tubes were left incubating overnight rotating at 4 ºC. The next day, the experiments were continued as in the primary ChIPs, with the addition of beads, etc.

Table 2.3. Antibodies used for ChIP analysis

Protein	**Antibody**	**Source**
Dr1	1162	In house
Dr1	1163	In house
Dr1	Ab28185	Abcam
DRAP1	TM-301A-55	Austral Biologicals
DRAP1	V18	Santa Cruz Biotechnology
TBP	MTBP-6	In house
Brf1	128	In house
TFIIIC220	Ab7	In house
Pol III	1900	In house
TFIIA	FL-109	Santa Cruz Biotechnology
TFIIB	C18	Santa Cruz Biotechnology

Table 2.4. Primers used in PCR analysis of ChIP samples

Transcript	Primers 5'-3': Forward Reverse	PCR Conditions (denaturing; cycling; final elongation)
5S rRNA	GGCCATACCACCCTGAACGC CAGCACCCGGTATTCCCAGG	95ºC-3min; 95ºC-30s; 58ºC-30s; 72ºC-1min; 72ºC-5min
ARPPP0	GCACTGGAAGTCCAACTACTTC TGAGGTCCTCCTTGGTGAACAC	95ºC-2 min; 95ºC-1min; 58ºC-30s; 72ºC-1min; 72ºC-5min
$tRNA^{LEU}$	GAGGACAACGGGGACAGTAA TCCACCAGAAAAACTCCAGC	95ºC-3min; 95ºC-30s; 68ºC-30s; 72ºC-30s; 72ºC-5min
$tRNA^{TYR}$	GGACTTGGCTTCCTCCATT GACCTAAGGATGTCCGCAAA	95°C-3min; 95°C-1min; 65°C-30s; 72°C-15s; 72°C-5 min
Hsp70	GGAGGTGCGGGAAGGTTCG TTCTTGTCGGATGCTGGA	95°C-3min; 95°C-30s; 58°C-30s; 72°C-30s; 72°C-5min
U1 snRNA	CACGAAGGAGTTCCCGTG CCCTGCCAGGTAAGTATG	95°C-3min; 95°C-30s ; 55°C-30s; 72°C-1min; 72°C-5min
U1 snRNA upstream control	GAACTTACTGGGATCTTGG GAGACAACTGAGCCACTTG	95°C-3min; 95°C-30s; 55°C-30s; 72°C-1min; 72°C-5min
U6 snRNA	TTCTTGGGTAGTTTGCAG GTTTCGTCCTTTCCACAAG	95°C-3min; 95°C-30s; 55°C-30s; 72°C-1min; 72°C-5min
U6 snRNA upstream control	GGAATGCTAAGAACTAGC GGCTGGGTTCAACTCTAC	95°C-3min; 95°C-30s; 55°C-30s; 72°C-1min; 72°C-5min
7SL-1	CCGTGGCCTCCTCTACTTG TTTACCTCGTTGCACTGCTG	95°C-2min; 95°C-1min; 58°C-30s; 72°C-1min; 72°C-3min
7SL-2	CGTCACCATACCACAGCTTC CGGGAGGTCACCATATTGAT	95°C-2 min; 95°C-1min; 58°C-30s; 72°C-1min, 72°C-3min
7SL-3	GTTGCCTAAGGAGGGGTGA TCTCTTGAGAGTCCAAAATTAA	95°C-2min; 95°C-1min; 58°C-30s; 72°C-1min; 72°C-3min
7SL-4	TTTTTGACACACTCCTCCAAGA ATCTGGTCAAAGCAACATACACTG	95°C-2min; 95°C-1min; 58°C-30s; 72°C-1min; 72°C-3min
7SL-5	TGCCTCCAGATAAAACTGCTC ACCCCACTAGAACCCTGACA	95°C-2min; 95°C-1min; 58°C-30s; 72°C-1min; 72°C-3min

CHAPTER 3

The Dr1-DRAP1 complex regulates expression of RNA polymerase III-transcribed genes

3.1 Introduction

3.1.1 The Dr1-DRAP1 (NC2) repressor

Dr1 (down-regulator of transcription 1, also known as negative cofactor 2 beta-NC2 beta) was first identified from HeLa nuclear extracts as an activity that could bind to TBP in electrophoretic mobility shift assays (EMSA) and repress Pol II transcription *in vitro* (Inostroza et al, 1992; Meisterernst & Roeder, 1991). Later, it was recognised that Dr1 interacts with DRAP1 (Dr1-associated protein 1, also known as NC2 alpha) and that the two proteins function together as a heterodimer (Goppelt et al, 1996; Mermelstein et al, 1996).

Human Dr1 is a 19 kD protein containing 176 amino acids (Inostroza et al, 1992). Structure-function studies indicated that it has at least three different domains, a TBP binding domain, a glutamine-alanine (QA)-rich region and a region with homology to the histone fold motif of H2B (Figure 3.1) (Goppelt et al, 1996; Mermelstein et al, 1996; Yeung et al, 1994). Human DRAP1 is a 28 kD protein with 205 amino acids (Goppelt et al, 1996; Mermelstein et al, 1996). It contains acidic and proline-rich stretches and also a histone fold motif similar to that of H2A (Figure 3.1) (Goppelt et al, 1996; Mermelstein et al, 1996). Dr1 and DRAP1 form a complex together through the histone fold motif, similar to H2A-H2B and a number of other proteins (Arents & Moudrianakis, 1995; Baxevanis et al, 1995; Kamada et al, 2001; Mermelstein et al, 1996). The histone motifs are essential for the interaction of Dr1 and DRAP1 and maximal repression of Pol II transcription by the complex (Mermelstein et al, 1996).

Genes encoding Dr1 and DRAP1 are absent in prokaryotes, including *Archaea*, but are conserved in all eukaryotes. In yeast the Dr1-DRAP1 complex is well conserved, as the *S.cerevisiae* homologs yDr1 and BUR6, the DRAP1 homolog in yeast, are both 37% identical to their human counterparts (Goppelt & Meisterernst, 1996; Kim et al, 1997). However, the yeast proteins are smaller, and the repression-related QA domain of Dr1 and most of the C-terminal region of DRAP1 are not present. Also, especially in the case of DRAP1, most gene identity corresponds to the histone fold motif (Figure 3.1) (Goppelt & Meisterernst, 1996; Kim et al, 1997). Both yDr1 and BUR6 are essential for yeast viability, and the complex interacts with TBP-DNA complexes in EMSA similarly to the human complex, indicating that it might function in an analogous manner (Kim et al, 1997).

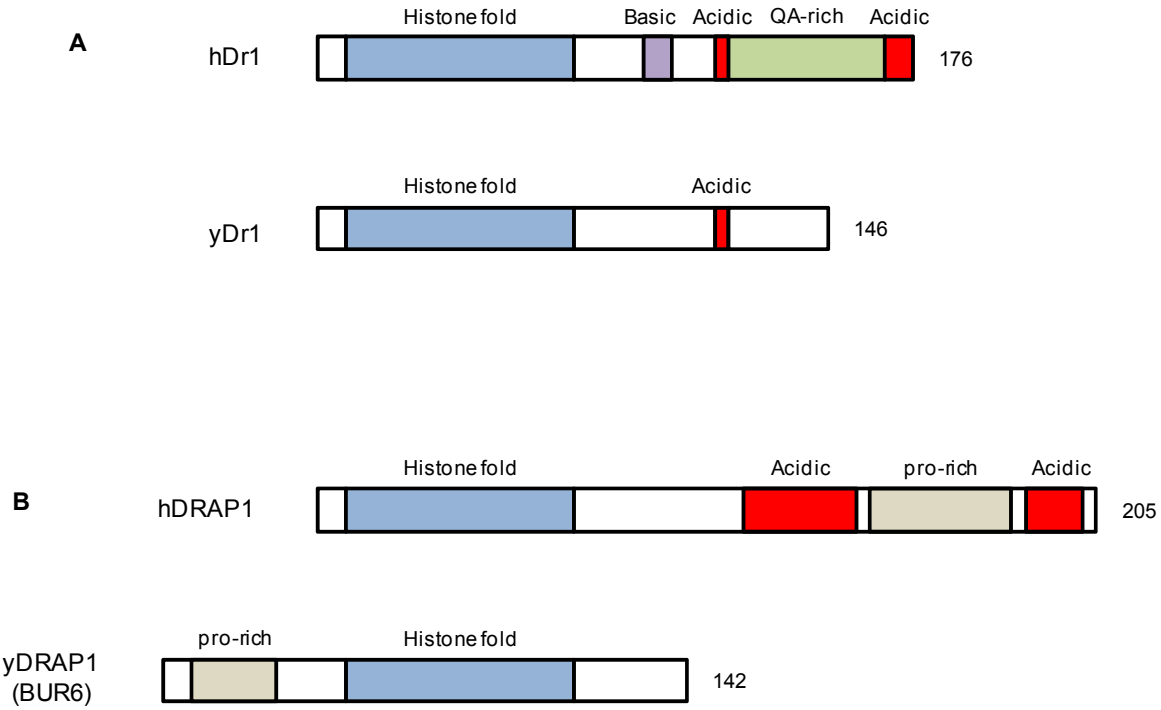


Figure 3.1. Schematic representation of Dr1 and DRAP1 domains from human (h) and *S. cerevisiae* (y). A. Dr1 **B.** DRAP1. Adapted from Goppelt and Meisterernst, 1996.

3.1.2 Transcriptional repression by Dr1-DRAP1

The Dr1-DRAP1 complex targets TBP and is considered to be a global repressor of transcription (Kim et al, 1997). Human Dr1-DRAP1 can repress Pol II transcription *in vitro* and *in vivo* from a number of different TATA-containing and TATA-less, viral and cellular promoters (Goppelt et al, 1996; Inostroza et al, 1992; Meisterernst & Roeder, 1991; Mermelstein et al, 1996; Yeung et al, 1994). The complex can also repress Pol II transcription *in vitro* and *in vivo* in yeast (Gadbois et al, 1997; Goppelt & Meisterernst, 1996; Kim et al, 1997; Lemaire et al, 2000; Prelich, 1997). Dr1 is the subunit that has the ability to repress transcription, with the QA domain, as well as the TBP-binding domain, being required for this function; DRAP1 cannot repress transcription, but significantly enhances Dr1-mediated transcriptional repression and is therefore considered a co-repressor (Mermelstein et al, 1996; Yeung et al, 1997).

The mechanism of transcriptional repression by Dr1-DRAP1 has been studied mainly *in vitro*. EMSA experiments have shown that Dr1-DRAP1 can bind to TFIIA-TBP-DNA, TFIIB-TBP-DNA and TFIIA-TFIIB-TBP-DNA complexes, and replace TFIIA and TFIIB (Goppelt et al, 1996; Inostroza et al, 1992; Meisterernst & Roeder, 1991; Mermelstein et al, 1996). It is therefore believed that the Dr1-DRAP1 complex binds to TBP at the promoter and disrupts the formation of a functional transcription preinitiation complex, by blocking recruitment of TFIIB and consequently the loading of the general transcription factors and Pol II (Goppelt et al, 1996; Mermelstein et al, 1996; Yeung et al, 1997). In support of this model, in yeast, TBP mutants defective for binding to Dr1-DRAP1 display increased transcription from core promoters *in vivo* and are resistant to Dr1-DRAP1 inhibition *in vitro* (Cang et al, 1999). The crystal structure of the Dr1-DRAP1 complex is also consistent with this concept. It revealed that the heterodimer acts as a molecular clamp gripping the upper and lower surfaces of the TBP-DNA complex (Figure 3.2) and thereby blocking binding by TFIIB and TFIIA (Figure 3.3) (Kamada et al, 2001).

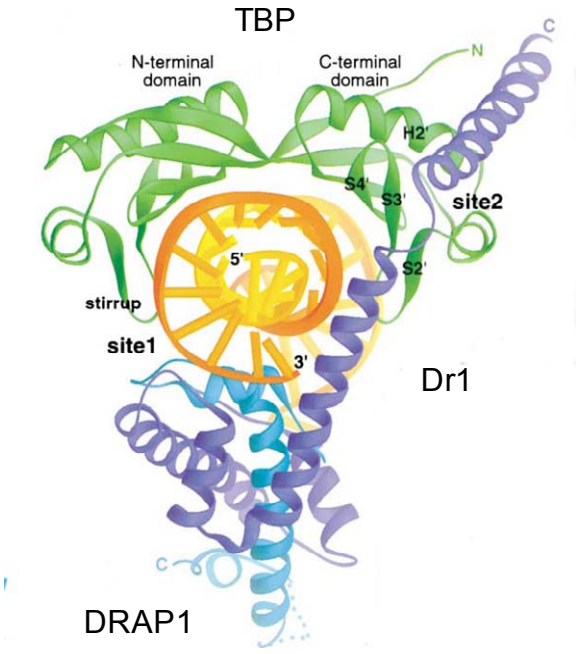


Figure 3.2. Schematic representation of the crystal structure of the Dr1-DRAP1-TBP-DNA complex. Dr1 is shown in dark blue, DRAP1 in light blue, TBP in green and DNA in yellow/orange (adapted from Kamada et al, 2001).

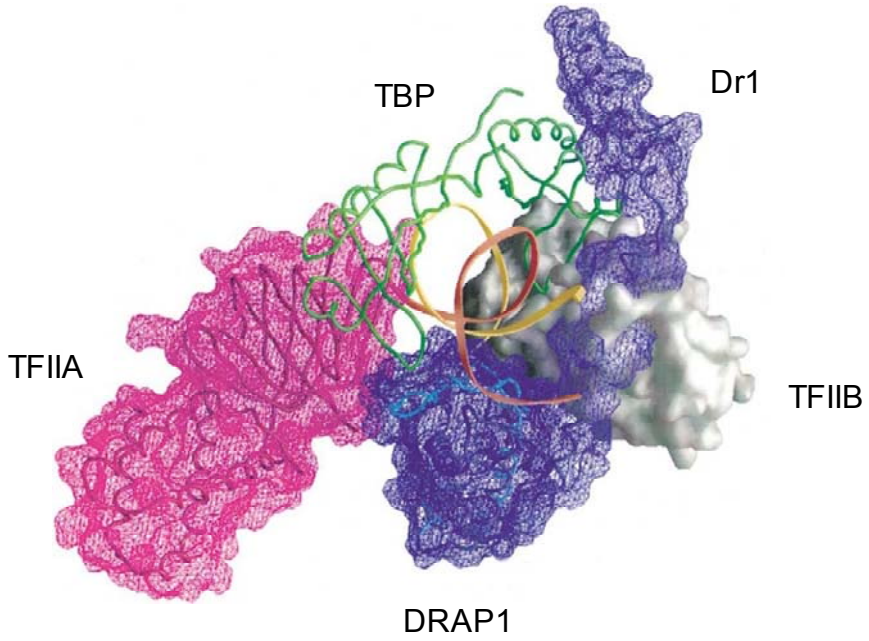


Figure 3.3. Dr1-DRAP1 binding to the TBP-DNA complex blocks recruitment of TFIIA and TFIIB. Dr1-DRAP1 is shown in blue, TFIIA in pink, TFIIB in gray, TBP in green and DNA in red/yellow ribbons. The image is a superposition of NC2-TBP-DNA, TFIIB-TBP-DNA and TFIIA-TBP-DNA complex structures (adapted from Kamada et al, 2001).

The Dr1-DRAP1 complex as a transcriptional repressor participates in an elaborate interplay network between negative and positive regulators that control transcriptional output. Genetic experiments involving Dr1-DRAP1 and subunits of the SRB/Mediator component of the Pol II holoenzyme in yeast, suggest that there is an intricate balance between positive and negative regulators *in vivo*; defects in the SRB/Mediator positive regulators and also TFIIH and Pol II can be suppressed by defects in Dr1-DRAP1 (Gadbois et al, 1997; Kim et al, 2000; Lee et al, 1998; Lemaire et al, 2000; Peiro-Chova & Estruch, 2007). Another example of finely balanced regulation includes factors that target TBP; Mot1 has been shown to compete with Dr1-DRAP1 for promoter occupancy *in vivo* (Geisberg et al, 2002), while TAF1 has been found to regulate a certain number of genes in common with Dr1-DRAP1 (Chitikila et al, 2002).

3.1.3 Potential positive role of the Dr1-DRAP1 complex

Interestingly, although Dr1-DRAP1 is a well documented transcriptional repressor, there are also reports suggesting a positive, direct or indirect, role in Pol II transcription. Yeast strains expressing mutant BUR6 increased transcription from certain promoters, but reduced transcription from others (Prelich, 1997) and yDr1 mutants revealed that Dr1-DRAP1 is required for transcription of the HIS3 TATA-less promoter, but also for repression of transcription of the HIS3 TATA promoter (Lemaire et al, 2000). Furthermore, whole genome expression analyses using two different mutant BUR6 strains revealed that 17% of all yeast genes are affected by two-fold or more (Cang & Prelich, 2002; Geisberg et al, 2001). In these studies, about 50% (Geisberg et al, 2001) or 60% (Cang & Prelich, 2002) of the at least 2-fold affected genes presented, increased expression while the expression of the rest was decreased. BUR6 was found at Pol II promoters of genes whose expression was increased, suggesting that this is a direct effect (Geisberg et al, 2001). Moreover, it was shown that TBP was also needed for activation *in vivo* and proposed that activation by the Dr1-DRAP1 complex is mediated by stimulating TBP binding in the presence of other activators, while inhibition due to blocking TBP interactions with TFIIA and TFIIB, as originally described (Cang & Prelich, 2002). Finally, another study in yeast, that examined both subunits, showed that yDr1 and BUR6 are not always associated in growing conditions, but form a tight complex when glucose is depleted (Creton et al, 2002). Furthermore, the presence of BUR6 without yDr1 at promoters correlated with transcriptional activity, while increased presence of yDr1 correlated with transcriptional repression, suggesting that the two subunits might play different roles *in vivo* (Creton et al, 2002).

Positive effects of Dr1-DRAP1 were also reported in other systems. In *Drosophila*, Dr1-DRAP1 was found to repress transcription by TATA promoters, but increase transcription by the, common in *Drosophila*, TATA-less DPE promoters in *in vitro* experiments (Willy et al, 2000). In humans, Dr1 has been found at active Pol II gene promoters in asynchronous cells and was displaced at some, but not all genes in mitotic cells (Christova & Oelgeschlager, 2002). Interestingly, TBP and TFIIB were also found at gene promoters in mitotic cells, indicating that TFIID complexes can withstand condensation of chromatin into transcriptionally silenced chromosomes (Christova & Oelgeschlager, 2002). Dr1 was also found to interact with the C-terminal domain-

phosphorylated IIO form of Pol II; Dr1 immunodepletion from HeLa nuclear extracts resulted in reduced transcription due to co-depletion of IIO Pol II, but transcription was restored by addition of this enzyme, suggesting that Dr1 might have a role in transcriptional activation (Castano et al, 2000).

3.1.4 The Dr1-DRAP1 complex and Pol III transcription

Not much is known about the effect of Dr1-DRAP1 in other than Pol II transcription systems. *In vitro* studies with human nuclear extracts revealed that Dr1 can repress Pol III, but not Pol I transcription, probably by blocking binding of Brf1 to TBP and formation of an active Pol III preinitiation complex (White et al, 1994). Dr1 overexpression in Xenopus embryos, resulted in inhibition of transcription of Pol II, but not Pol III or Pol I (Nagano & Shiokawa, 1999). In yeast, overexpression of yDr1-BUR6 also resulted in reduced Pol III and Pol II, but not Pol I transcription, confirming the findings in human cell extracts (Kim et al, 1997); this was most probably due to yDr1, as overexpression of yDr1 alone impaired growth, while overexpression of BUR6 alone was without effect (Kim et al, 1997). Furthermore, ChIP experiments in yeast revealed absence of BUR6 at rRNA genes and presence at very low level compared to TBP (BUR6/TBP ratio < 0.05) at tRNA genes, and therefore, it was suggested that yDr1-BUR6 complex does not associate with Pol I- or Pol III- transcribed genes (Geisberg et al, 2001).

It would be interesting to investigate if Dr1 can affect Pol III transcription and is not found at Pol III-transcribed genes *in vivo*, in human cells.

3.2 Results

3.2.1 Validation of Dr1 custom made antibodies

Due to commercial unavailability, two Dr1 antibodies were custom made against peptides ASSSGNDDDLTIPRA and SNQAESSQDEEDDDDI. The peptides were selected to be found uniquely in the Dr1 protein from human, mouse and rat origin. They were injected in two rabbits and polyclonal antibodies were generated according to the company's protocol (Eurogentec Ltd). The sera produced from the two rabbits, named Dr1 1162 and Dr1 1163, were tested by western analysis to test their efficacy to detect the Dr1 protein (Figure 3.4, A and B). Bands of the expected 19 kD, corresponding to the Dr1 protein were detected in whole and nuclear cell extracts from HeLa cells; Dr1 purified from HeLa cells (hDr1) and recombinant Dr1 (rDr1) protein were used as positive controls (White et al, 1994). Except from the 19 kD band, a number of two or more slower migrating bands were observed, which were also present in the rDr1 lane when higher concentrations of the recombinant protein were used. Although these antibodies clearly recognise Dr1, they also seem to recognise additional proteins of higher molecular weight. Taking into account the molecular weight of these bands, as well as their presence in the recombinant protein lanes (Figure 3.4, A), it is tempting to hypothesise that they might belong to Dr1 complexes that failed to dissociate, even though the samples were denatured and run in SDS-PAGE gels, as Dr1 is known to form homodimeric and homotetrameric complexes (Inostroza et al, 1992).

3.2.2 Dr1 regulates Pol III transcription

Dr1 has been shown to be a Pol II transcriptional repressor *in vitro* and *in vivo*, in yeast and mammalian systems (Geisberg et al, 2001; Kim et al, 1997; Mermelstein et al, 1996). In reference to Pol III, Dr1 has been shown to repress Pol III transcription *in vitro* in human extracts (White et al, 1994) and also *in vivo* in yeast (Kim et al, 1997). To further extend these studies and test whether Dr1 has an effect on Pol III transcription *in vivo* in human cells, an RNAi approach was employed. HeLa cells were transiently transfected by electroporation, either with pSUPER-Dr1 plasmid, which encodes a shRNA against the second exon of Dr1, or the empty pSUPER vector as control (Brummelkamp et al, 2002).

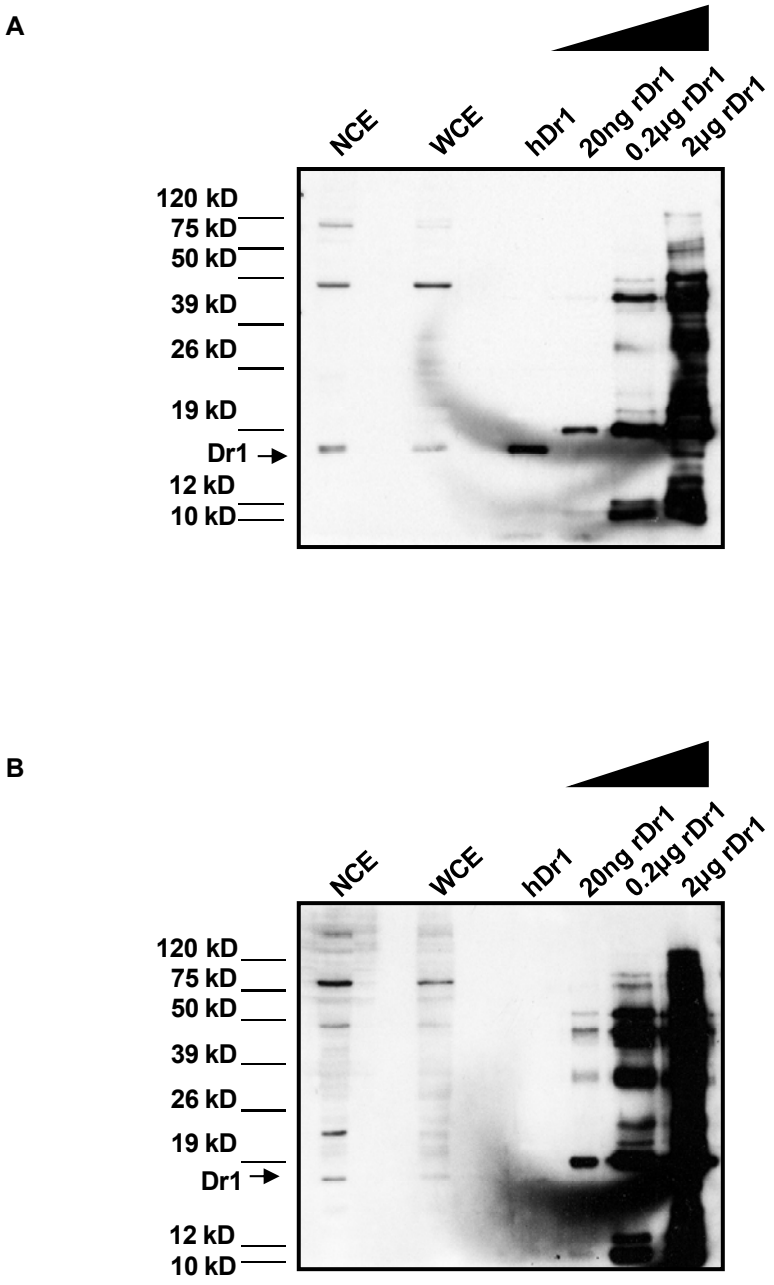


Figure 3.4. Dr1 custom made antibody validation. Western blotting was employed to test the ability of the antibodies to recognise Dr1 protein. Nuclear cell extract (NCE) and whole cell extract (WCE) from HeLa cells were used as source. Human purified Dr1 (hDr1) and recombinant Dr1 (rDr1) protein were used as positive controls. 30 µg of WCE and NCE, 30 ng hDr1 and 20 ng, 0.2 µg and 2 µg of rDr1 were loaded in the respective lanes. **A.** Dr1 1162 serum. **B.** Dr1 1163 serum. The arrow indicates the Dr1 protein.

The cells were harvested 48 hours later, whole cell extracts were prepared and RNA was isolated. Western blotting showed that there was a partial knock-down of Dr1 at the protein level (Figure 3.5.A), which was in agreement with Dr1 reduction in the mRNA level, as determined by RT-PCRs (Figure 3.5.B). A total of three independent experiments showed that on average, there was about 60% reduction in the mRNA level of Dr1 and 40% in the protein level as compared to the control (Figure 3.5.C). Any attempts to achieve higher knock-down efficiency have failed, resulting in high toxicity and cell death.

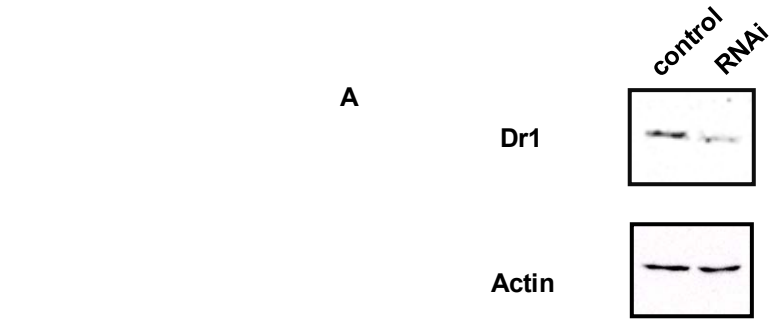
Having achieved a partial Dr1 depletion, the next step was to evaluate the effect that this might have on Pol III transcription. RT-PCRs revealed that RNAi-treated samples had enhanced tRNA expression compared to the control (Figure 3.6.A). Other Pol III templates like 5S rRNA, 7SK RNA and U6 snRNA were unaffected (Figure 3.6.A). Quantification of three different experiments showed that tRNAs are increased by a factor of about 2-fold, when Dr1 is partially depleted, while the other Pol III transcripts are not (Figure 3.6.B).

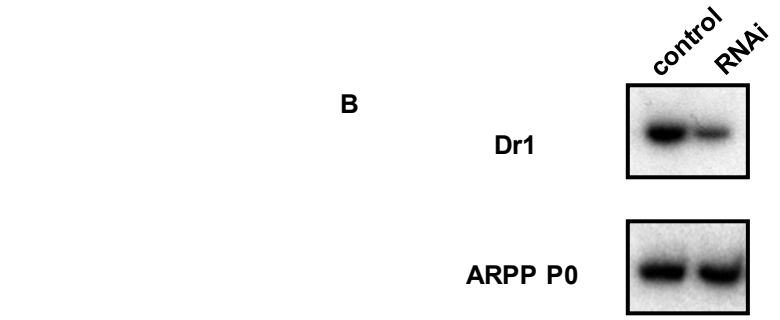
To further confirm and validate the results of the pSUPER-Dr1 vector shRNA experiments, Dr1 was also depleted by siRNA. Two different siRNAs, targeting the first and third exon of Dr1, were transfected into cells. 48 hours later the cells were harvested and cell extracts and RNA were analysed. Both siRNAs partially depleted the Dr1 protein and RNA levels at an equivalent degree to shRNA (Figure 3.7, A and B). These Dr1 knock-downs also resulted in an about 2-fold enhancement of tRNA expression, but left unaffected the 5S rRNA and U6 snRNA transcripts (Figure 3.8).

In conclusion, all three exons of Dr1 were targeted by RNAi (shRNA or siRNA) in three independent experiments and the results obtained were similar and reproducible. Even a 40% decrease in the protein level of Dr1 seems to be enough to increase tRNA expression by two-fold, while other Pol III transcripts remain unchanged. These data suggest that Dr1 can repress Pol III transcription *in vivo*, in human cells.

3.2.3 TFIIIB, TFIIIC and Pol III are not affected by Dr1 depletion

Since the Dr1-DRAP1 complex is a Pol II transcriptional repressor, the increased tRNA expression observed after Dr1 depletion could potentially be attributed to enhanced





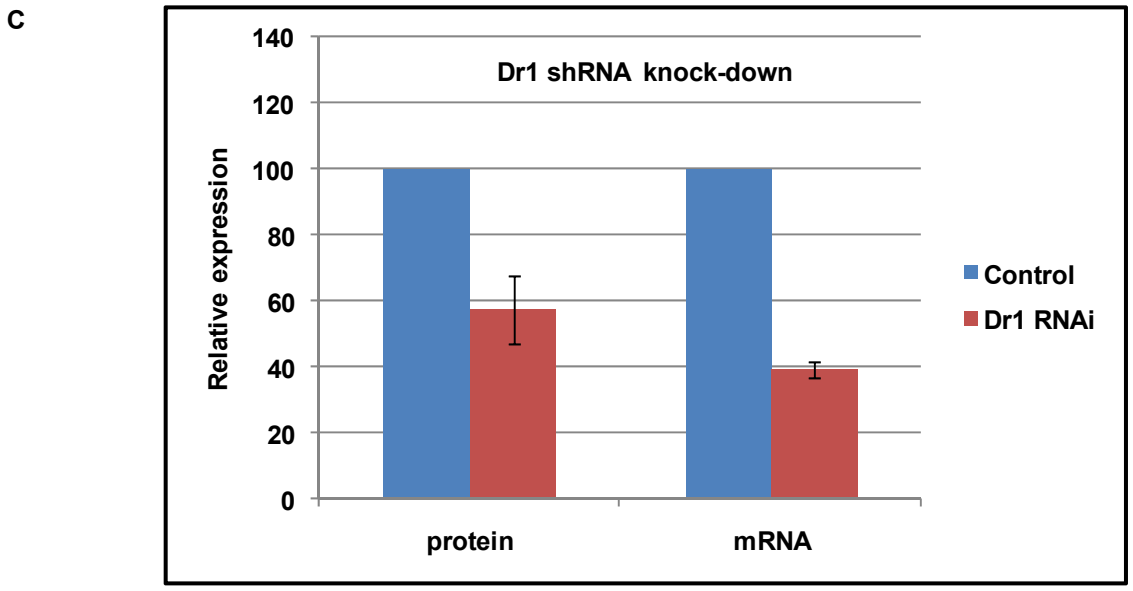
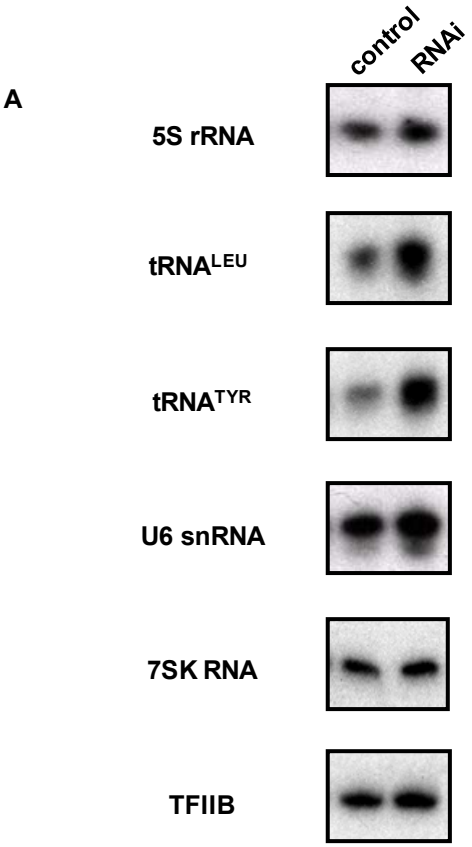


Figure 3.5. Dr1 knock-down by shRNA. HeLa cells were transfected with the pSUPER-Dr1 vector by electroporation and harvested 48 hours later. An empty pSUPER vector was used as control. **A.** Western analysis for Dr1 from whole cell extracts of control and Dr1 targeted cells. Actin was used as loading control. **B.** RT-PCRs for Dr1. The Pol II-transcribed gene ARPP P0 was used as control. **C.** Quantification of Dr1 signals from A and B. n=3.



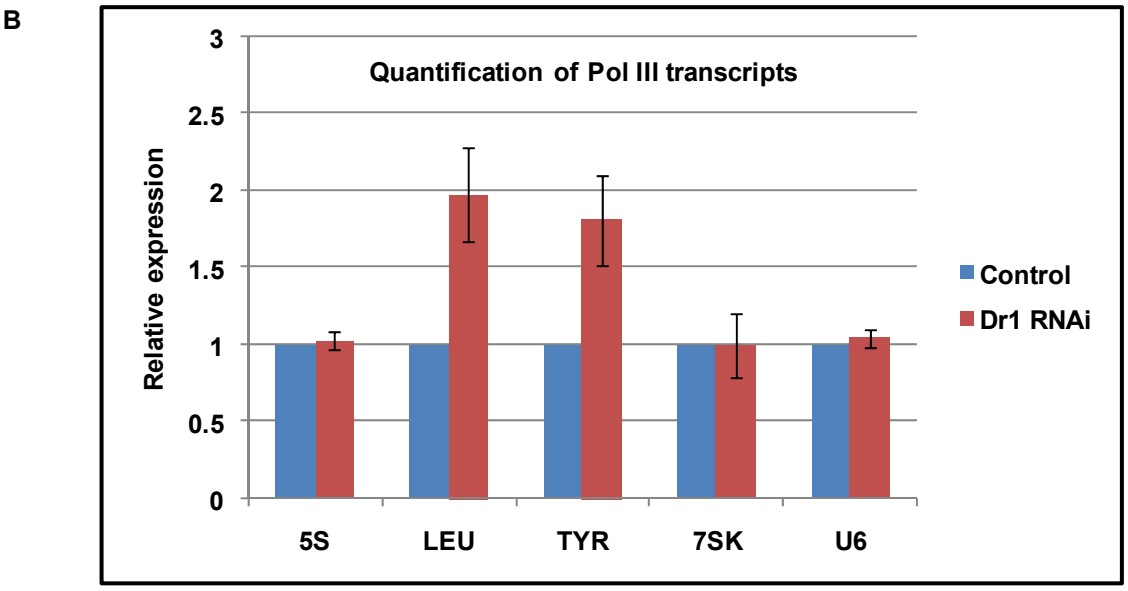


Figure 3.6. Effect of Dr1 shRNA knock-down on Pol III transcripts. A. RT-PCR analysis for a number of Pol III templates. The Pol II-transcribed genes TFIIB and ARPP P0 (data not shown) were used as controls. **B.** Quantification of the signals of Pol III transcripts from A. n=3.

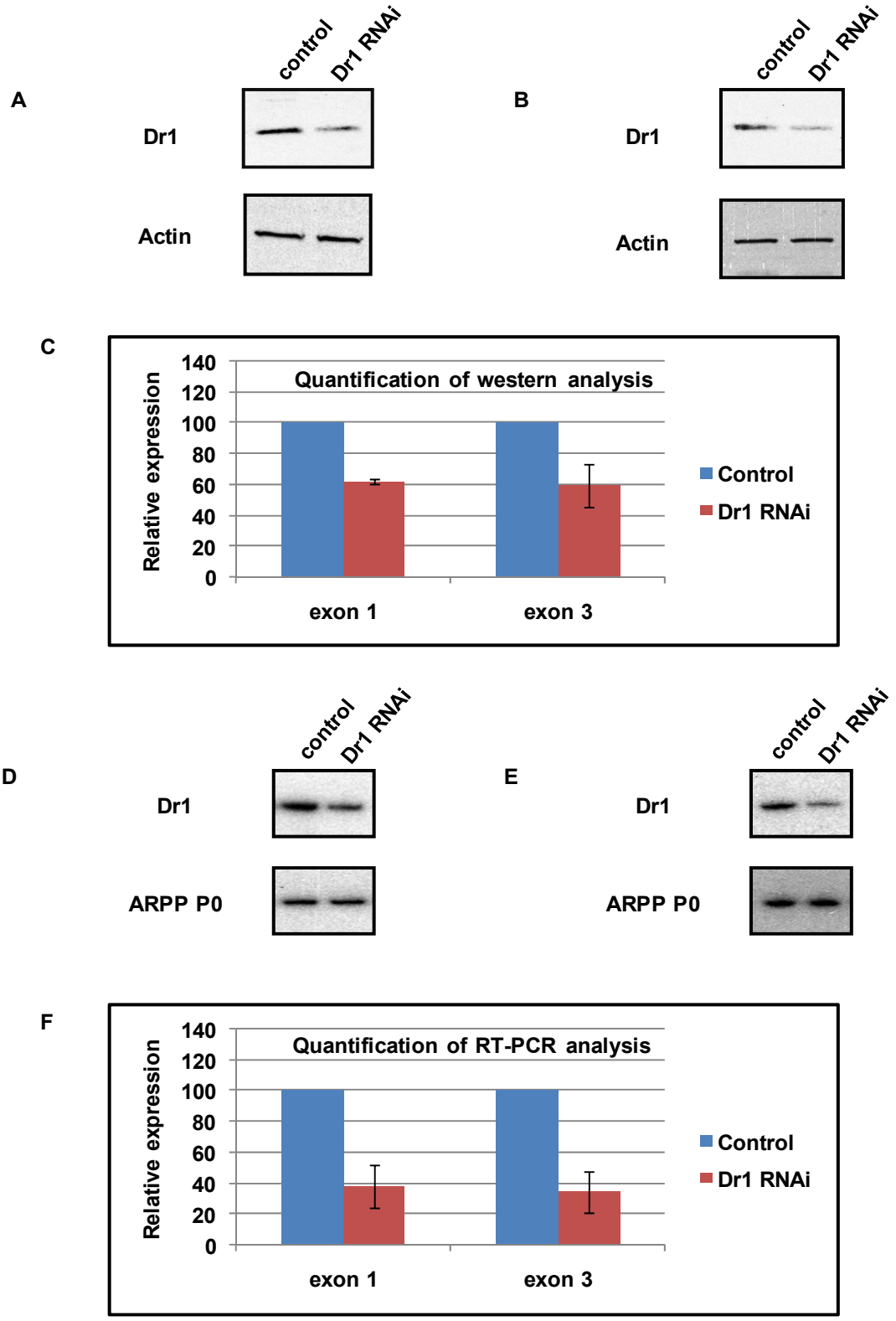


Figure 3.7. Dr1 knock-down by siRNA. Dr1 was knocked-down by siRNA in HeLa cells. A validated, non-targeting siRNA was used as negative control. **A.** Western for Dr1 siRNA against exon 1. **B.** Western for Dr1 siRNA against exon 3. **C.** Quantification of Dr1 signals from A and B. n=2. **D.** RT-PCR for Dr1 depletion by siRNA against exon 1. **E.** RT-PCR for Dr1 depletion by siRNA against exon 3. **F.** Quantification of Dr1 signals from D and E. n=2.

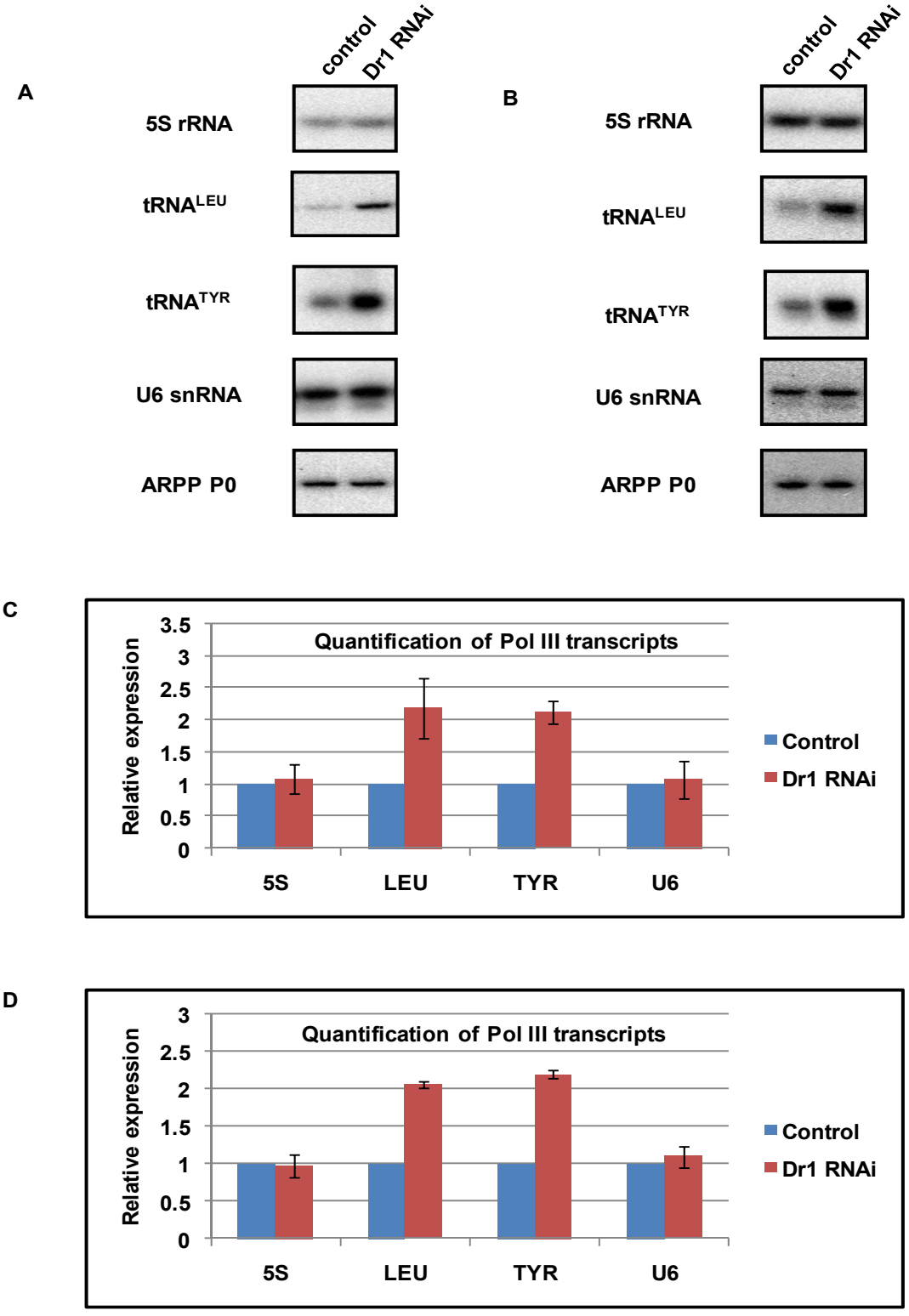


Figure 3.8. Effect of Dr1 siRNA knock-down on Pol III transcripts. A. RT-PCR analysis for Pol III templates in cells treated either with control or siRNA against Dr1 exon 1. **B.** RT-PCR analysis for Pol III templates in cells treated either with control or siRNA against Dr1 exon 3. **C.** Quantification of the signals of Pol III transcripts from A. n=2. **D.** Quantification of the signals of Pol III transcripts from B. n=2.

Pol II transcription and increased levels and availability of TFIIIB, TFIIIC and/or Pol III. To investigate this possibility, the protein levels of the TFIIIB subunits, a number of TFIIIC subunits and a Pol III subunit, from control and Dr1-depleted cells were tested by western blotting analysis. No differences were found between the two conditions (Figure 3.9) and the same conclusion was reached for the mRNA levels of TFIIIB and TFIIIC (Figure 3.10). The mRNA levels of TFIIIB and TFIIIC subunits in cells treated with control or siRNAs against exons 1 and 3, were also tested and found to be unaffected (Figure 3.11).

Since the levels of TFIIIB, TFIIIC and Pol III remain stable and are not affected by the Dr1 RNAi, the enhanced tRNA expression cannot be attributed to changes in these factors; therefore, these data would suggest that this increase is not due to raised Pol II transcription, but probably due to alleviation of direct repression of Pol III transcription by Dr1.

3.2.4 Dr1 is found on Pol III-transcribed genes

Experiments in yeast have shown the DRAP1 occupancy at tRNA promoter to be negligible compared to TBP, and it was concluded that the Dr1-DRAP1 complex is absent from Pol III promoters (Geisberg et al, 2001). In order to investigate if Dr1-DRAP1 is occupying Pol III-transcribed genes in human, chromatin immunoprecipitation (ChIP) experiments were performed. It was found that endogenous Dr1 is present at Pol III-transcribed genes, like 5S rDNA, $tRNA^{LEU}$ and $tRNA^{TYR}$ and U6 snRNA in HeLa cells (Figure 3.12). Both 1162 and 1163 Dr1 sera were able to precipitate DNA for Pol III-transcribed genes (Figure 3.12 and data not shown). Dr1 was also found at Pol II-transcribed genes like U1 snRNA and Hsp70; the promoter of the latter can be repressed by Dr1 (Kraus et al, 1994). No signal was detected when primers were used for the upstream, not transcribed DNA region of the U6 snRNA gene, as well as for an internal coding region of the Pol II-transcribed gene ARPP P0. These data indicate specific binding of the antibodies to the promoter regions of specific genes and minimal promiscuous binding to DNA.

To further validate the above results and minimise the possibility that they might be artifacts of the custom-made antibodies, a recently available commercial antibody for Dr1

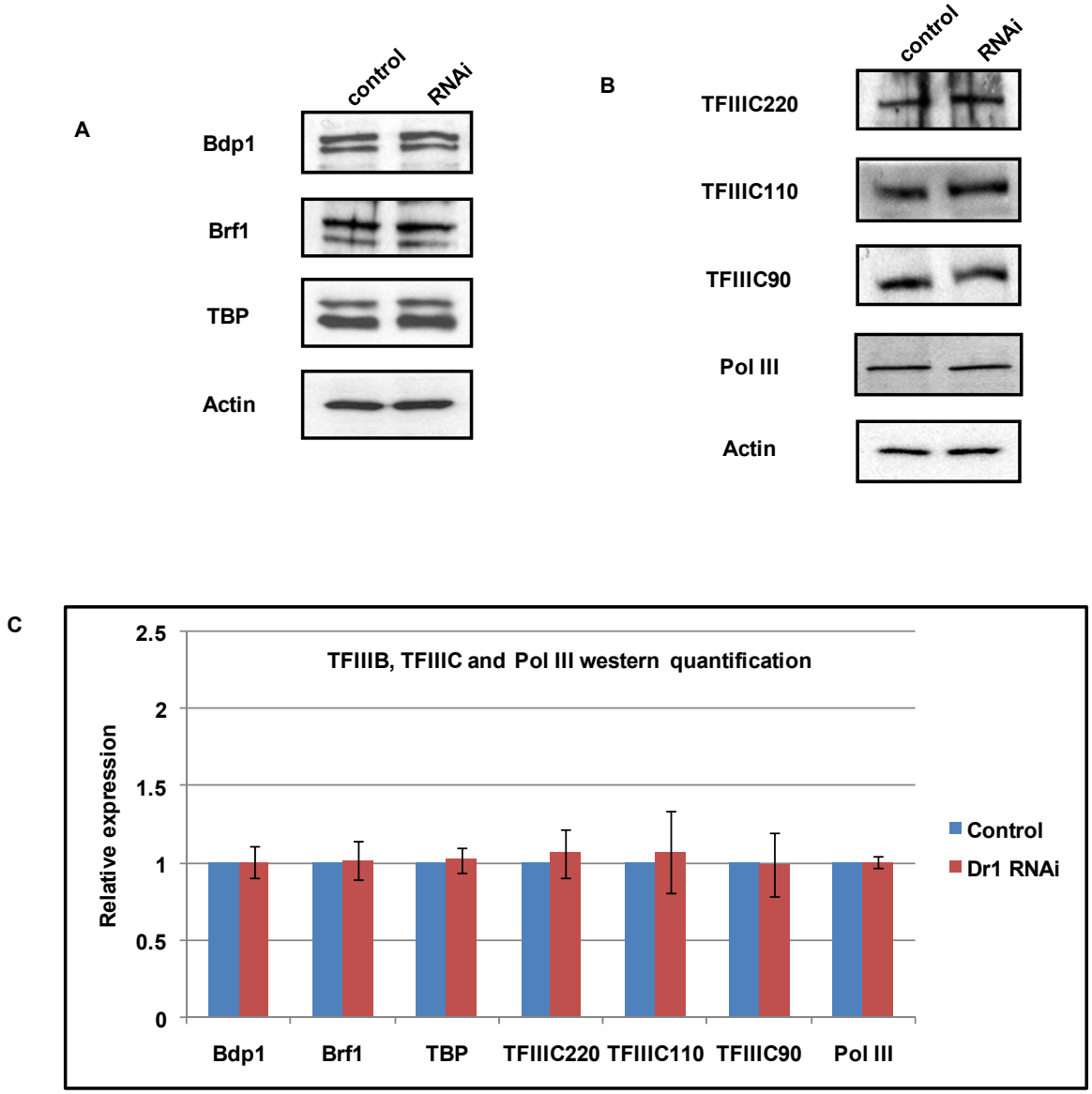
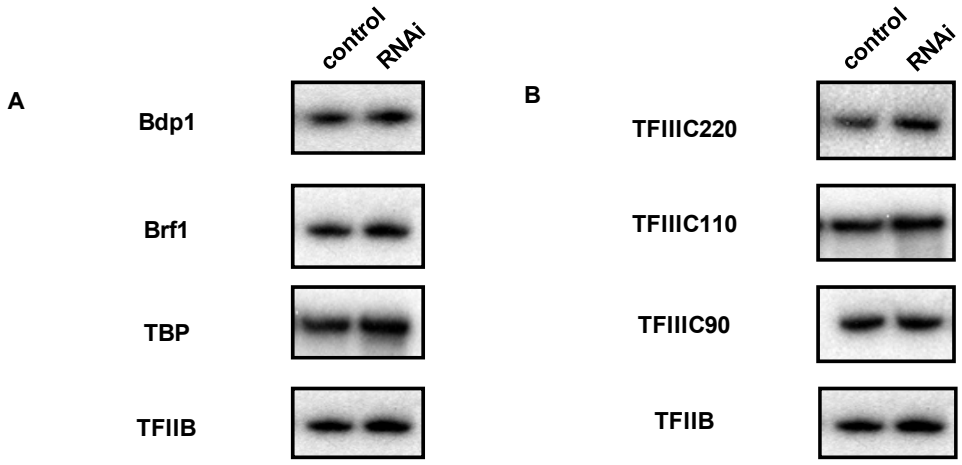


Figure 3.9. Dr1 shRNA knock-down does not affect the protein levels of TFIIIB, TFIIIC and Pol III. Western analysis was employed to determine the protein levels of TFIIIB, TFIIIC and Pol III (RPC 155) in control cells and cells treated with shRNA. **A.** The protein levels of the TFIIIB subunits were not altered by Dr1 RNAi. **B.** The protein levels of TFIIIC subunits 220,110, and 90 were not altered after Dr1 RNAi. **C.** Quantification of signals of TFIIIB and TFIIIC subunits from A and B. n=3.



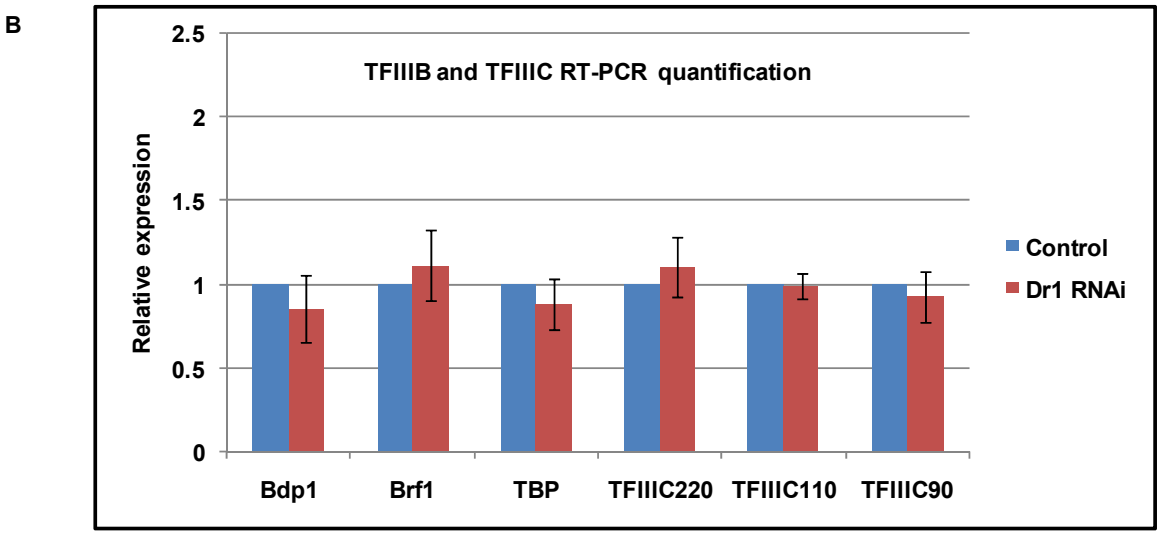


Figure 3.10. Dr1 shRNA knock-down does not affect the mRNA levels of TFIIIB and TFIIIC. RT-PCR analysis was employed to determine the RNA levels of TFIIIB and TFIIIC in control cells and cells treated with shRNA. **A.** The mRNA levels of the TFIIIB subunits were not altered by Dr1 RNAi. **B.** The mRNA levels of TFIIIC subunits 220,110, and 90, were not altered after Dr1 depletion. **C.** Quantification of signals of TFIIIB and TFIIIC subunits from A and B. n=3.

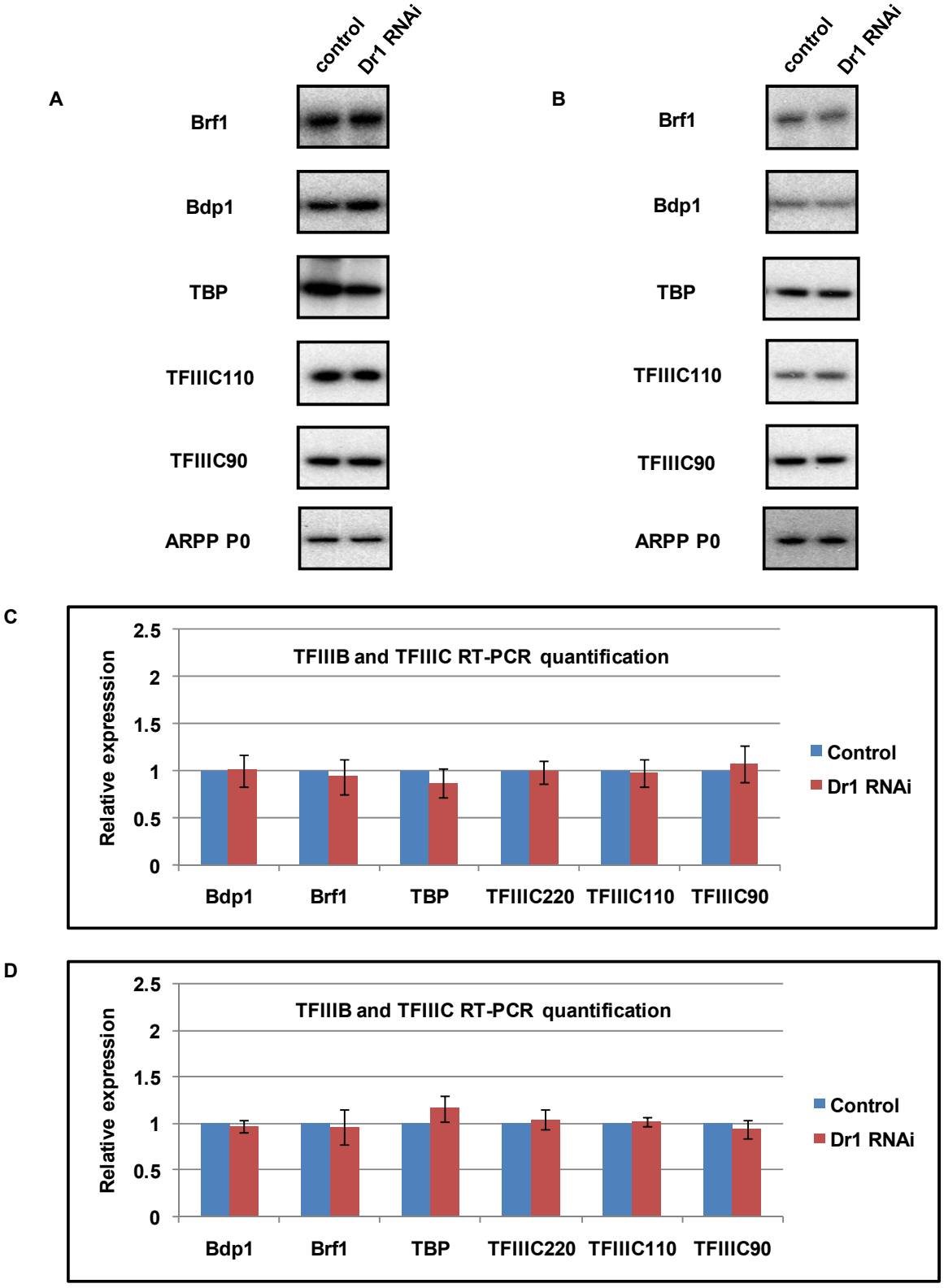


Figure 3.11. The mRNA levels of TFIIIB and TFIIIC are not affected by Dr1 siRNA knock-down. RT-PCR analysis was employed to determine the RNA levels of TFIIIB and TFIIIC in control cells and cells treated with siRNA. **A**. siRNA against Dr1 exon 1. **B.** siRNA against Dr1 exon 3. **C.** Quantification of signals from A. n=2. **D.** Quantification of signals from B. n=2.

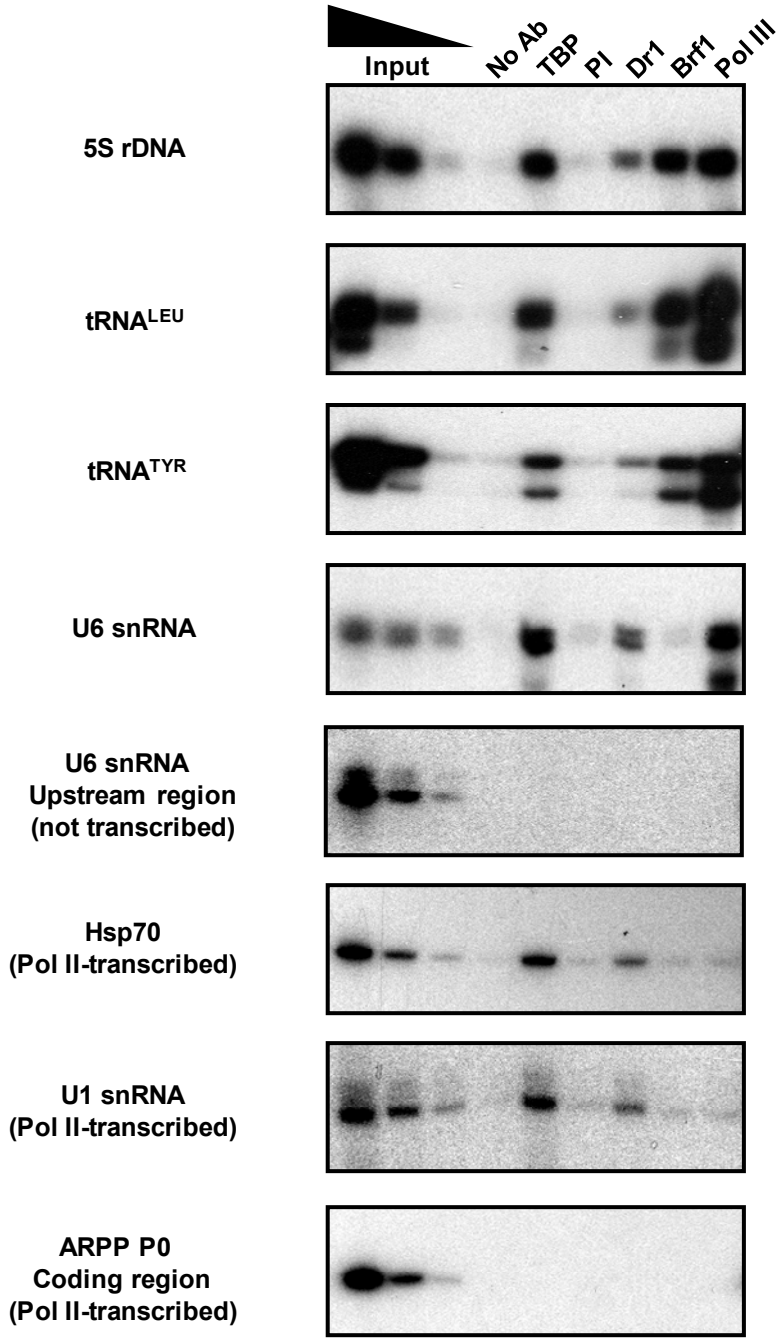


Figure 3.12. Dr1 occupancy at Pol III-transcribed genes. HeLa cells were used for ChIP. The Dr1 1162 antibodies were used together with the 1162 pre-immune (PI) serum and mock (no antibody) as negative controls. Brf1 (128) and Pol III (1900) antibodies were used as positive controls for Pol III-transcribed genes and TBP (MTBP-6) as positive control for Pol III- and Pol II-transcribed genes.

was used in ChIP experiments, in parallel with Dr1 1162 serum. Dr1 was found again to be present at 5S rDNA, $tRNA^{LEU}$ and $tRNA^{TYR}$ genes (Figure 3.13). The Pol II transcription factor TFIIA was used as a negative control and revealed no presence at Pol III genes, as did the mock (no antibody) and the 1162 pre-immune serum controls. Interestingly, it has been reported that TFIIA can regulate Pol III transcription, at least *in vitro* (Meissner et al, 1993). PCR analysis from ChIP experiments showed, however, that this does not seem to the case *in vivo*, as TFIIA is absent from 5S rDNA, $tRNA^{TYR}$, 7SK RNA and U6 snRNA genes, but present at Pol II-transcribed genes encoding U1 snRNA (Figure 3.14).

As previously mentioned, the DRAP1/TBP ratio from ChIP experiments in yeast was found to be below 0.05 and considered negligible (Geisberg et al, 2001). However, the Dr1/TBP ratio at human Pol III-transcribed genes was found to be 0.4-0.5, very similar to the ratio of Pol II-transcribed genes 0.5-0.7 (Table 3.1). These results suggest that in humans Dr1 is found at Pol III-transcribed genes at about the same ratio to TBP as at the Pol II-transcribed genes.

Table 3.1. Dr1/TBP ratio at Pol III-transcribed genes

Genes	Dr1/TBP
5S rRNA	0.5 ± 0.03
$tRNA^{LEU}$	0.4 ± 0.20
$tRNA^{TYR}$	0.5 ± 0.10
U6 snRNA	0.5 ± 0.05
Hsp70	0.5 ± 0.08
U1 snRNA	0.7 ± 0.13

ChIP signals were normalised to input and the value of the Dr1 signal was divided by the TBP signal. The ratio shown is the average of two independent experiments with standard variation.

In summary, ChIP experiments employing different antibodies against Dr1 revealed *in vivo* occupancy of the Dr1 protein at Pol III-transcribed genes. If Dr1 can repress Pol III activity in a way similar to that of repressing Pol II, i.e. by disrupting the preinitiation complex, then one would not expect to find Dr1 at these genes. However, it could be argued that Dr1 is present at inactive/repressed Pol III genes and not the active ones. In this case ChIP experiments, due to their inherent limitations, would not distinguish between the two states.

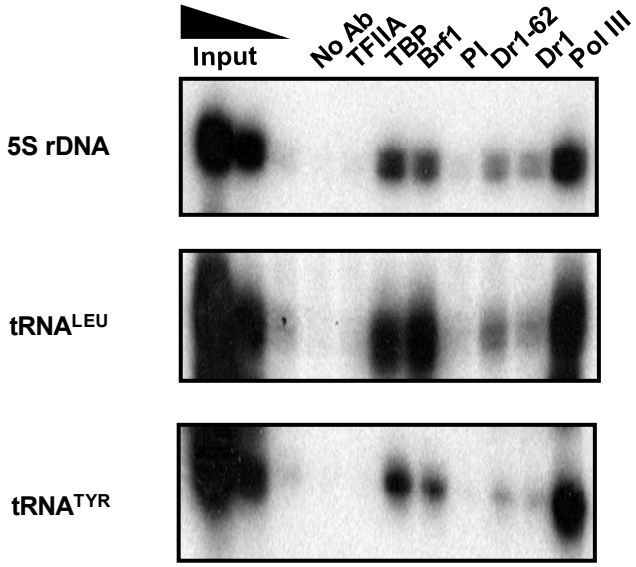


Figure 3.13. Dr1 is found at Pol III-transcribed genes. HeLa cells were used for ChIP. The Dr1 1162 and Dr1 (ab28185) antibodies were used together with 1162 pre-immune serum (PI) and mock (no antibody) as negative controls. The Pol III (1900) antibody was used as positive control for Pol III-transcribed genes and the TBP (MTBP-6) antibody as positive control for Pol III- and Pol II- transcribed genes.

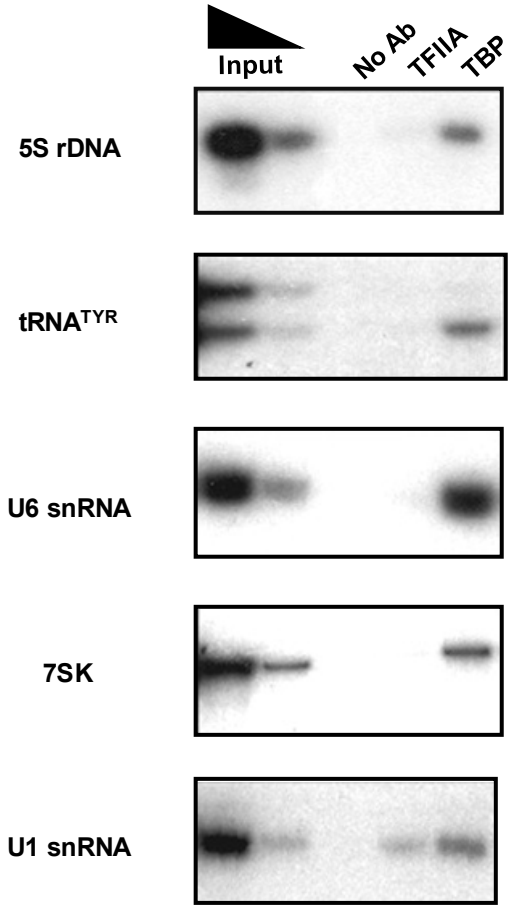


Figure 3.14. TFIIA is not found at Pol III-transcribed genes. HeLa cells were used for ChIP. Antibodies against TFIIA (FL-109) and positive control TBP (MTBP-6) were used.

To further clarify if Dr1 is actually present at active Pol III-transcribed genes, sequential ChIP experiments were performed. Antibodies against Pol III, TBP and TFIIB (as negative control) were used in the primary ChIP, while antibodies against Dr1 (1162 and 1163) and pre-immune serum (as negative control) were used in the secondary ChIP. These experiments revealed co-occupancy of Dr1 and Pol III at Pol III-transcribed genes (Figure 3.15). Dr1 can also be found together with TBP at Pol II- and Pol III-transcribed genes, but is not present at not-transcribed DNA regions, indicating the antibodies' specificity. Antibodies against TFIIB reveal that Dr1 can be found together with TFIIB at Pol II-, but not Pol III-transcribed genes. These results, suggest that Dr1 might actually be able to occupy formed Pol II and Pol III preinitiation complexes with a number of other factors. This comes in contrast with the proposed mechanism of Dr1 repression that has been shown in *in vitro* experiments, and might indicate a more complicated repression mechanism *in vivo*.

3.2.5 Dr1 can be found in complexes with TFIIIB and TFIIIC

Since Dr1 can be found at Pol II- and Pol III-transcribed genes, it must be recruited to them by specific factors. It has been shown that Dr1 can bind TBP and this interaction seems to stabilise the complex at DNA (Gilfillan et al, 2005; Inostroza et al, 1992; Kim et al, 1995). TBP is therefore considered responsible for bringing Dr1 to promoters. However, this interaction does not exclude the possibility of other factors being involved, recruiting Dr1 specifically to Pol III promoters.

To test if Dr1 can interact with TFIIIB and TFIIIC, ^{35}S co-immunoprecipitation (co-IP) experiments were employed. TFIIIB and TFIIIC subunits were *in vitro* translated and radio-labelled with ^{35}S, mixed with HeLa nuclear extract and then used in co-IP assays. Antibodies against Dr1 were capable of bringing down the radio-labelled subunits Brf1 and TBP (Figure 3.16, A), as well as TFIIIC90 and TFIIIC110 (Figure 3.16, B). Very little or no signal was detected when the pre-immune serum was used instead of the antibody, indicating that the interactions were specific. These data suggest that the Dr1 protein can interact with TFIIIB and TFIIIC. Interactions of Dr1 with endogenous subunits of TFIIIB or TFIIIC were also observed by co-IP experiments. Dr1 antibodies were used to bring down Dr1-binding proteins in HeLa nuclear extracts. In these experiments the Dr1

antibodies can immunoprecipitate Dr1 and also co-immunoprecipitate TBP and TFIIIC90 (Figure 3.17, A). Other subunits of TFIIIB (Brf1 and Bdp1) and TFIIIC (110 and 220) were also detected in this kind of analysis (Figure 3.17, B). Using antibodies against Brf1 it was also possible to co-immunoprecipitate endogenous Dr1 (Figure 3.17, C). These interactions were specific, as they were not detected when using the pre-immune serum.

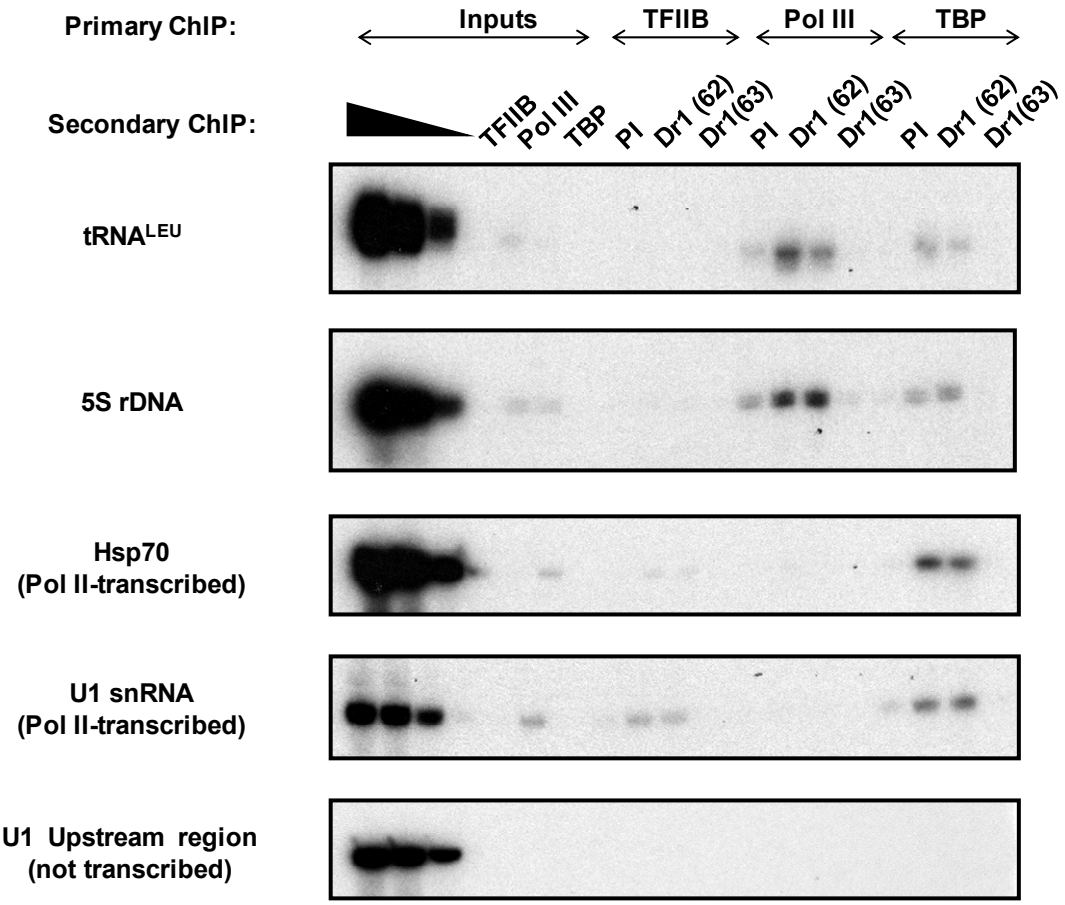


Figure 3.15. Dr1 and Pol III co-occupancy at Pol III-transcribed genes. HeLa cells were used for sequential ChIP experiments. TFIIB (C-18), Pol III (1900) and TBP (MTBP-6) antibodies were used for the primary ChIP. The immunoprecipitated DNA was subjected to secondary ChIP with antibodies against 1162 PI serum, Dr1 1162 and Dr1 1163. The inputs of the primary and the three secondary ChIP experiments are shown at the left.

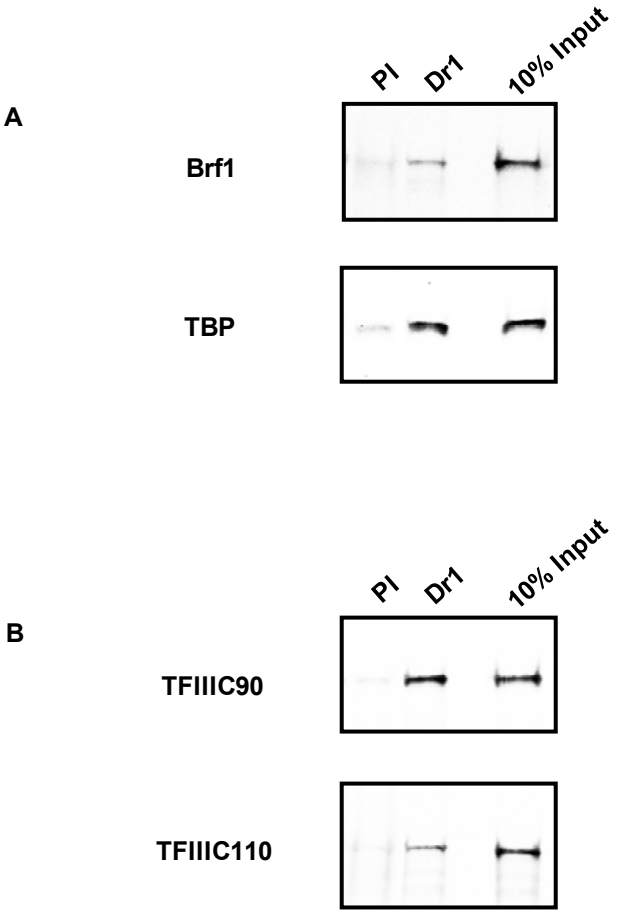


Figure 3.16. ^{35}S co-immunoprecipitation experiments reveal interaction of Dr1 with TFIIIB and TFIIIC subunits. A. Brf1 and TBP were *in vitro* translated and radio-labelled with ^{35}S, mixed with HeLa nuclear extracts and then used in co-IP experiments with antibodies for Dr1 (1162) and 1162 pre-immune serum as control. **B.** TFIIIC90 and TFIIIC110 were *in vitro* translated and radio-labelled with ^{35}S, mixed with HeLa nuclear extract and then used in co-IP experiments with antibodies for Dr1 (1162) and 1162 PI serum as control.

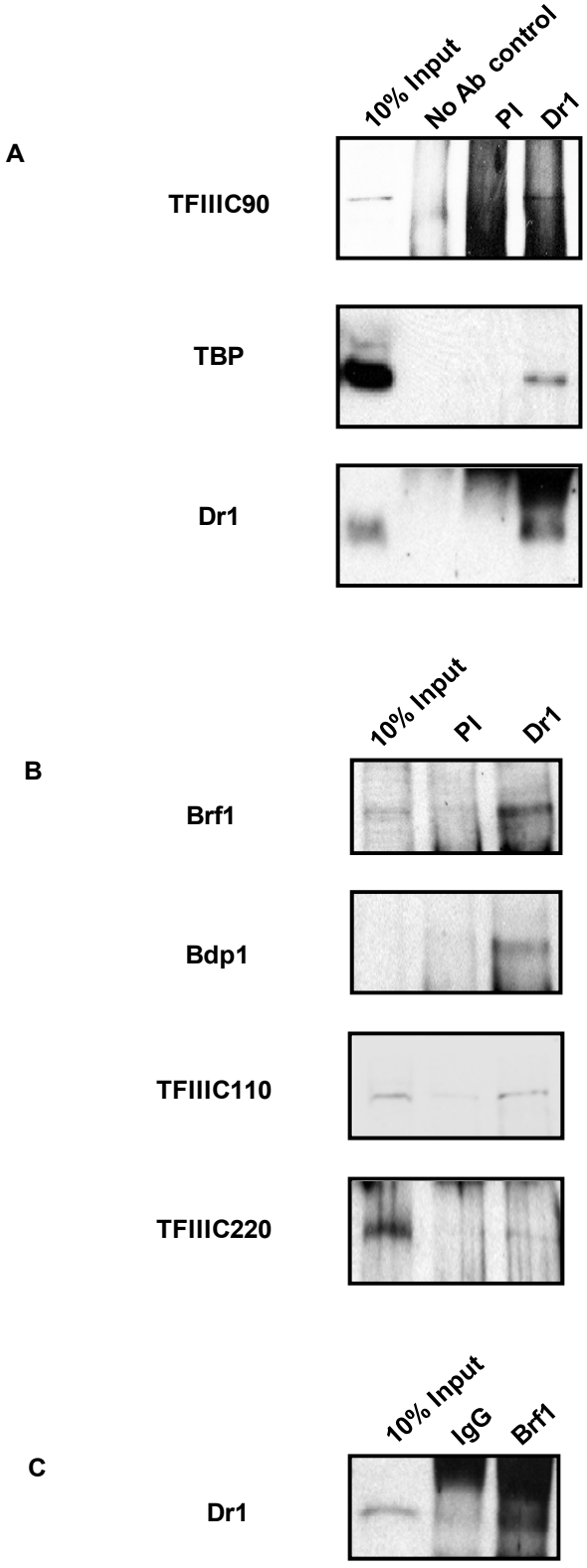


Figure 3.17. Co-immunoprecipitation experiments reveal interaction of Dr1 with TFIIIB and TFIIIC subunits. HeLa nuclear extracts were used for co-IP experiments. **A.** Antibodies for Dr1 (1162) were used to immunoprecipitate Dr1 and co-immunoprecipitate TBP and TFIIIC90 **B.** Antibodies for Dr1 (1162) were used to co-immunoprecipitate Brf1 and Bdp1 as well as TFIIIC110 and TFIIIC220 **C.** Antibodies for Brf1 (128) were used to co-immunoprecipitate Dr1.

The above findings demonstrate interaction of Dr1 with endogenous TFIIIB and TFIIIC. Dr1 might be able to bind not only TBP, but also other subunits of TFIIIB, i.e. with Brf1 and Bdp1 and subunits of TFIIIC, like TFIIIC90, TFIIIC110 and TFIIIC220. By binding to TFIIIB and/or TFIIIC subunits, Dr1 could be specifically recruited to Pol III-transcribed gene promoters and form a stable complex. It must be noted, however, that these assays do not distinguish between direct and indirect binding; it is possible that Dr1 directly binds only to TBP and through TBP, indirectly to the other TFIIIB and TFIIIC subunits, although this is unlikely for the ^{35}S radio-labelled *in vitro* translated Brf1 and TFIIIC subunits. Nevertheless, this is a surprising result, as it comes in contrast to *in vitro* data where Dr1 could clearly inhibit the TBP-Brf1 interaction in pull-down assays (White et al, 1994).

3.2.6 Brf1 overexpression results in Dr1 recruitment to Pol III-transcribed genes

A probable Brf1-Dr1 interaction could potentially recruit the Dr1-DRAP1 complex to Pol III promoters. According to this hypothesis, overexpression of Brf1 would then result in enhanced recruitment of Dr1 at these promoters. To test this, a Brf1-inducible "TET-OFF" CHO cell line was employed. The cells were either induced, or not, to over-express Brf1 and 48 hours later were harvested and prepared for ChIP assays. PCRs using the precipitated DNA revealed increased occupancy of Pol III-transcribed genes by Dr1 after overexpression of Brf1, compared to control (un-induced) (Figure 3.18).

These results suggest Dr1 recruitment to Pol III-transcribed genes by TFIIIB. It remains unclear, whether TBP, Brf1 or both are responsible for recruiting Dr1. Interestingly, however, increased TFIIIB presence at Pol III-transcribed genes correlates with activation of Pol III transcription (Marshal and White, personal communication). The enhanced presence of Dr1 then contrasts with both its role as a repressor and its postulated repression mechanism, according to the current model of inhibiting formation of a complete transcription preinitiation complex.

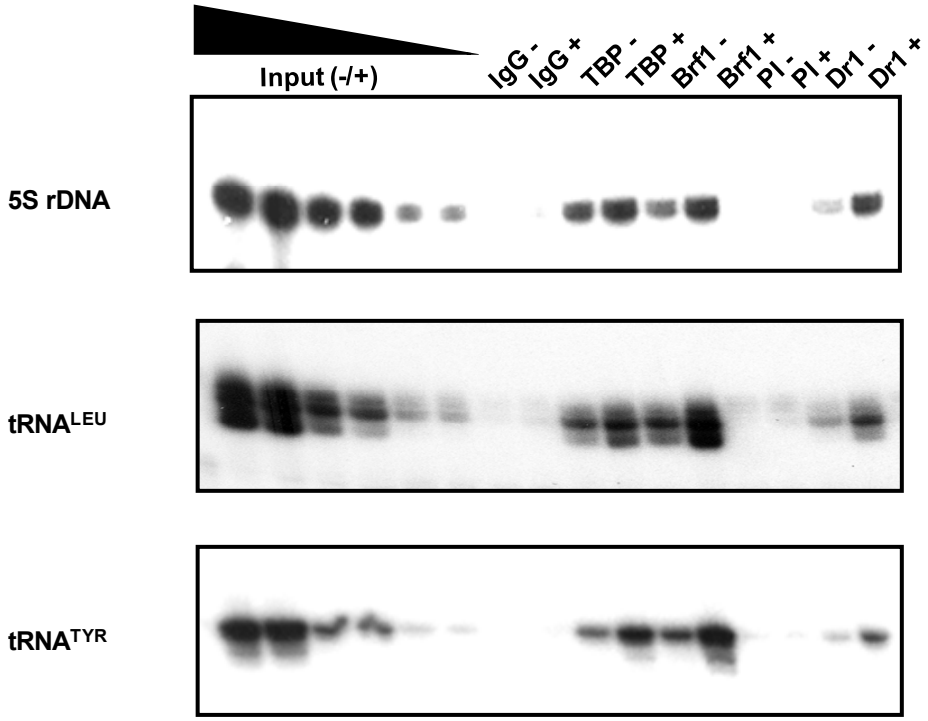


Figure 3.18. Overexpression of Brf1 results in increased Dr1 presence at Pol III-transcribed genes. CHO cells, stably transfected with an inducible Brf1 TET-OFF expression system, were induced to express Brf1 for 48 hours before harvesting. ChIP experiments were performed using antibodies against TBP (MTBP-6), Brf1 (128) and Dr1 (1162). Pre-immune (PI 1162) serum and rabbit IgGs (sc-2027, Santa Cruz Biotechnology) were used as negative controls. The "+" denotes induction of Brf1 compared to the un-induced "-" control.

3.2.7 Hypoxia results in increased occupancy of Pol III-transcribed genes by Dr1.

The Dr1 recruitment at Pol III-transcribed genes after induction of Brf1, casts doubts as to whether increased presence of Dr1 at promoters correlates with transcriptional repression. Therefore, it would be interesting to investigate if increased occupancy of Pol III-transcribed genes by Dr1 can also be detected when Pol III transcription is repressed.

Dr1 has been reported to repress Pol II transcription under extreme hypoxic conditions (Denko et al, 2003) and it has also been shown that hypoxia represses Pol III transcription (Ernens et al, 2006). To test whether Dr1 can be found at Pol III-transcribed genes in hypoxia, U2OS cells, grown under normal (20% O_2) or hypoxic conditions (1% O_2) for 24 hours, were used for ChIP assays. PCRs from these ChIPs revealed increased Dr1 recruitment at Pol III-transcribed genes under hypoxic conditions, while Brf1 and TBP were displaced (Figure 3.19, A). The protein levels of Dr1, as well as TBP and Brf1, did not change (Figure 3.19, B), indicating that the TFIIIB and Dr1 occupancy changes at the Pol III-transcribed genes were not due to reduced or increased protein levels. Repression of Pol III transcription under these conditions was confirmed in later experiments (Figure 5.14).

These results demonstrate that Dr1 can be recruited to Pol III-transcribed genes under repressing conditions. These data, combined with the observation of increased Dr1 occupancy at Pol III-transcribed genes when Pol III transcription is activated due to Brf1 overexpression, suggest that mere presence of Dr1 at gene promoters is not indicative of transcriptional repression.

3.2.8 Rapamycin treatment displaces Dr1 from Pol III-transcribed genes

To further investigate the presence of Dr1 at Pol III-transcribed genes under repressive conditions, HeLa cells were treated with the drug rapamycin (100 nM) for 4 hours. Under these conditions, Pol III transcription is repressed in cells treated with the drug compared to untreated cells (Ramsbottom and White, personal communication and Figure 5.20). ChIP experiments revealed that in rapamycin-treated cells Dr1 occupancy at Pol III-transcribed genes was reduced, along with that of Brf1, TFIIIC, and Pol III (Figure 3.20, A). This decrease cannot be attributed to lower protein levels, as western

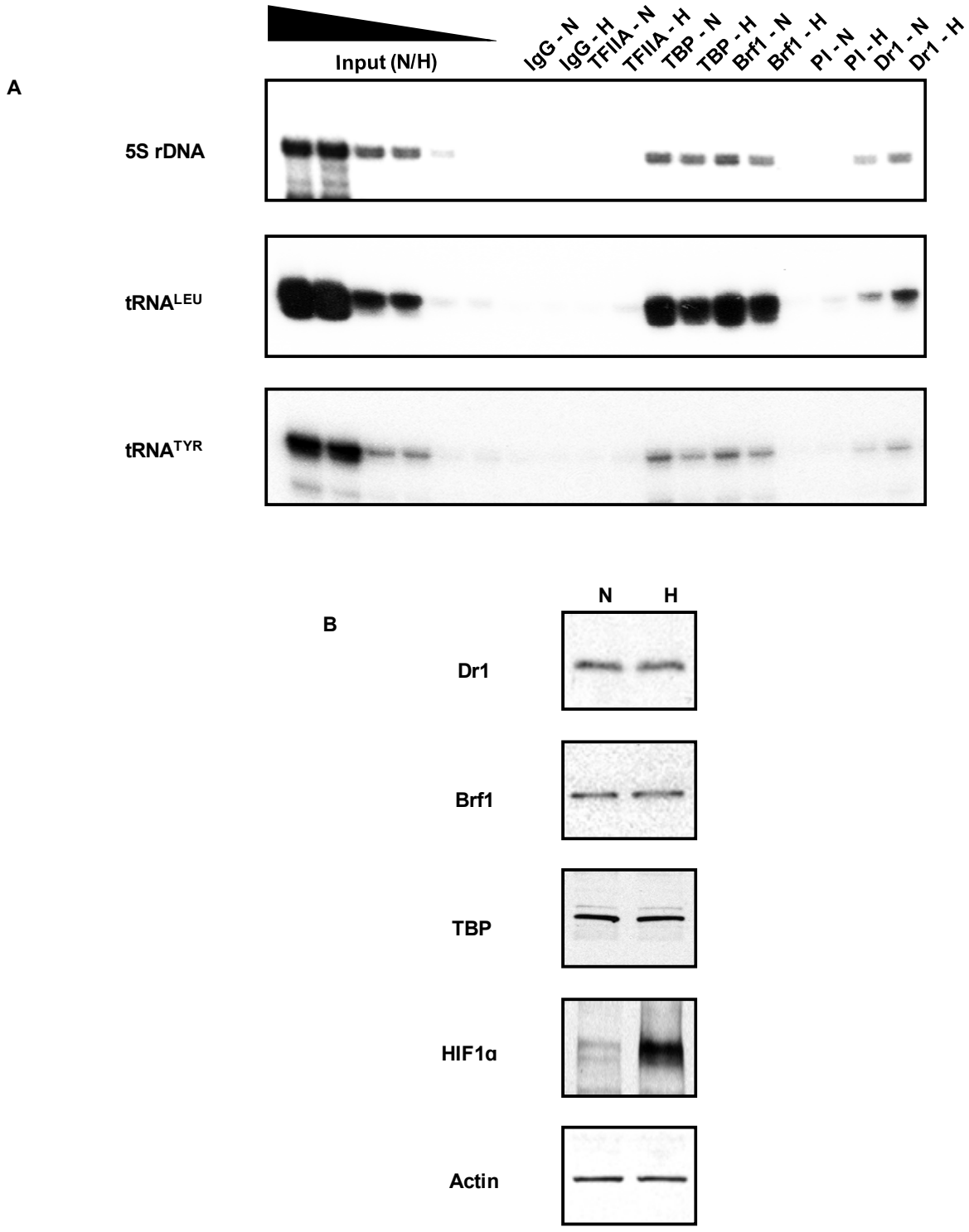


Figure 3.19. Dr1 is recruited at Pol III-transcribed genes under hypoxic conditions. U20S cells were grown under normoxia (N) or 1% O_2 hypoxia (H) for 24 hours, before they were harvested. **A.** ChIP experiments were performed using antibodies against TBP (MTBP-6), Brf1 (128) and Dr1 (1162). Pre-immune serum (PI 1162), rabbit IgGs (sc-2027) and TFIIA (FL-109) antibodies were used as negative controls. **B.** Western blotting shows that the total protein levels of Dr1, Brf1 and TBP have not changed under hypoxic conditions. HIF1α was used as a positive control and actin as a loading control.

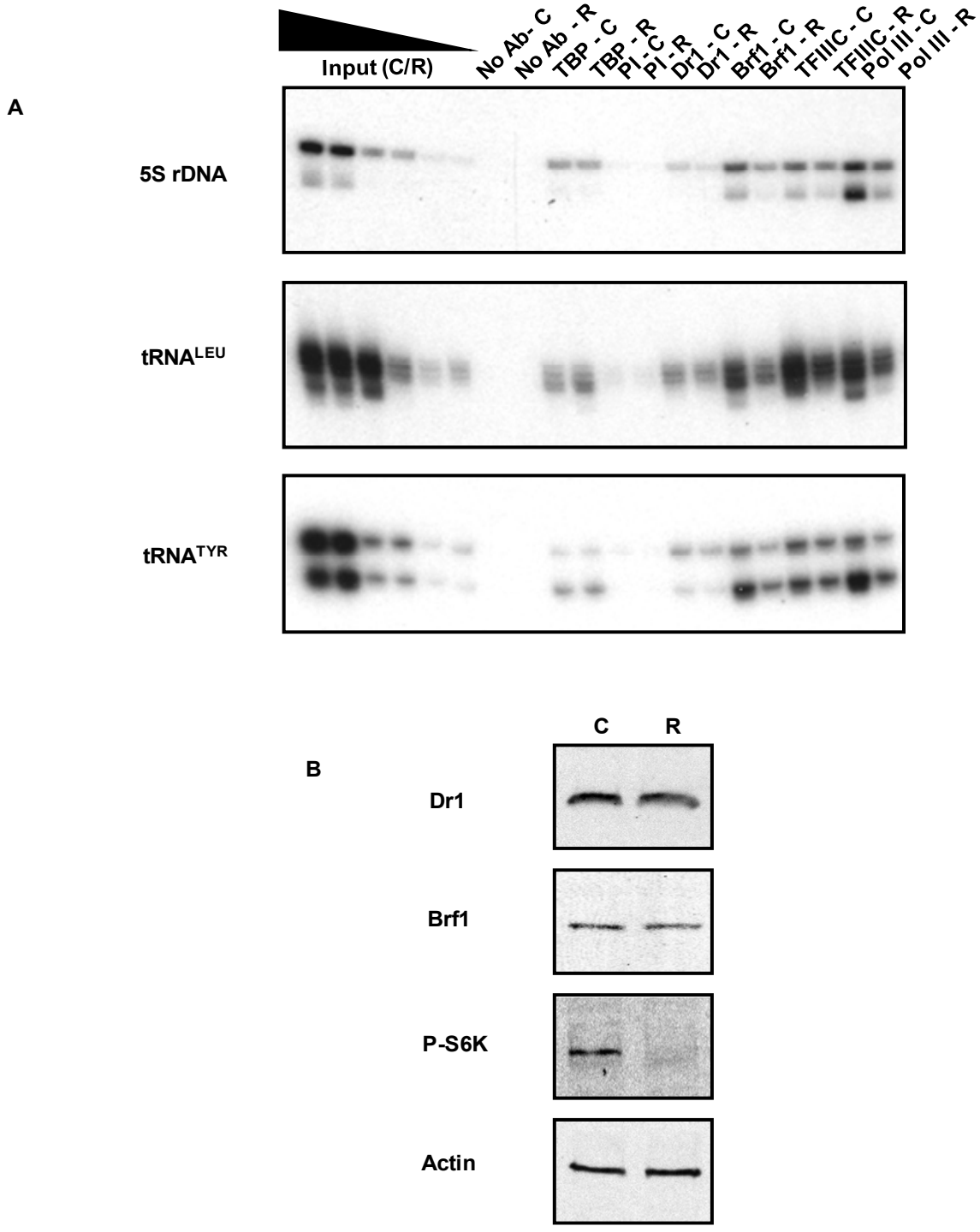


Figure 3.20. Rapamycin treatment results in displacement of Dr1 from Pol III-transcribed genes. HeLa cells were treated with rapamycin (100 nM) or with DMSO control for 4 hours before harvesting. **A.** ChIP experiments were performed using antibodies against TBP (MTBP-6), Brf1 (128), TFIIIC220 (Ab7), Pol III (1900) and Dr1 (1162). Pre-immune (PI 1162) and mock (no antibody) were used as negative controls. **B.** Western blotting shows that the total protein levels of Dr1 and Brf1 do not change after rapamycin treatment. p-S6K was used as a positive control and actin as a loading control. The "R "denotes treatment with rapamycin and the "C" treatment with DMSO control.

blotting analysis reveals that the total Brf1 and Dr1 protein levels remain unaltered (Figure 3.20, B). Interestingly, the TBP levels do not decrease, suggesting that Brf1 might have a role in Dr1 recruitment at Pol III-transcribed genes.

3.2.9 Dr1 is found at Pol III promoters together with TFIIIB rather than TFIIIC

Protein co-IP experiments revealed possible, direct or indirect, interactions of Dr1 with TFIIIB and TFIIIC. ChIP experiments in cells overexpressing Brf1 also suggested that Dr1 might be recruited to Pol III promoters by TFIIIB. However, a role of TFIIIC in recruiting Dr1 at Pol III promoters cannot be excluded. To investigate if Dr1 is preferentially associated with TFIIIB rather than TFIIIC at Pol III promoters, a 7SL gene promoter mapping approach was followed. PCR reactions using five sets of primers, designed for DNA regions at and around the Pol III-transcribed 7SL gene promoter (Figure 3.21, A), amplified the DNA immunoprecipitated from ChIP assays. It was revealed that the presence of transcription factors TFIIIB and TFIIIC at different positions at and around the 7SL gene promoter is not uniform (Figure 3.21, B). More specifically, increased occupancy of Dr1 and TFIIIB was observed at the start of the gene (position 2), but is relatively decreased at the end of it (position 3) (Figure 3.21, C). On the other hand, TFIIIC and Pol III occupancies remain stable throughout the gene (positions 2 and 3) (Figure 3.21, C). According to these results, the presence of Dr1 at the 7SL gene seems to relate more to the presence of TFIIIB, rather than TFIIIC, implying that Dr1 is bound to and recruited by TFIIIB.

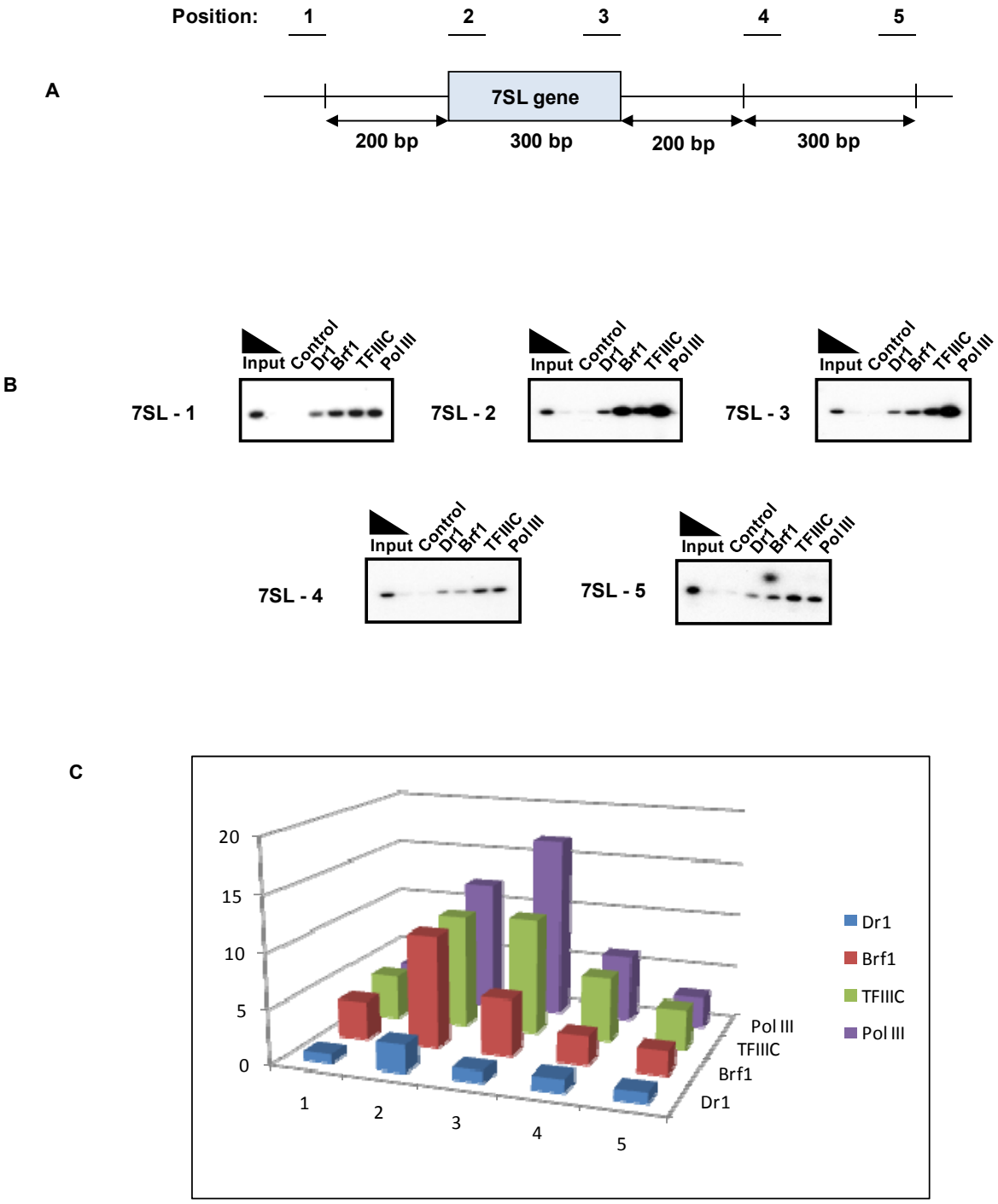


Figure 3.21. Dr1 occupancy at the 7SL gene locus. ChIP assays were performed in HeLa cells. **A.** Schematic diagram showing the relative positions of the five primer sets (1-5) at the 7SL gene. **B.** ChIPs were performed using the Dr1 (1162), Brf1 (128), TFIIIC (Ab7) and Pol III (1900) antibodies together with the 1162 pre-immune (PI) serum and mock (no antibody) as negative controls. **C.** Schematic representation of the ChIP signals. The x-axis represents the five primer sets. The Y-axis shows the mean relative strengths of ChIP signal expressed in arbitrary units after normalisation against input and the signal with pre-immune serum. n=3.

3.3 DISCUSSION

3.3.1 Dr1 can regulate tRNA expression *in vivo*

It has been previously shown that Dr1 can affect Pol III transcription *in vitro* in HeLa nuclear extracts (White et al, 1994) and *in vivo* in yeast (Kim et al, 1997). yDr1 shares 37% identity with its human homolog, most of it is in the histone fold domain, while its C-terminal region does not contain the QA domain, which is important for repression in human (Goppelt & Meisterernst, 1996; Kim et al, 1997). Therefore, it would be interesting to test if Dr1 can regulate Pol III transcription *in vivo* in human cells. In order to investigate this, an RNAi approach was employed and each of the three human Dr1 exons was targeted either by shRNA (exon 2) or siRNA (exons 1 and 3). The targeting of all three different exons by two different RNAi approaches should minimise off-target effects and provide reliable results.

RNAi for Dr1 in human HeLa cells resulted in an about 40% decrease of Dr1 protein levels and about 60% decrease in mRNA levels compared to the control (Figures 3.5 and 3.7). It is interesting that all three different experiments resulted in very similar levels of Dr1 reduction, both in protein and mRNA level. Attempts to achieve a better knock-down by fine-tuning the transfection methods, the amount of shDNA or siRNA used or the time before harvesting the cells, did not result in a better outcome. Taking into account that Dr1 is considered to have a general role in transcription affecting, perhaps, as many as 25% of the human Pol II-transcribed genes (Albert et al, 2007) and is important for cell growth in yeast (Kim et al, 1997), this might indicate a need for the presence of a certain lower limit amount of Dr1 protein in cells, essential for cell growth and survival.

Nevertheless, the RNAi-mediated depletion of Dr1 did have an effect on tRNA expression. More specifically, tRNA expression was elevated about two-fold, both for $tRNA^{LEU}$ and $tRNA^{TYR}$, in all three experiments (Figures 3.6 and 3.8). This result indicates that Dr1 can regulate Pol III transcription *in vivo* and argues for a repressive role of Dr1 in human cells, in accordance with the *in vitro* data (White et al, 1994), and its well documented role as a repressor in yeast (Kim et al, 1997).

Interestingly, the effect was specific for tRNAs and not other Pol III templates, like 5S rRNA, 7SK RNA and U6 snRNA. This could imply that Dr1 particularly affects

promoter type 2 (tRNA) genes and not type 1 (5S rRNA) or type 3 (7SK, U6 snRNA). However, the failure to detect increased transcripts of all templates might also be attributed to technical issues. One cannot exclude the possibility that more efficient Dr1 depletion and consequently lower Dr1 protein levels, could result in increased expression of the other Pol III products. Furthermore, it should be noted that the primers designed for detection of 5S rRNA, 7SK RNA and U6 snRNA recognise the final mature RNA product (after its reverse transcription to cDNA), which can be relatively stable. In contrast, the primers for $tRNA^{LEU}$ and $tRNA^{TYR}$ are designed to detect the unspliced short-lived tRNA precursor, which allows for the detection of newly synthesised tRNAs, and better represent the transcription product. Therefore, the two-fold increase in tRNAs would suggest an increase in Pol III transcription due to relief from Dr1 repression.

Since Dr1 can repress both Pol II and Pol III transcription (Inostroza et al, 1992; White et al, 1994), it is possible that the two-fold increase in tRNA after depletion of Dr1, is not directly due to alleviation of Pol III transcriptional repression, but rather of Pol II. Indeed, since the Pol II-transcribed TFIIIB and TFIIIC can be limiting factors for Pol III transcription (White, 1998), increased Pol II transcription could result in increased levels of TFIIIB and TFIIIC and subsequent elevation of Pol III transcription. However, the protein and RNA levels of all three subunits of TFIIIB did not change after Dr1 RNAi (Figure 3.9, 3.10 and 3.11). The same is true for the three TFIIIC subunits that were examined (Figure 3.9, 3.10 and 3.11). Pol III protein levels were also not affected, indicating that Pol III remained stable too (Figure 3.9, B). These data demonstrate that TFIIIB, TFIIIC and Pol III were not affected in Dr1-depleted cells and, therefore, the observed effect in tRNA expression cannot be attributed to elevated Pol II transcription, but rather to relief of Pol III repression.

3.3.2 Dr1 occupancy at Pol III-transcribed genes

The Dr1-DRAP1 complex is thought to repress transcription through binding to TBP and blocking recruitment of TFIIB or TFIIIB (Inostroza et al, 1992; White et al, 1994). Interestingly, the Dr1-DRAP1 subunits have been found at Pol II-transcribed gene promoters and it was suggested that the Dr1-DRAP1 complex might have a positive role in transcription (Christova & Oelgeschlager, 2002; Creton et al, 2002; Geisberg et al, 2001).

ChIP experiments were employed to investigate if Dr1 is found at Pol III-transcribed genes.

Indeed, Dr1 is found at a number of Pol III templates like 5S rRNA, $tRNA^{LEU}$ and $tRNA^{TYR}$ and U6 snRNA genes (Figure 3.12). The binding of Dr1 at these Pol III genes seems to be specific, as no signal could be detected in a mock ChIP with no antibody or when the Dr1 pre-immune sera was used. Dr1 was also found at Pol II-transcribed genes encoding U1 snRNA and Hsp70; the latter acted as positive control, as the presence of Dr1 at Hsp70 promoters has been previously reported (Christova & Oelgeschlager, 2002). Neither Brf1 nor Pol III were detected at Pol II-transcribed genes, acting as internal controls. Furthermore, no signal was detected at the non-transcribed region upstream of the U6 snRNA promoter and also in the coding region of the Pol II-transcribed ARPP P0 gene, suggesting specificity for the gene promoter regions.

The ChIP experiments revealed Dr1 occupancy at Pol III-transcribed genes (Figure 3.12). In these experiments the Dr1 1162 serum was used, but identical results were obtained also by Dr1 1163 serum (data not shown). However, the validation of both Dr1 1162 and 1163 sera revealed a couple of extra bands of unknown origin and higher molecular weight than the one expected for Dr1 (Figure 3.4). Since one cannot exclude the possibility that these Dr1 antibodies can recognise proteins other than Dr1, the ChIP experiments were repeated, using an additional commercial Dr1 (ab28185) antibody that became available recently. Dr1 was detected by both antibodies at the Pol III-transcribed genes tested, i.e. 5S rRNA, $tRNA^{LEU}$ and $tRNA^{TYR}$, confirming that Dr1 can occupy Pol III-transcribed genes (Figure 3.13). It must be noted that in this experiment the signal of Dr1 in comparison to the other factors is lower when compared to the one obtained in a number of previous experiments (compare TBP, Brf1, Pol III signal to Dr1 in Figures 3.12 and 3.13). However, the signal from both Dr1 (ab28185) and Dr1 1162 antibodies was clearly above the background level as defined by the mock (no antibody) control, as well as with the TFIIA and pre-immune sera controls. Therefore the differences are probably due to the experimental conditions of this specific experiment. Taken together, these results show that Dr1 can be found at Pol III-transcribed genes in human cells.

An antibody recognising TFIIA was employed as negative control in ChIP experiments and revealed absence of TFIIA at Pol III-transcribed genes (Figure 3.13). However, *in vitro* experiments have suggested that TFIIA can regulate Pol III transcription

(Meissner et al, 1993; Waldschmidt & Seifart, 1992). Experiments with better purified factors suggested that TFIIA was not essential for Pol III transcription (Hu et al, 2003). To exclude an *in vivo* supporting role of TFIIA in Pol III transcription, ChIP experiments were employed. TFIIA was found to occupy the Pol II-transcribed U1 snRNA gene, but not the Pol III-transcribed 5S rRNA, $tRNA^{TYR}$, 7SK and U6 snRNA genes (Figure 3.11). These data suggest that TFIIA does not have an *in vivo* role in Pol III transcription regulation.

It has been demonstrated that in yeast cells the level of Pol II transcriptional activity strongly correlates with the level of TBP association at promoters (Kuras & Struhl, 1999; Li et al, 1999b) and that the TBP/TFIIA and TBP/TFIIB occupancy ratios are essentially constant at promoters (Kuras et al, 2000). ChIP experiments showed that BUR6 (the yeast homolog of DRAP1) was found at Pol II promoters and that the ratio of BUR6/TBP was very similar to that of TFIIB/TBP; enhanced BUR6/TBP ratio at specific genes was interpreted as a direct, positive transcriptional role of the Dr1-DRAP1 complex (Geisberg et al, 2001). In the same experiments DRAP1, was found at a very low level at a tRNA promoter; the signal ratio of BUR6/TBP was <0.05 and it was concluded that Dr1-DRAP1 is not found at Pol III-transcribed genes (Geisberg et al, 2001). However, the results presented in this thesis argue that the Dr1-DRAP1 complex is present at human Pol III-transcribed genes (Figure 3.9). The ratio of Dr1/TBP at all Pol III promoters tested is ~0.5, the same as that of Dr1/TBP at Pol II-transcribed genes (Table 3.1). These data demonstrate that Dr1 is found at human Pol II and Pol III at a similar ratio, in contrast to findings in yeast (Geisberg et al, 2001).

Dr1 RNAi affected the expression of tRNA (promoter type 2), but not of other Pol III templates (Figures 3.6 and 3.8). On the other hand, ChIP experiments showed the presence of Dr1 at all Pol III-transcribed genes tested (promoter types 1, 2 and 3) (Figure 3.12). Technical reasons that might inhibit the detection of 5S rRNA and U6 snRNA products have already been discussed. The finding of Dr1 at 5S rRNA and U6 snRNA gene promoters, might argue for a general repressive role of Dr1 at all Pol III target genes.

DRAP1 occupancy at Pol II-transcribed genes has been interpreted as evidence of transcriptional activation (Creton et al, 2002; Geisberg et al, 2001). In this light, Dr1 occupancy at Pol III-transcribed genes contradicts the RNAi evidence of Dr1 repressing tRNA expression. However, Dr1 occupancy at promoter regions as shown by ChIP experiments might not necessarily be indicative of transcriptional activation or repression.

It might be that ChIP experiments recognize TBP and Dr1 at transcriptionally silent genes; it has been shown that in yeast, TBP and Pol II can been found associated with heterochromatin (Sekinger & Gross, 2001), and also that human TBP and Dr1 can be found at mitotic chromosomes (Christova & Oelgeschlager, 2002). Moreover, it is possible that the signal detected by ChIP does not originate from active, but rather from transcriptionally repressed genes, considering that in a cell population, not all the genes coding for a transcript are necessarily active or inactive at the same given time.

In an attempt to address these arguments, sequential ChIP experiments were employed to determine co-occupancy of Dr1 and Pol III. The idea behind this experiment was that after the first round of ChIP, by using antibodies against Pol III, only active Pol III-bound genes would be precipitated; from that pool, the second ChIP with Dr1 antibodies would select those genes that are bound both by Pol III and Dr1.

As it is shown in figure 3.15, Dr1 was found to co-occupy the tested 5S rRNA and $tRNA^{LEU}$ genes together with Pol III or TBP, but not TFIIB. The Pol II-transcribed Hsp70 and U1 snRNA genes were occupied by TBP and TFIIB, but not Pol III, which in this case acts as negative control. No signal was detected form the non-transcribing region upstream of the U1 snRNA gene promoter, indicating the specificity of the experiment. There was a higher background signal in the pre-immune sera in the case of Pol III antibody, compared to that of TFIIB and TBP antibodies used in the primary ChIP, but this was clearly of much less intensity than the signal given by the Dr1 1162 and 1163 sera in both 5S rRNA and $tRNA^{LEU}$ genes. It must be noted that the amount of the immunoprecipitated material after the primary ChIP, as indicated by the secondary TFIIB, Pol III and TBP inputs, was not always equal, representing the different immunoprecipitating efficiencies of the different antibodies. Nevertheless, these data show that Dr1 can be found at Pol III promoters together with Pol III, suggesting that Dr1 can be found at active Pol III-transcribed genes. Furthermore, they also suggest that Dr1 can be found together with TFIIB at Pol II-transcribed genes, which contrasts with the proposed repression mechanism of Dr1-DRAP1 (Cang & Prelich, 2002; Goppelt et al, 1996; Mermelstein et al, 1996), but is in agreement with studies in yeast (Creton et al, 2002; Geisberg et al, 2001).

These surprising findings might indicate that the Dr1-DRAP1 complex can be found at promoters in an inactive, non-repressing form, as part of an active Pol II, or in an analogous way Pol III, transcription complex. In this case Dr1-DRAP1 might be bound to

TBP, but still allow TFIIB, or analogously Brf1, to productively bind the Dr1-DRAP1-TBP-promoter complex. This possibility, that would provide an explanation for the ChIP (Figure 3.12) and sequential ChIP results (Figure 3.15), in this and previous studies (Creton et al, 2002; Geisberg et al, 2001; Gilfillan et al, 2005), has already been suggested as a potential strategy for upregulation of Pol II transcription initiation (Kamada et al, 2001); transcriptional activators and/or positive cofactors might bind and alter the conformation of the Dr1 helices that contact the DNA backbone and upper surface of TBP (Figure 3.3), allowing efficient binding of TFIIB, while the histone-like portion of Dr1-DRAP1 remains bound to the core promoter (Kamada et al, 2001). Possible advantages for the presence of the Dr1-DRAP1 complex at the promoter might be the more efficient binding of TBP to DNA, especially at TATA-less promoters (Gilfillan et al, 2005), as well as the potential for rapid repression of transcription.

It is well established that Dr1 can bind to TBP and inhibit binding of TFIIB (Goppelt et al, 1996; Inostroza et al, 1992; Mermelstein et al, 1996; Yeung et al, 1994). It has also been shown that GST-fused Brf1 can bind to TBP, but this interaction is lost if rDr1 is included in the reaction mix, indicating that Dr1 can disrupt TFIIIB by blocking binding of TBP and Brf1 (White et al, 1994). To further investigate the interaction of Dr1 with TFIIIB and TFIIIC, co-immunoprecipitation experiments were employed. Dr1 was found to interact with radio-labelled subunits of TFIIIB and TFIIIC (Figure 3.16), as well as with endogenous subunits of TFIIIB and TFIIIC (Figure 3.17). These data suggest that *in vivo*, Dr1 can be found in complexes with TFIIIB and TFIIIC, in contrast to previous reports *in vitro* (White et al, 1994). Dr1-TFIIIB-TFIIIC interactions come in agreement with the ChIP data showing Brf1 at Pol III-transcribed gene promoters and suggest that perhaps Brf1 or subunits of TFIIIC could recruit Dr1 to Pol III promoters. Co-IP experiments with TFIIB have not been reported and it is possible that *in vivo*, but not *in vitro*, TFIIB can be found in complexes with Dr1 and TBP. The presence of TFIIB in a similar ratio as DRAP1 at the same Pol II promoters in yeast (Geisberg et al, 2001), and the co-occupancy of TFIIB and Dr1 at Pol II promoters in human cells (Figure 3.15) would support this idea.

3.3.3 Dr1 presence at Pol III promoters does not necessarily correlate with repression or activation of Pol III-dependent transcription

The findings that Dr1 associates with Pol III-transcribed genes (Figure 3.12), co-occupies Pol III templates with Pol III (Figure 3.15), and forms a complex with TFIIIB and TFIIIC *in vivo* (Figure 3.16 and 3.17), demonstrate recruitment of Dr1 to Pol III promoters. To investigate if TFIIIB is responsible for recruiting Dr1, a Brf1-inducible cell line was employed. ChIP analysis revealed that cells overexpressing Brf1 had increased Dr1 occupancy at Pol III-transcribed genes, compared to cells expressing physiological amounts of Brf1 (Figure 3.18). TBP occupancy was also elevated, suggesting that overexpression of Brf1 results in TFIIIB and Dr1 recruitment to Pol III promoters. Since TFIIIC is recruited at Pol III promoters previously to TFIIIB, it seems that the latter might be recruiting Dr1, although a role for TFIIIC cannot be excluded. Interestingly, this Dr1 recruitment correlates with increased Pol III transcription (Marshal and White, personal communication), offering further support to the argument that Dr1 can bind active Pol III-transcribed genes.

However, RNAi experiments revealed a negative role of Dr1 in Pol III transcription (Figures 3.6. and 3.8). To test if Dr1 presence at Pol III-transcribed genes is also enhanced under repressive conditions, ChIP assays were performed with cells growing under normal or hypoxic conditions. Under hypoxia, TFIIIB is displaced from Pol III-transcribed genes, but Dr1 occupancy is increased (Figure 3.19, A). These results agree with previous findings that associated Dr1 with Pol II transcriptional repression under hypoxic conditions (Denko et al, 2003). However, in contrast to that report, under these conditions Dr1 protein levels were not altered (Figure 3.19, B). A possible explanation might be the different cells lines (human U2OS and murine hepatoma) used or that the current experiments used mild hypoxia of 1% O_2, in contrast to the almost anoxic conditions of 0.01% O_2 used previously (Denko et al, 2003). Nevertheless, these data, taken together with those from the Brf1 overexpression, suggest that enhanced occupancy of Pol III promoters by Dr1 can correlate both with transcriptional activation and repression, suggesting that other factors might be implicated and dictate the final transcriptional outcome.

To further examine the Dr1 status at Pol III-transcribed genes under stress conditions, the drug rapamycin was used to inhibit the mTOR pathway and repress Pol III transcription. ChIP analysis revealed that in cells treated with rapamycin, Dr1 occupancy at

Pol III-transcribed genes was reduced (Figure 3.20, A), while the protein levels of Dr1 were not affected (Figure 3.20, B). Brf1, TFIIIC and Pol III were also displaced, but interestingly TBP occupancy was not affected (Figure 3.20, A). This finding suggests that Dr1 presence at the Pol III promoters does not necessarily correlate with TBP, implying that other factors might have a more active role in the recruitment and association of Dr1 with Pol III promoters.

Potential factors that could recruit Dr1 to Pol III promoters might include Brf1 and TFIIIC, as well as Pol III itself. Promoter mapping experiments were employed in an attempt to map the presence of Dr1 at a Pol III promoter and distinguish how this relates with the presence of the previously mentioned factors. These experiments revealed that Dr1 is present at the beginning of the Pol III-transcribed 7SL gene, but not at the end of it, following the same pattern with Brf1 (TFIIIB), but not TFIIIC or Pol III and suggesting that Dr1 is rather associated with Brf1 than TFIIIC or Pol III (Figure 3.21). This finding therefore implies that *in vivo* Brf1 might have a role in the recruitment of Dr1 to Pol III promoters.

In summary, this chapter presented data that Dr1 can repress Pol III transcription *in vivo*, and that this effect seems to be direct, rather than mediated by changes in expression of TFIIIB, TFIIIC or Pol III. ChIP experiments revealed Dr1 occupancy at Pol III promoters and sequential ChIP experiments showed co-occupancy of Dr1 with Pol III, suggesting the presence of Dr1 at active Pol III-transcribed genes. Dr1 can be associated with TFIIIB, probably through both TBP and Brf1, and through these interactions might be recruited to Pol III promoters. Promoter occupancy by Dr1, however, is a subject of regulation and can be related to either transcriptional activation or repression. These data contradict the model of Dr1-DRAP1 repression by inhibition of an active preinitiation complex (Goppelt et al, 1996; Inostroza et al, 1992; Mermelstein et al, 1996) and come in partial agreement with reports suggesting the presence of Dr1-DRAP1 at active genes (Creton et al, 2002; Geisberg et al, 2001).

CHAPTER 4

Studies on the role of DRAP1 in RNA polymerase III transcription

4.1 Introduction

4.1.1 DRAP1 is found at Pol II promoters and might have roles independent of Dr1

As previously described, DRAP1 is considered to be a co-repressor that cannot repress transcription independently, but significantly enhances Dr1-mediated transcriptional repression (Mermelstein et al, 1996; Yeung et al, 1997). A number of yeast studies, with strains expressing mutant BUR6 (the yeast homolog of DRAP1), reported increased transcription of specific genes and the presence of BUR6 at active Pol II promoters, suggesting a positive role for the Dr1-DRAP1 complex (Cang & Prelich, 2002; Geisberg et al, 2001). Moreover, it was observed that the presence of BUR6 at Pol II promoters correlated with transcriptional activity, while increased presence of yDr1 correlated with transcriptional repression, suggesting that the two subunits might play different roles *in vivo* (Creton et al, 2002).

DRAP1 has also been found at TATA-containing and TATA-less promoters of Pol II genes in human cells (Gilfillan et al, 2005). A recent genome-wide promoter association study of DRAP1 in human cells revealed that DRAP1 was present at more than 25% of human Pol II promoters, supporting the original hypothesis of a general role of Dr1-DRAP1 in gene transcription regulation (Albert et al, 2007). DRAP1 occupancy positively correlated with mRNA levels, probably reflecting the Dr1-DRAP1 capacity to stabilise TBP on promoter regions (Albert et al, 2007). In contrast to a previous study in *Drosophila* (Willy et al, 2000), genome-wide DPE promoter binding in human was not confirmed (Albert et al, 2007), neither activation of the human DPE-dependent IRF1 gene (Lewis et al, 2005); however, there is some correlation of Dr1-DRAP1 occupancy with the initiator element, which was also independently confirmed (Albert et al, 2007; Malecova et al, 2007). DRAP1 was also found to interact with BTAF1, the mammalian homolog of yeast Mot1 (Klejman et al, 2004). The role of this interaction is not clear, but it seems that BTAF1 can compete with Dr1 for binding at overlapping surfaces of TBP and DRAP1; it was proposed that BTAF1 may first contact DRAP1 and then disrupt the Dr1-DRAP1 complex from TBP, relieving repression and contributing to transcriptional activation (Klejman et al, 2004).

Interestingly, as was suggested by yeast studies (Creton et al, 2002; Kim et al, 2000), DRAP1 seems to have Dr1-independent roles. Knock-out of the DRAP1 gene

resulted in embryonic lethality and it was found that DRAP1, independently of Dr1, can interact with and inhibit binding of DNA by the transcription factor FoxH1, a critical component of a positive feedback loop for *Nodal* signalling (Iratni et al, 2002). Furthermore, in rice OsDRAP1 is the main repressor of the complex, with Os Dr1 having a co-repressor role (Song et al, 2002).

4.2 Results

4.2.1 DRAP1 RNAi and Pol III transcription

Nothing has been reported so far concerning the role of DRAP1 in Pol III transcription. Therefore, it would be interesting to investigate if DRAP1 has a role in affecting Pol III transcription in human cells. To this end, shRNA was employed in an attempt to knock down DRAP1 and study the effects on Pol III-transcribed genes. HeLa cells were transiently transfected by electroporation, with either a pSUPER-DRAP1 plasmid or the empty pSUPER vector as control (Brummelkamp et al, 2002). The cells were harvested 48 hours later, whole cell extracts were prepared and RNA was isolated. RT-PCRs showed that the DRAP1 mRNA levels were depleted by more than 90% (Figure 4.1, B and C); however the protein levels were decreased by an average of only 25% (Figure 4.1, A and C). Under these conditions the levels of $tRNA^{LEU}$ and $tRNA^{TYR}$ tend to increase (Figure 4.2, A), but the experimental errors do not allow for a sound conclusion (Figure 4.2, B).

In an attempt to clarify the effect of DRAP1 knock-down on Pol III transcription, a siRNA approach was also employed. siRNA targeting DRAP1 or control siRNA targeting Oct-1 were transfected into cells. 48 hours later, the cells were harvested and cell extracts and RNA were analysed. Western blotting revealed that at the protein level, the positive control Oct-1 was reduced by more than 55% (Figure 4.3, A). RT-PCRs revealed that DRAP1 mRNA levels were decreased by more than 80% (Figure 4.3, B and C), but still the protein levels remained quite high, reduced by about 35% (Figure 4.3, A and C). Under these condition the 5S rRNA and U6 snRNA Pol III-transcripts were not affected, but $tRNA^{LEU}$ and $tRNA^{TYR}$ tended to decrease by about 28% compared to the control (Figure 4.4, A and B). However, the experimental errors were not optimal, casting doubt on the validity of these results (Figure 4.4, B).

In summary, the two RNAi approaches employed to investigate the effect of DRAP1 knock-down on Pol III transcripts gave confusing results. The shRNA experiments revealed a tendency for tRNA expression to increase, while the siRNA experiments showed tRNA expression to decrease. In both cases the experimental errors do not allow

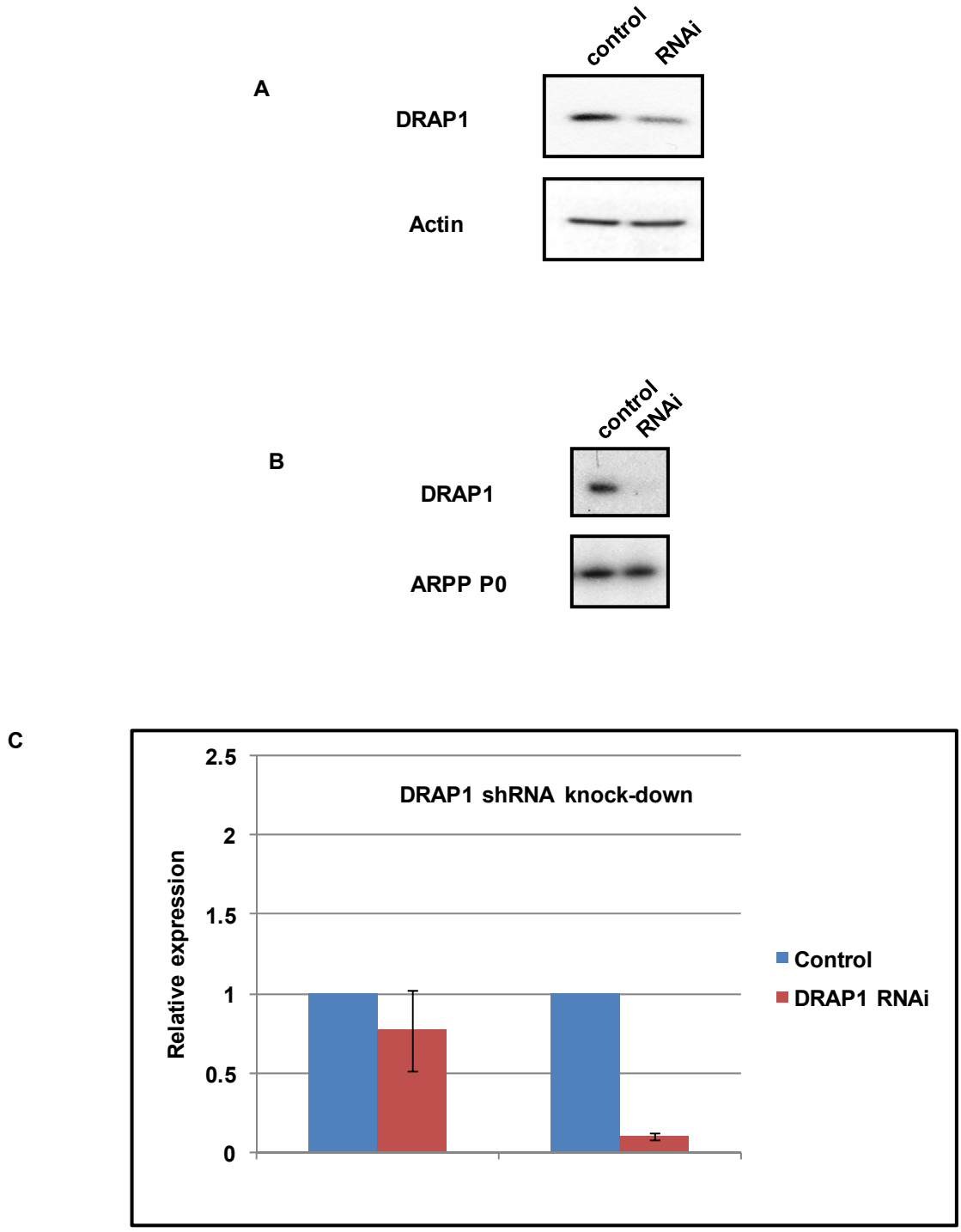


Figure 4.1. DRAP1 knock-down by shRNA. HeLa cells were transfected with the pSUPER-DRAP1 vector by electroporation and harvested 48 hours later. An empty pSUPER vector was used as control. **A.** Western analysis for DRAP1 from whole cell extracts. Actin was used as loading control. **B.** RT-PCRs for DRAP1 mRNA. The Pol II-transcribed gene ARPP P0 was used as control. **C.** Quantification of DRAP1 signals from A and B. n=2.

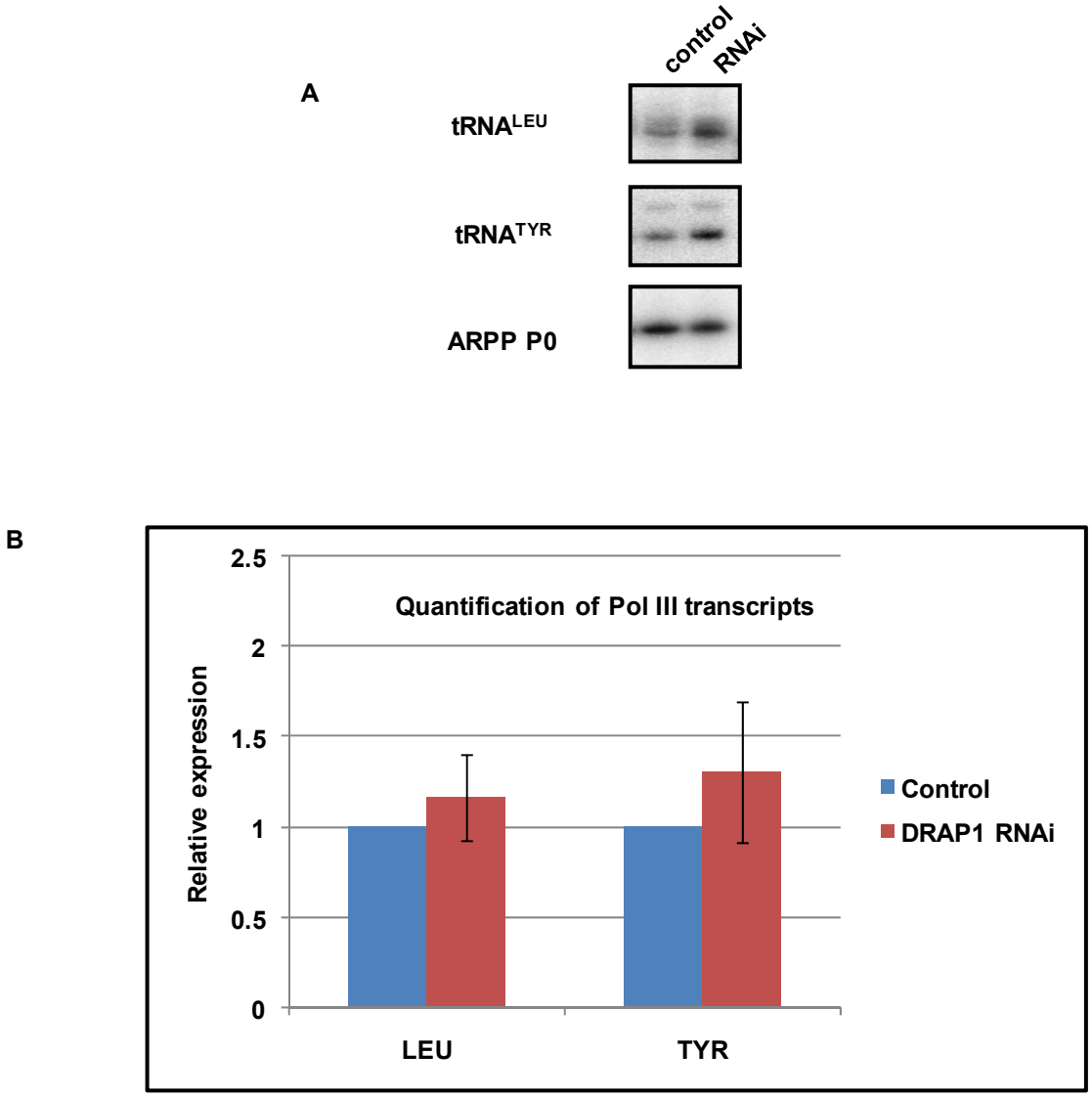


Figure 4.2. tRNA expression levels tend to increase after DRAP1 shRNA knock down. HeLa cells were transfected with the pSUPER-DRAP1 vector by electroporation and harvested 48 hours later. An empty pSUPER vector was used as control. **A.** RT-PCRs for $tRNA^{LEU}$ and $tRNA^{TYR}$. The Pol II-transcribed gene ARPP P0 was used as control. **B.** Quantification of tRNA signals from A. n=2.

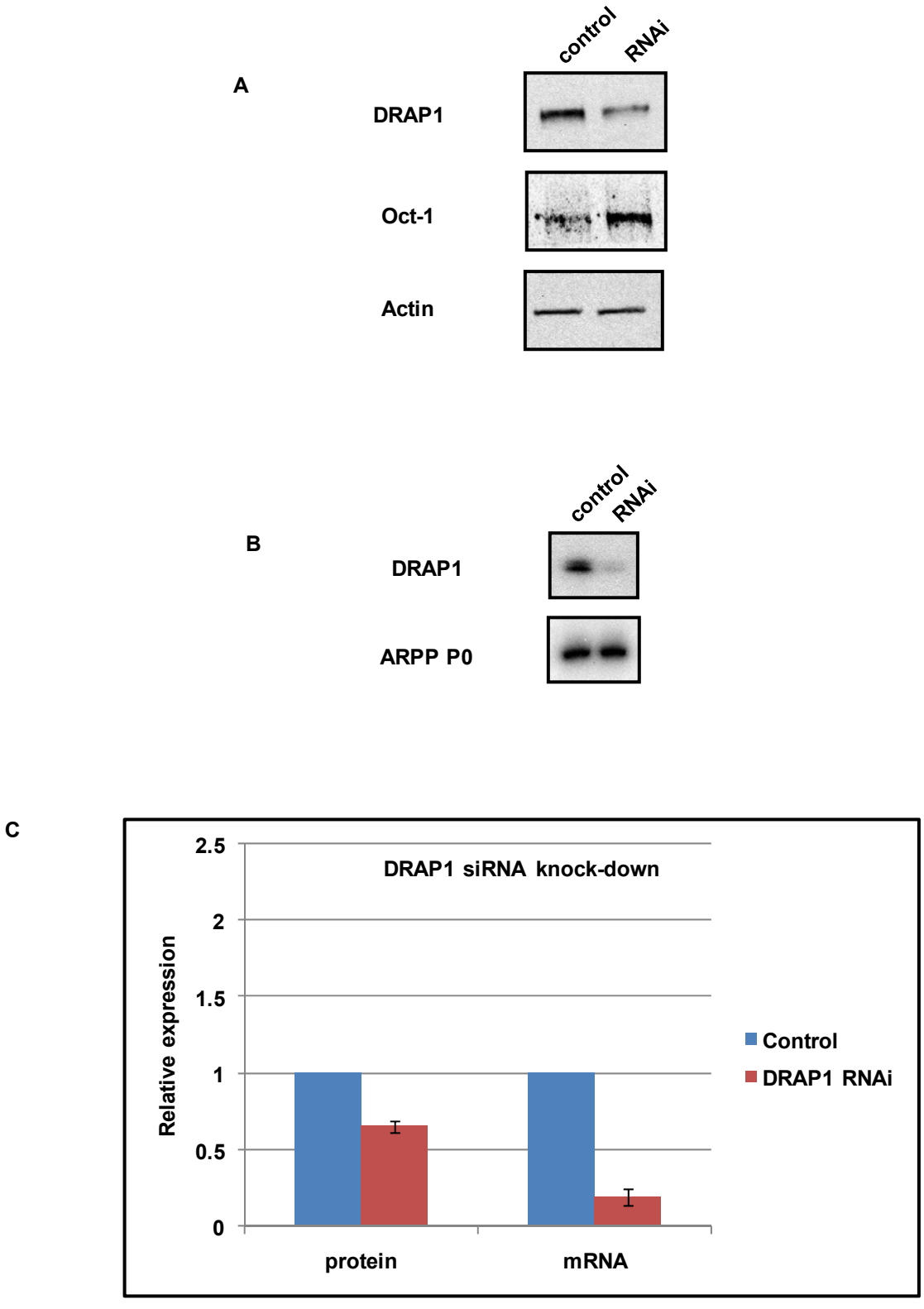


Figure 4.3. DRAP1 knock-down by siRNA. HeLa cells were transfected with DRAP1 siRNA by electroporation and harvested 48 hours later. siRNA targeting Oct-1 was used as control. **A.** Western analysis for DRAP1 from whole cell extracts. Actin was used as loading control. **B.** RT-PCRs for DRAP1. The Pol II-transcribed gene ARPP P0 was used as control. **C.** Quantification of DRAP1 signals from A and B. n=2.

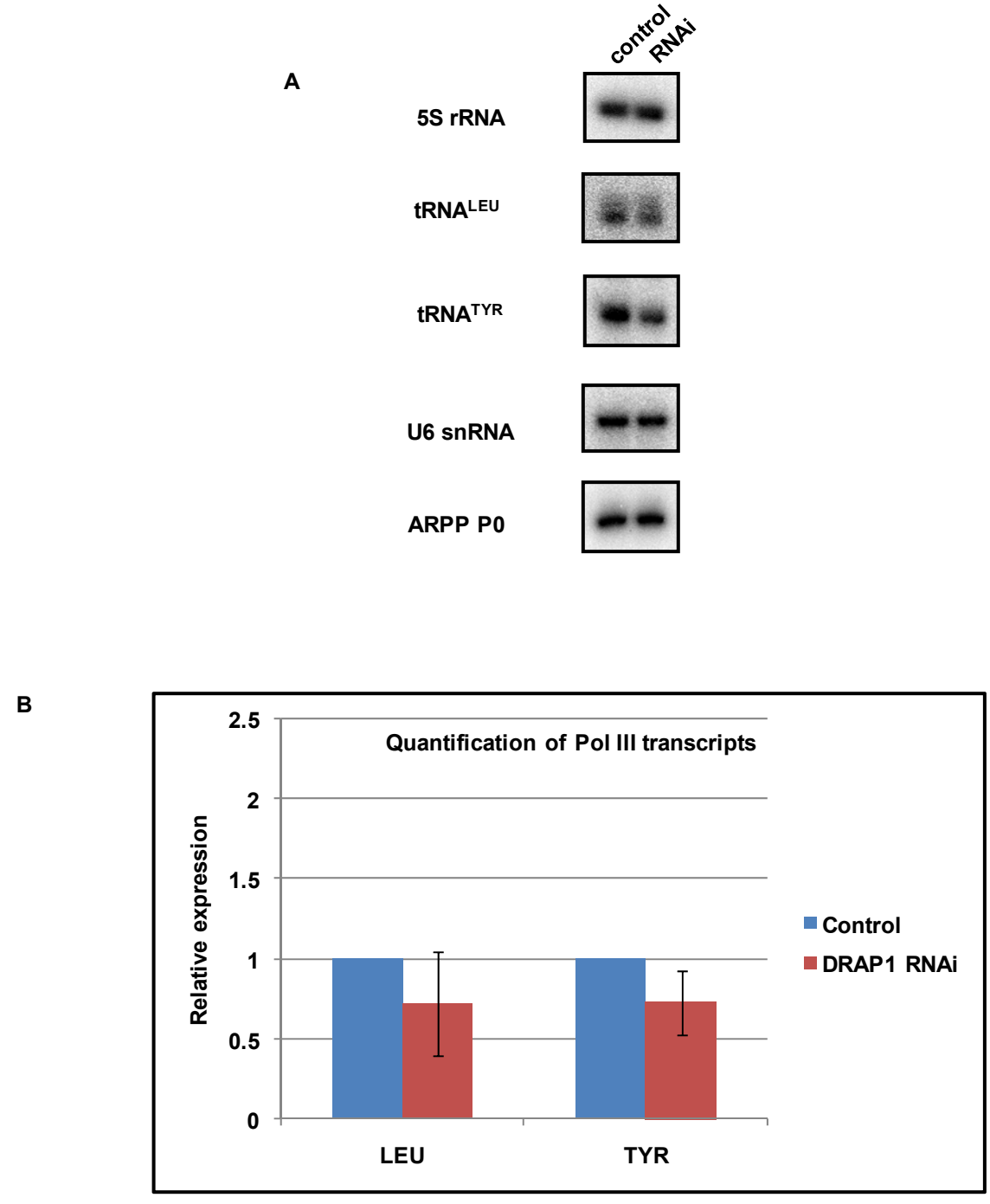


Figure 4.4. tRNA expression levels tend to decrease after DRAP1 siRNA knock-down. HeLa cells were transfected with DRAP1 siRNA by electroporation and harvested 48 hours later. siRNA targeting Oct-1 was used as control. **A.** RT-PCRs for $tRNA^{LEU}$ and $tRNA^{TYR}$. The Pol II-transcribed gene ARPP P0 was used as control. **B.** Quantification of tRNA signals from A. n=2.

for safe interpretations of the results obtained. Moreover, in both cases, DRAP1 depletion at the protein level proved to be difficult, in contrast to the mRNA level, suggesting that DRAP1 protein can be quite stable under these conditions.

4.2.2 DRAP1 is found at Pol III-transcribed genes

DRAP1 has been found at Pol II promoters in yeast (Creton et al, 2002; Geisberg et al, 2001) and human (Albert et al, 2007; Gilfillan et al, 2005; Lewis et al, 2005). DRAP1 occupancy at Pol III-transcribed genes has been investigated in yeast, but found to be present at very low levels compared to TBP and therefore, DRAP1 (and the Dr1-DRAP1 complex) was proposed to be absent from Pol III promoters (Geisberg et al, 2001). However, the current study previously showed that Dr1 is found at Pol III-transcribed genes in human cells (Figure 3.9 and 3.10), implying that DRAP1 might also be present there.

To investigate if DRAP1 can be found at Pol III-transcribed genes, ChIP experiments were employed. Endogenous DRAP1 can be found at the Pol III-transcribed 5S rDNA, $tRNA^{LEU}$, $tRNA^{TYR}$ and U6 snRNA genes in HeLa cells (Figure 4.5). DRAP1 can also be found at the Pol II-transcribed Hsp70 and U1 snRNA genes, but not in the internal ARPP P0 region or the non-transcribed U6 snRNA upstream region, showing that under these experimental conditions the antibodies specifically recognise proteins bound at, or near to the promoters of the specific genes (Figure 4.5). As expected, Brf1 and Pol III are not present at Pol II genes, working as internal controls for the specificity of the antibodies (Figure 4.5). To further validate these results, the ChIP experiments were repeated with a different DRAP1 antibody together with Dr1 (Figure 4.6). These experiments confirmed the presence of DRAP1, as well as Dr1, at Pol III-transcribed genes and minimised the possibility of recognition of other proteins at Pol III-transcribed genes by a DRAP1 antibody.

As previously mentioned, the DRAP1/TBP ratio from ChIP experiments in yeast was found to be below 0.05 and compared to ratios of over 0.8 for Pol II-transcribed genes, considered negligible (Geisberg et al, 2001). However, this does not seem to be the situation in humans. The DRAP1/TBP ratios at Pol III-transcribed genes were found to be 0.4-0.6 and the ratio at Pol II-transcribed genes 0.7-0.8 (Table 4.1). These results suggest

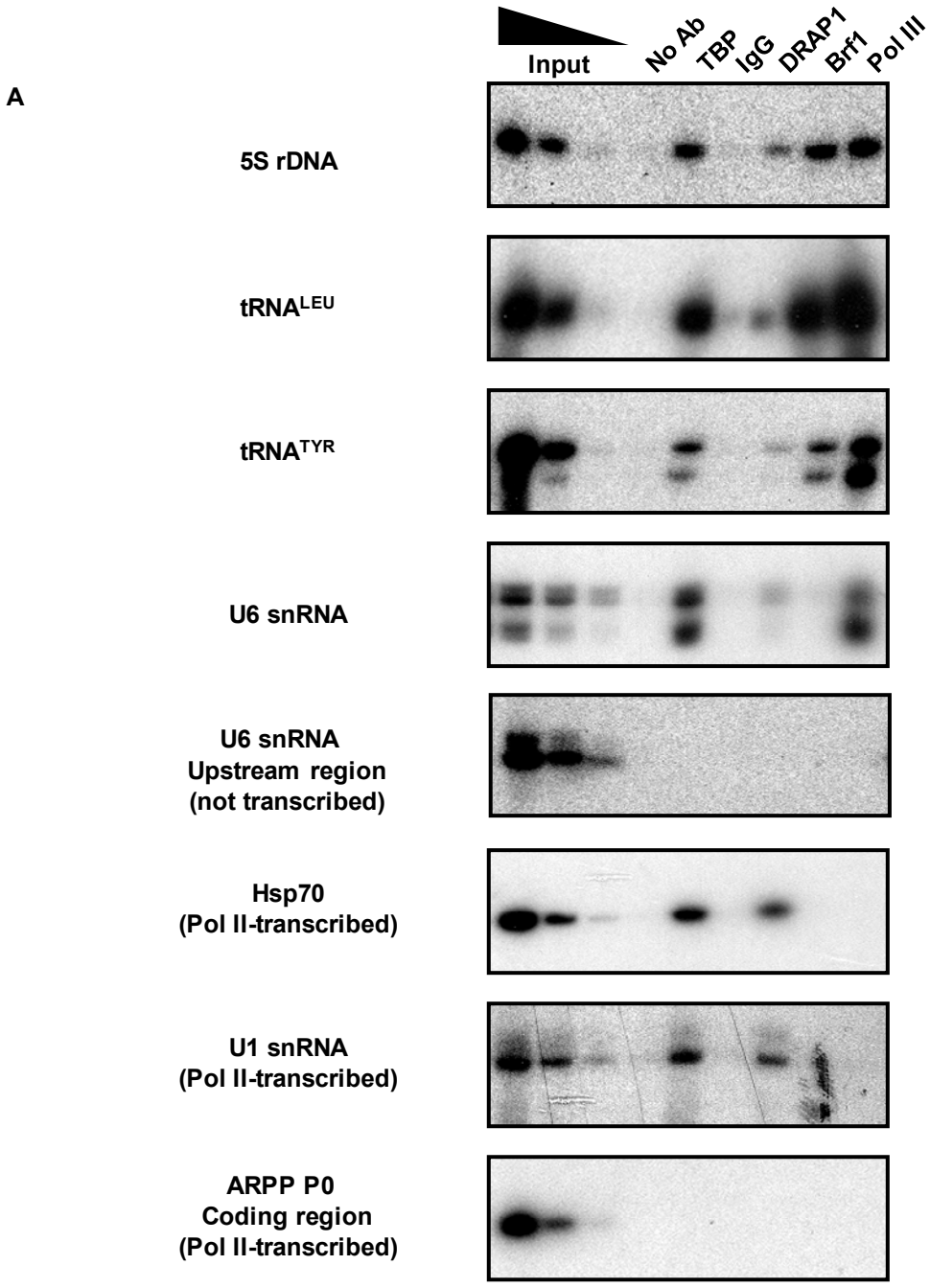


Figure 4.5. DRAP1 occupancy at Pol III-transcribed genes. HeLa cells were used for ChIP. A polyclonal DRAP1 antibody (sc-17272) was used to test for DRAP1 presence at Pol III-transcribed genes. TBP (MTBP-6), Brf1 (128) and Pol III (1900) antibodies were used as positive controls. Goat IgGs (sc-2028, Santa Cruz Biotechnology) and mock (no antibody) were used as negative controls.

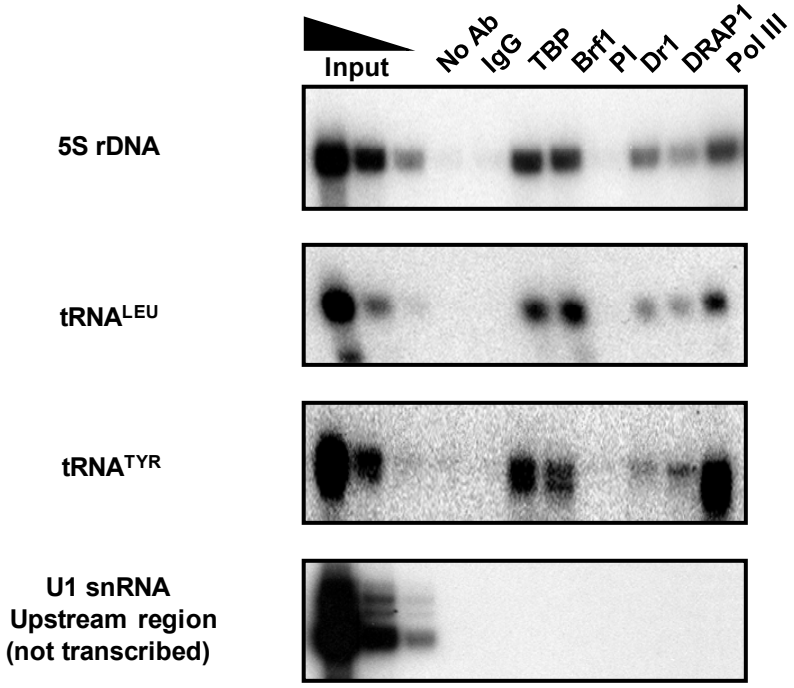


Figure 4.6. DRAP1 is found at Pol III-transcribed genes. HeLa cells were used for ChIP. A monoclonal DRAP1 antibody (TM-301B-55) was used to confirm DRAP1 presence at Pol III-transcribed genes, along with the Dr1 (1162). TBP (MTBP-6), Brf1 (128) and Pol III (1900) antibodies were used as positive controls; mouse IgGs (sc-2025) and pre-immune (PI 1162) serum were used as negative controls.

Table 4.1. DRAP1/TBP ratio at Pol III-transcribed genes

Genes	DRAP1/TBP
5S rRNA	0.6 ± 0.04
$tRNA^{LEU}$	0.6 ± 0.37
$tRNA^{TYR}$	0.6 ± 0.16
U6 snRNA	0.4 ± 0.08
Hsp70	0.8 ± 0.40
U1 snRNA	0.7 ± 0.06

ChIP signals were normalised to input and the value of the DRAP1 signal was divided by the TBP signal. The ratio shown is the average of two independent experiments with standard variation.

that in humans DRAP1 is found at Pol III-transcribed genes at about the same ratio to TBP as at the Pol II-transcribed genes.

Since DRAP1 can be found at Pol III-transcribed genes, it is of interest to investigate where DRAP1 is found at Pol III promoter regions. The 7SL promoter was mapped by five pairs of primers in order to examine the positions of DRAP1 (Figure 4.7, A). As in the case of Dr1 (Figure 3.18), DRAP1 was found to correlate with the presence of Brf1 at the promoter, rather than TFIIIC or Pol III (Figure 4.7, B and C). This finding suggests that DRAP1 might be found at Pol III promoters with Brf1 and also Dr1.

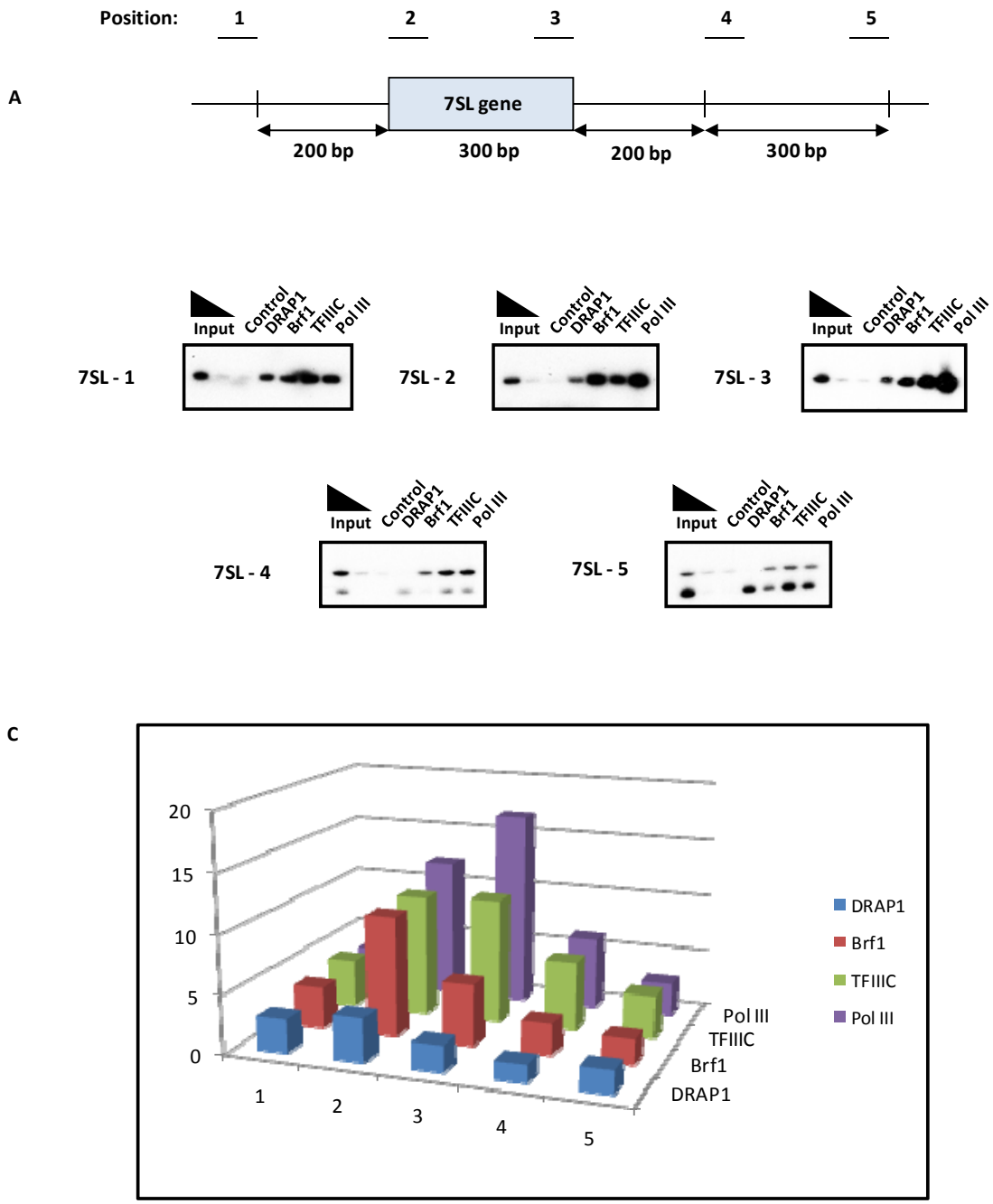


Figure 4.7. DRAP1 occupancy at the 7SL gene locus. ChIP assays were performed in HeLa cells. **A.** Schematic diagram showing the relative positions of the five primer sets (1-5) at the 7SL gene. **B.** ChIPs were performed using the DRAP1 (TM-301B-55), Brf1 (128), TFIIIC (Ab7) and Pol III (1900) antibodies. Mouse IgGs (sc-2025) were used as negative control. **C.** Schematic representation of the ChIP signals. The x-axis represents the five primer sets. The Y-axis shows the mean relative strengths of ChIP signal expressed in arbitrary units after normalisation against input and the signal with the negative control. n=2.

4.3 Discussion

4.3.1 DRAP1 RNAi and Pol III transcription

RNAi experiments showed that knock-down of Dr1 results in enhanced expression of tRNA, indicating that Dr1 has a role in Pol III transcription regulation. It is not clear, however, if DRAP1 has a role as well, either independently or as a co-repressor. To investigate a possible role of DRAP1 in Pol III transcription in human cells, DRAP1 was knocked-down by RNAi using either shRNA (Figures 4.1) or siRNA (Figure 4.3). Both RNAi approaches resulted in almost complete depletion of DRAP1 mRNA, as detected by RT-PCRs, but the protein levels of DRAP1 were only moderately affected (Figures 4.1 and 4.3). Expanding the RNAi incubation period from 48 up to 96 hours, or increasing the amount of the transfected shDNA (1-3 μg) or siRNA (50-200 nM) did not result in a better protein knock down, but rather in reduced cell viability (data not shown).

Under these conditions, the two approaches led to contradicting results concerning Pol III transcription. In the case of shRNA, $tRNA^{LEU}$ and $tRNA^{TYR}$ expression seems to be elevated (Figure 4.2, A and B), while when siRNA was used, $tRNA^{LEU}$ and $tRNA^{TYR}$ expression seems to be reduced (Figure 4.4, A and B). If DRAP1 works as a co-repressor, enhancing Dr1-mediated repression, then depletion of DRAP1 would be expected to result in increased Pol III transcripts, as seems to be indicated by the shRNA experiments (Figure 4.2, A and B). If, however, DRAP1, as has been suggested (Creton et al, 2002; Geisberg et al, 2001), has a positive role *in vivo*, either direct or indirect by stabilising TBP to the promoters, then knock-down of DRAP1 might result in decreased expression of Pol III transcripts, as is suggested by the siRNA experiments (Figure 4.4., A and B). In both cases, however, the mildly depleted protein levels of DRAP1 and the high experimental errors do not allow for safe conclusions. Future experiments with a sound DRAP1 knock-down should reveal to what degree DRAP1 can affect Dr1 repression and if there is a positive role for DRAP1 in Pol III transcription regulation.

4.3.2 DRAP1 is found at Pol III-transcribed genes

It has been previously reported that DRAP1 can be found at the promoters of Pol II-transcribed genes in yeast (Creton et al, 2002; Geisberg et al, 2001) and in human (Gilfillan et al, 2003; Lewis et al, 2005). In yeast, DRAP1 occupancy of Pol III promoters was found to be negligible, and it was inferred that Dr1-DRAP1 is not present at Pol III-transcribed genes (Geisberg et al, 2001). However, ChIP experiments revealed that Dr1 is present at Pol III-transcribed genes in human cells (Figure 3.9). By expanding these studies to DRAP1, it was shown that DRAP1 also occupies Pol III-transcribed genes (Figure 4.5). This was further confirmed by obtaining the same result with the use of an alternative DRAP1 antibody, thus minimising the possibility of antibody-related unspecific effects (Figure 4.6). Mapping experiments of DRAP1 occupancy at the 7SL gene promoter indicate that DRAP1 is mainly found in the beginning of the gene (Figure 4.7). This finding suggests that DRAP1 is found at the same region as Dr1, as expected.

The ratio of DRAP1/TBP occupancy of Pol III-transcribed genes is ~0.6 (Table 4.1), in contrast to the <0.05 ratio found in yeast (Geisberg et al, 2001), demonstrates that endogenous DRAP1 is present at Pol III-transcribed genes in human cells. These findings suggest that there might be substantial differences between the two organisms, especially since the identity between the two DRAP1 homologs mostly occurs in the histone motif.

CHAPTER 5

Dr1 and DRAP1 can be differentially regulated under stress conditions that affect RNA polymerase III transcription

5.1 Introduction

A number of stress conditions, such as heat shock (Allen et al, 2004; Liu et al, 1995) and hypoxia (Ernens et al, 2006), have been shown to affect Pol III transcription in murine or human cells. It has also been shown that DRAP1 can be recruited at heat shock gene promoters after heat shock in yeast (Geisberg et al, 2001) and suggested that in yeast Dr1 and DRAP1 might not always work together, but cooperate under stress conditions, such as a diauxic shift (Creton et al, 2002). In this chapter a number of stress conditions were investigated with regards to Pol III transcription and the Dr1-DRAP1 complex.

5.2 Results

5.2.1 Heat shock, Pol III transcription and Dr1-DRAP1

Heat shock has been shown to affect Pol III transcription by increasing the levels of Alu RNA in human cells, but not of 5S rRNA, 7SL RNA, 7SK RNA and U6 snRNA (Liu et al, 1995). Similarly, in mouse cells, B1 and B2 RNAs (Allen et al, 2004; Li et al, 1999a) increase after heat shock and it has been proposed that the B2 elements have a role in repressing Pol II transcription (Allen et al, 2004). In yeast it has also been shown that under heat shock, DRAP1 was recruited to heat shock gene promoters (Geisberg et al, 2001).

In order to investigate how heat shock affects Pol III transcription in human cells, HeLa cells were subjected to heat shock at 45 ºC for 30 minutes, and were then left to recover at 37 ºC, for different periods of time. Western blotting showed that while the total protein levels of Dr1 remained stable, DRAP1 levels were increased, even just after the heat shock, and kept increasing for the next 2-4 hours, while they remained high even after 8 hours (Figure 5.1, A and B). The Hsp70 heat shock protein was also induced after 2 hours and started going down after 4 hours, in accordance with previous studies in the same cell line (Liu et al, 1995). p53 levels were also increased in a similar pattern to DRAP1, i.e. increased immediately after the heat shock and peaked after 2-4 hours of

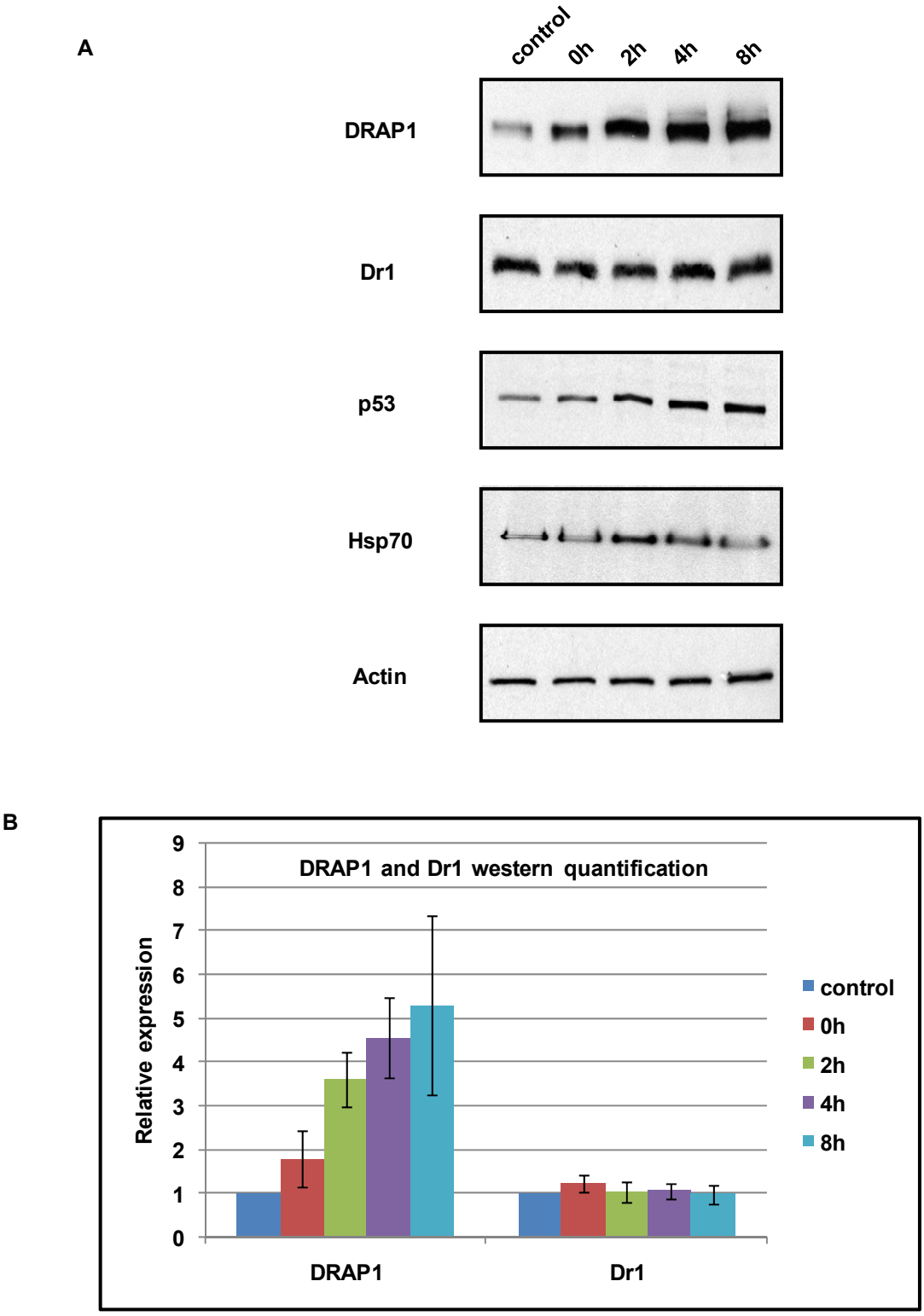


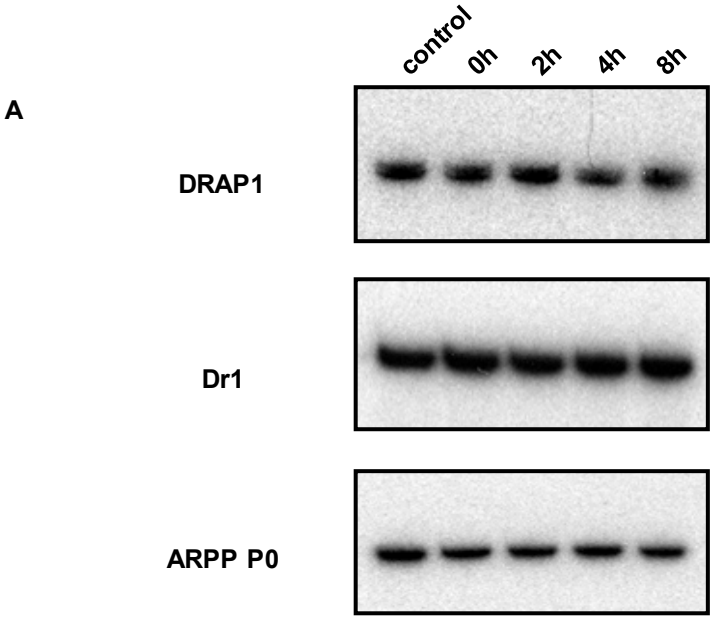
Figure 5.1. Induction of DRAP1, but not Dr1, after heat shock in HeLa cells. HeLa cells were subjected to heat shock at 45 ºC for 30 minutes and left to recover for 0, 2, 4 or 8 hours before being harvested. Control cells were not subjected to heat shock **A.** Western blotting for total protein levels. Actin was used as loading control**. B.** Quantification of DRAP1 and Dr1 signals from A. n=3.

recovery (Figure 5.1, A). Quantification of 3 different experiments revealed that the Dr1 protein levels remained stable, while DRAP1 increased up to 3-5 times (Figure 5.1, B).

The rapid increase in the protein levels of DRAP1 would suggest that this is probably a posttranscriptional effect, perhaps due to protein stabilisation and decreased degradation. Indeed, the mRNA levels of DRAP1 and Dr1 remained unaffected, even after 8 hours of recovery (Figure 5.2). The RNA levels of Pol III-transcribed genes were also examined. 5S rRNA and U6 snRNA levels were stable (Figure 5.3, A), as expected from previous studies (Liu et al, 1995). Although, tRNA expression levels initially seemed to be increased (Figure 5.3A), analysis of three different experiments showed that there was no consistent increase of the $tRNA^{LEU}$ levels. However, $tRNA^{TYR}$ seemed to be two-fold increased after the 2 hour time point (Figure 5.3, B).

To further verify the above results, the experiments were repeated with a U2OS cell line (human osteosarcoma). In this cell line, the levels of DRAP1 were also increased about 2.5-fold after heat shock, while the Dr1 levels remained stable (Figure 5.4). Interestingly, DRAP1 protein was found to be at levels comparable to the control at the 8 hour time point, in contrast to the experiments in HeLa cells (Figure 5.4, A and B). p53 and Hsp70 were also increased after the 2 hour time point (Figure 5.4, A). The increase at the protein level was not accompanied with an increase at the mRNA levels of DRAP1, as was the case in HeLa cells (Figure 5.5), while the mRNA levels of Hsp70 seemed to be elevated after 2 hours from the heat shock (Figure 5.5), in accordance with previous studies (Liu et al, 1995). The RNA levels of Pol III transcripts (Figure 5.6), and especially of $tRNA^{LEU}$ and $tRNA^{TYR}$ (Figure 5.6, A and B), were also examined and found not to change significantly (Figure 5.6, A and B).

These results indicate that heat shock in human cells results in increased protein levels of DRAP1, but not Dr1. This does not seem to be a transcriptional effect as the RNA levels of both proteins remain unaffected. Furthermore, the heat shock treatment, as well as the physiological response of DRAP1 induction, do not seem to affect Pol III transcription, as the examined 5S rRNA, tRNAs and U6 snRNA transcripts do not change significantly.



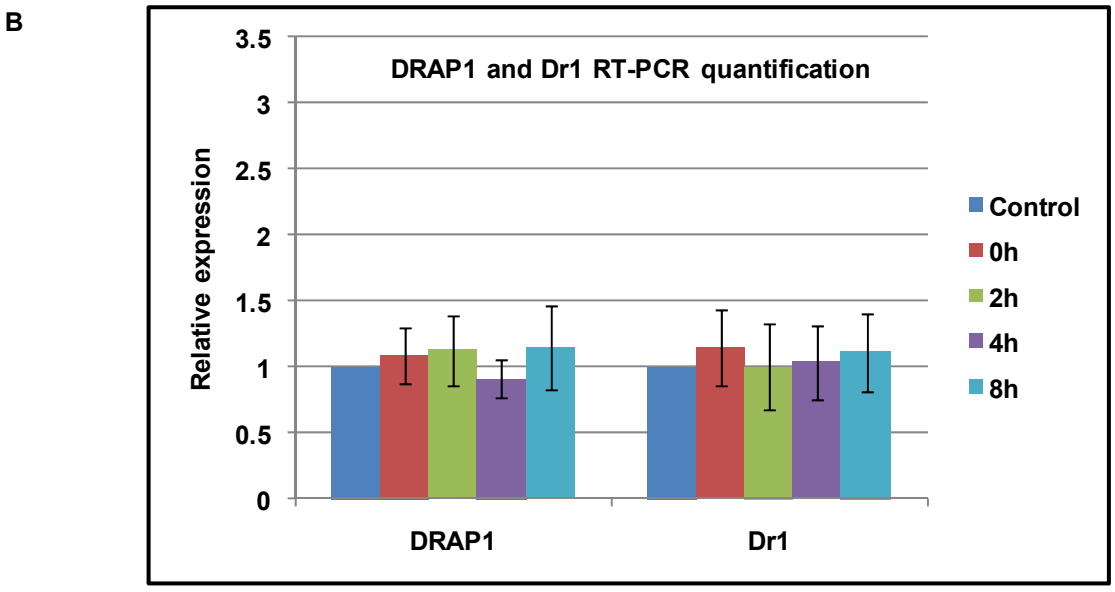


Figure 5.2. DRAP1 and Dr1 mRNA levels remain stable after heat shock in HeLa cells. HeLa cells were subjected to heat shock at 45 ºC for 30 minutes and left to recover for 0, 2, 4 or 8 hours before being harvested. Control cells were not subjected to heat shock. **A.** RT-PCRs for DRAP1 and Dr1 mRNA. ARPP P0 was used as a control. **B.** Quantification of DRAP1 and Dr1 signals from A. n=3.

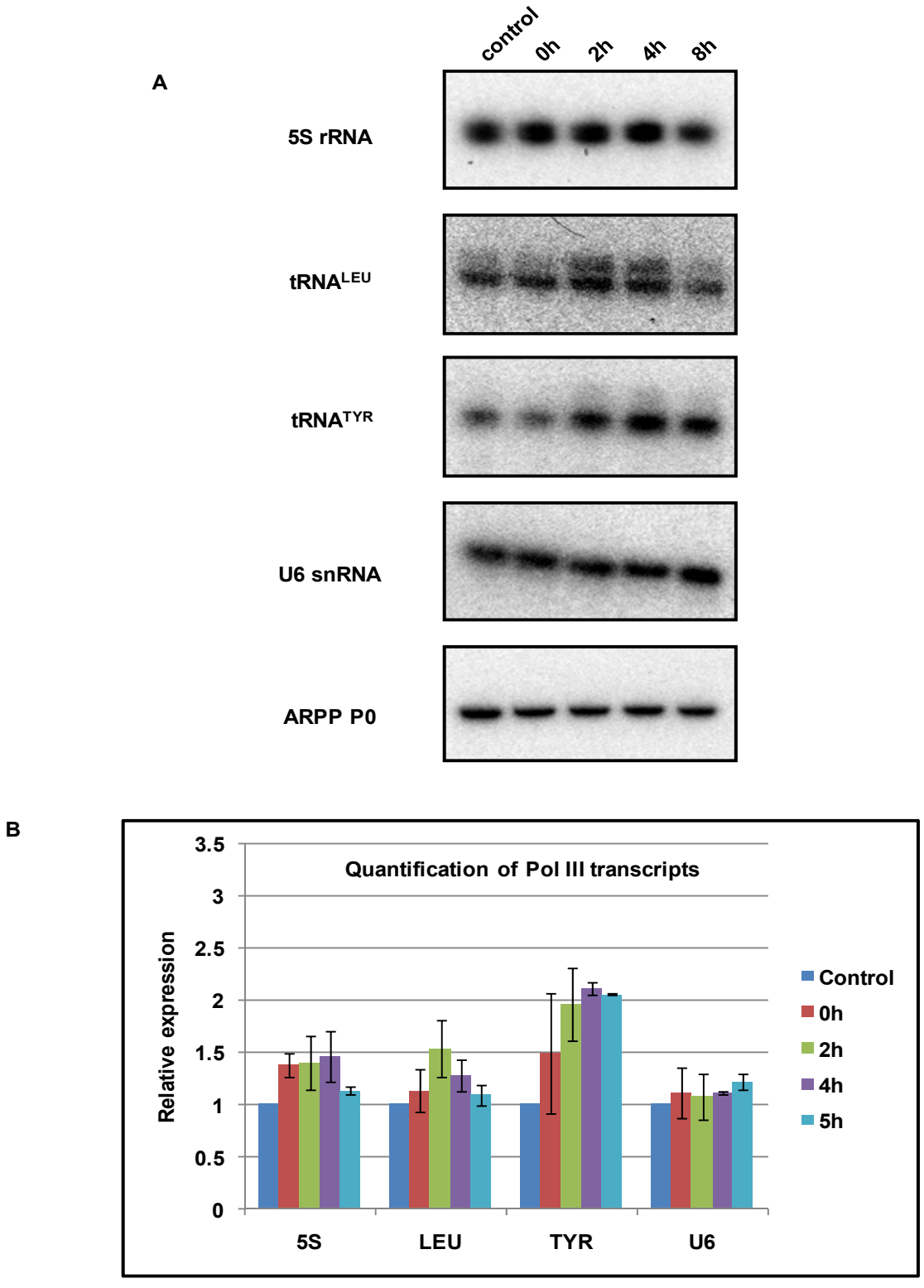


Figure 5.3. RNA levels of Pol III-transcribed genes after heat shock in HeLa cells. HeLa cells were subjected to heat shock at 45 ºC for 30 minutes and left to recover for 0, 2, 4 or 8 hours before being harvested. Control cells were not subjected to heat shock. **A.** RT-PCRs for Pol III templates. ARPP P0 was used as a control. **B.** Quantification of the signals of Pol III transcripts from A. n=3.

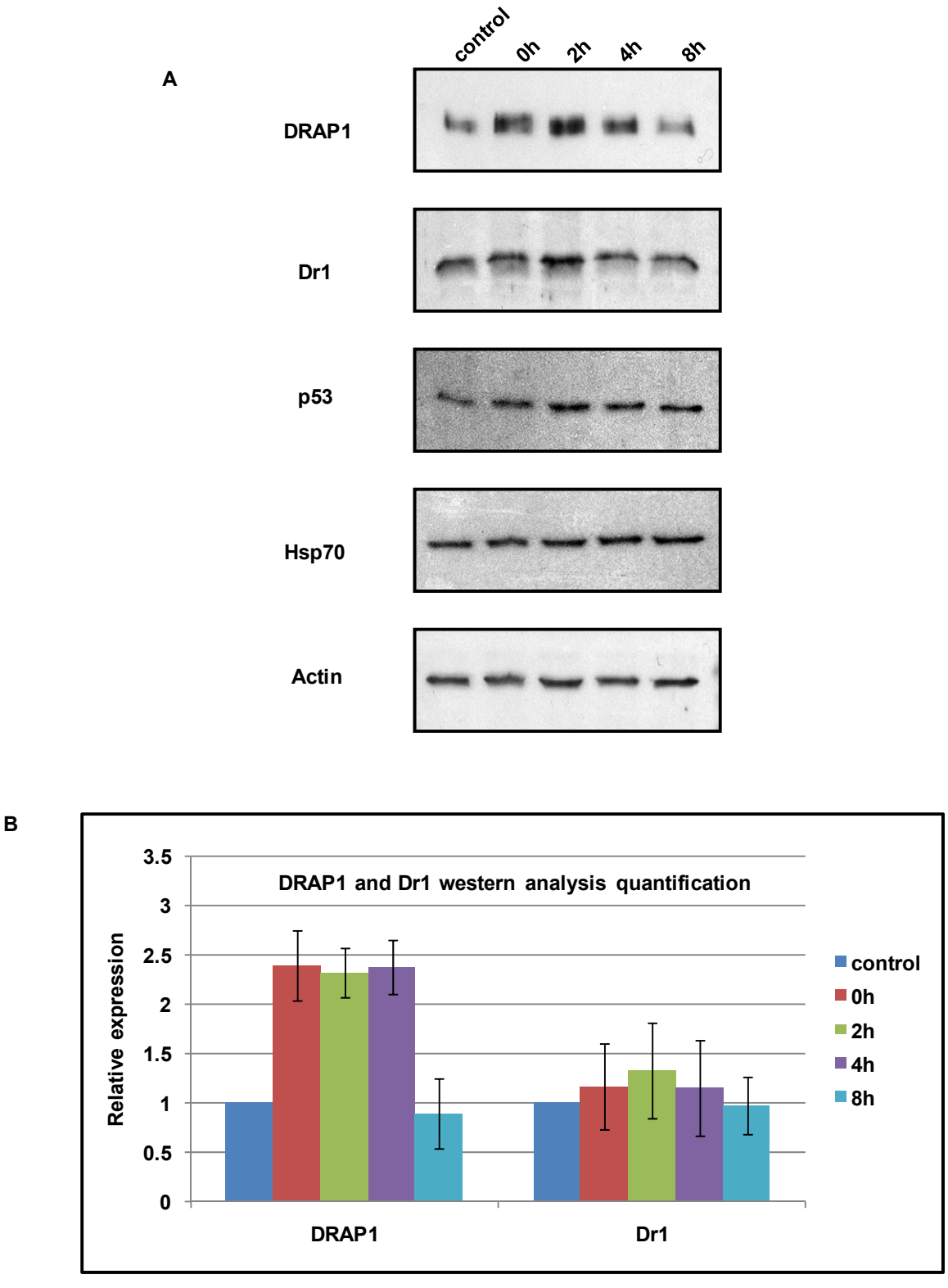


Figure 5.4. Induction of DRAP1, but not Dr1, after heat shock in U2OS cells. U2OS cells were subjected to heat shock at 45 ºC for 30 minutes and left to recover for 0, 2, 4 or 8 hours before being harvested. Control cells were not subjected to heat shock **A.** Western blotting from whole cell extracts. Actin was used as loading control. **B.** Quantification of DRAP1 and Dr1 signals from A. n=3.

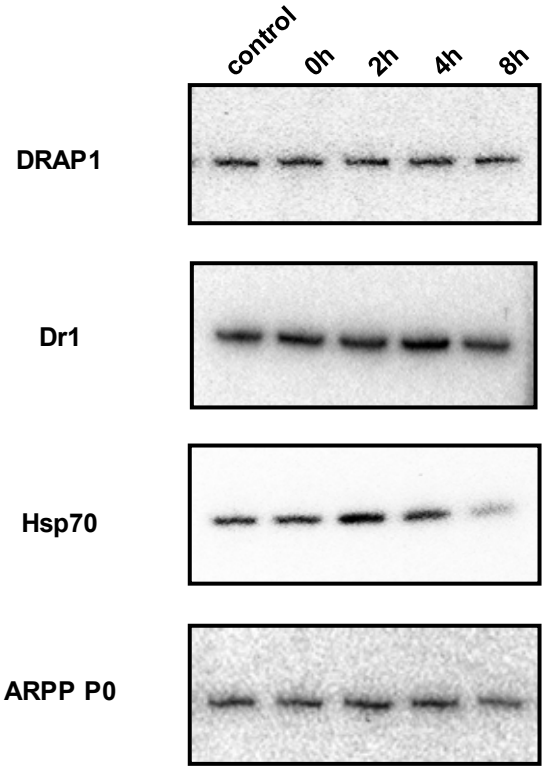


Figure 5.5. DRAP1 and Dr1 mRNA levels remain stable after heat shock in U2OS cells. U2OS cells were subjected to heat shock at 45 ºC for 30 minutes and left to recover for 0, 2, 4 or 8 hours before being harvested. Control cells were not subjected to heat shock. ARPP P0 was used as a control.

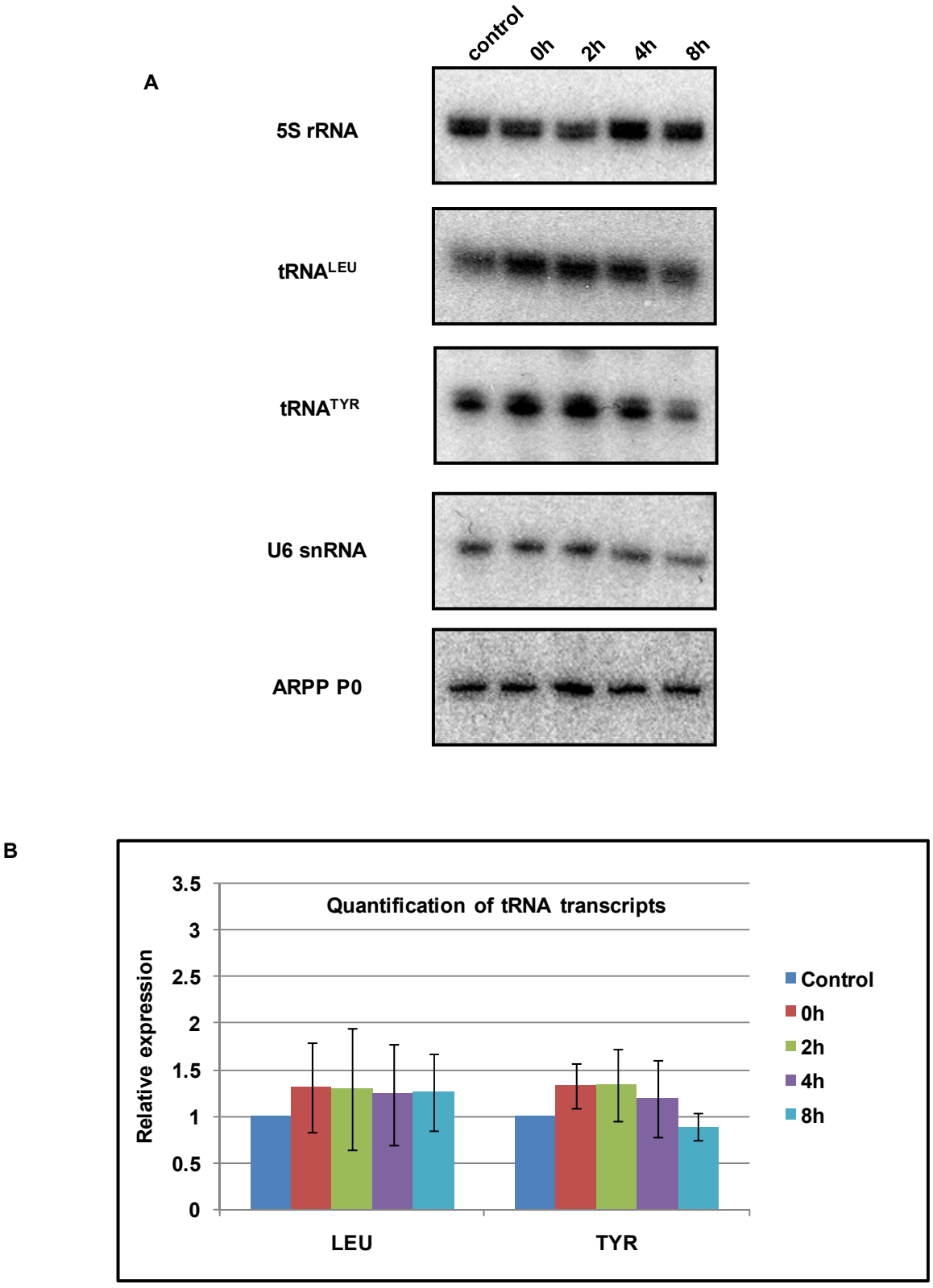


Figure 5.6. RNA levels of Pol III-transcribed genes after heat shock in U2OS cells. U2OS cells were subjected to heat shock at 45 ºC for 30 minutes and left to recover for 0, 2, 4 or 8 hours before being harvested. Control cells were not subjected to heat shock. **A.** RT-PCRs for Pol III templates. ARPP P0 was used as a control. **B.** Quantification of tRNA signals from A. n=3.

5.2.2 Hypoxia, Pol III transcription and Dr1-DRAP1

Dr1 has been reported to repress Pol II transcription under extreme hypoxic conditions (Denko et al, 2003) and it has also been shown that hypoxia represses Pol III transcription (Ernens et al, 2006). Previous experiments revealed Dr1 being recruited to Pol III-transcribed genes in 1% O_2 hypoxia (Figure 3.19, A), while under these conditions the protein levels of Dr1 remain unaffected (Figure 3.19, B). It would be interesting to investigate if, as is the case in heat shock, the protein levels of Dr1 and DRAP1 are differentially regulated under hypoxic conditions.

To this end, HeLa cells were subjected to hypoxic conditions (1% O_2) for 24 hours or normal conditions (20% O_2) for the same amount of time. Western blotting revealed that the protein levels of DRAP1 in cells under hypoxia were elevated two-fold compared to the control (20% O_2) cells, while Dr1 levels remained stable (Figure 5.7, A and B). Protein levels of p53 and HIF1α, genes known to be induced in hypoxic conditions (Graeber et al, 1994; Wang & Semenza, 1993), were increased, as expected (Figure 5.7, A). Under these conditions, the mRNA levels of Dr1 and DRAP1, as tested by RT-PCRs, were not affected, while p53 was induced (Figure 5.8). The RNA levels of the Pol III products, at least in the case of tRNAs, were reduced about two-fold (Figure 5.9), in accordance with previously published results in rat cardiomyocytes (Ernens et al, 2006).

Similar results were obtained from experiments where HeLa cells were subjected to chemically-induced severe hypoxia/anoxia. Cells treated with 150 μM deferoxamine (DFX) were found to have 2.5-fold more DRAP1 protein than the untreated control (Figure 5.10). Dr1 remained stable, while HIF1α and p53 were induced, as expected (Figure 5.10). As also seen in HeLa cells with 1% O_2 hypoxia, the mRNA levels of DRAP1 and Dr1 remained stable, p53 mRNA was induced, while tRNAs were reduced (Figure 5.11).

In order to further validate the above results, the experiments were replicated with the human U2OS cell line. In these cells, 1% O_2 hypoxia also resulted in two-fold increased protein levels of DRAP1, while Dr1 remained stable (Figure 5.12, A and B). In accordance with the results obtained from the HeLa cells, the mRNA levels of Dr1 and DRAP1 were not altered, while p53 was increased (Figure 5.13, A and B). Pol III transcription was also affected similarly to HeLa cells, i.e. tRNAs were reduced about two-fold (Figure 5.14, A and B).

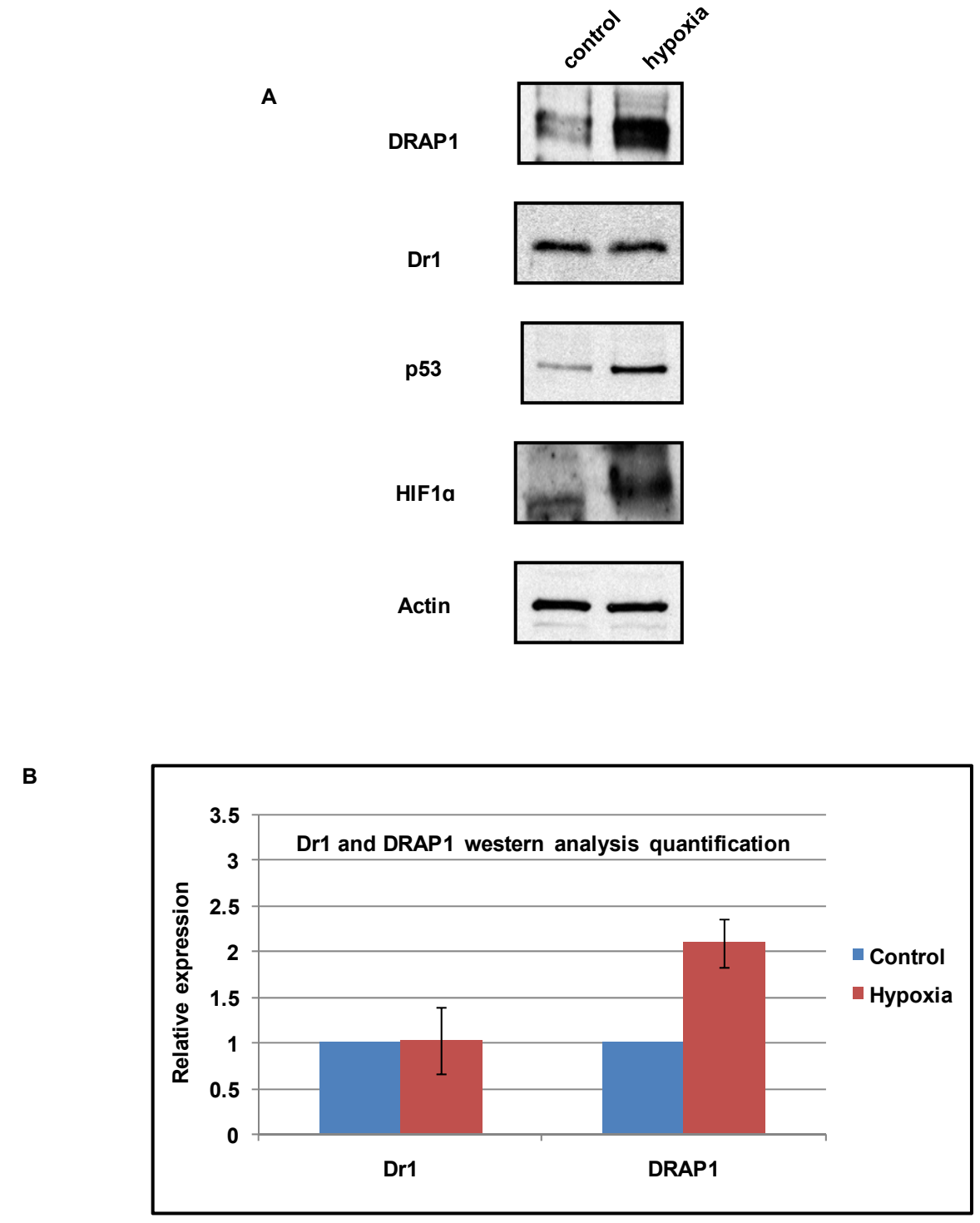


Figure 5.7. Induction of DRAP1, but not Dr1, under hypoxic conditions in HeLa cells. HeLa cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** Western blotting from whole cell extracts. HIF1α and p53 were used as positive controls and actin as loading control. **B.** Quantification of Dr1 and DRAP1 signals from A. n=3.

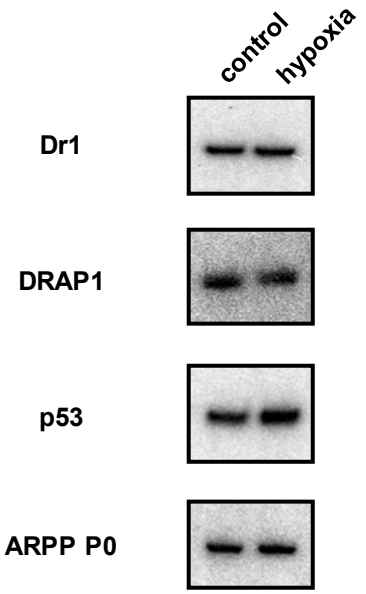


Figure 5.8. Dr1 and DRAP1 mRNA levels remain stable under hypoxic conditions in HeLa cells. HeLa cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. Total RNA was isolated, reverse transcribed to cDNA and analysed by PCRs. ARPP P0 was used as a control.

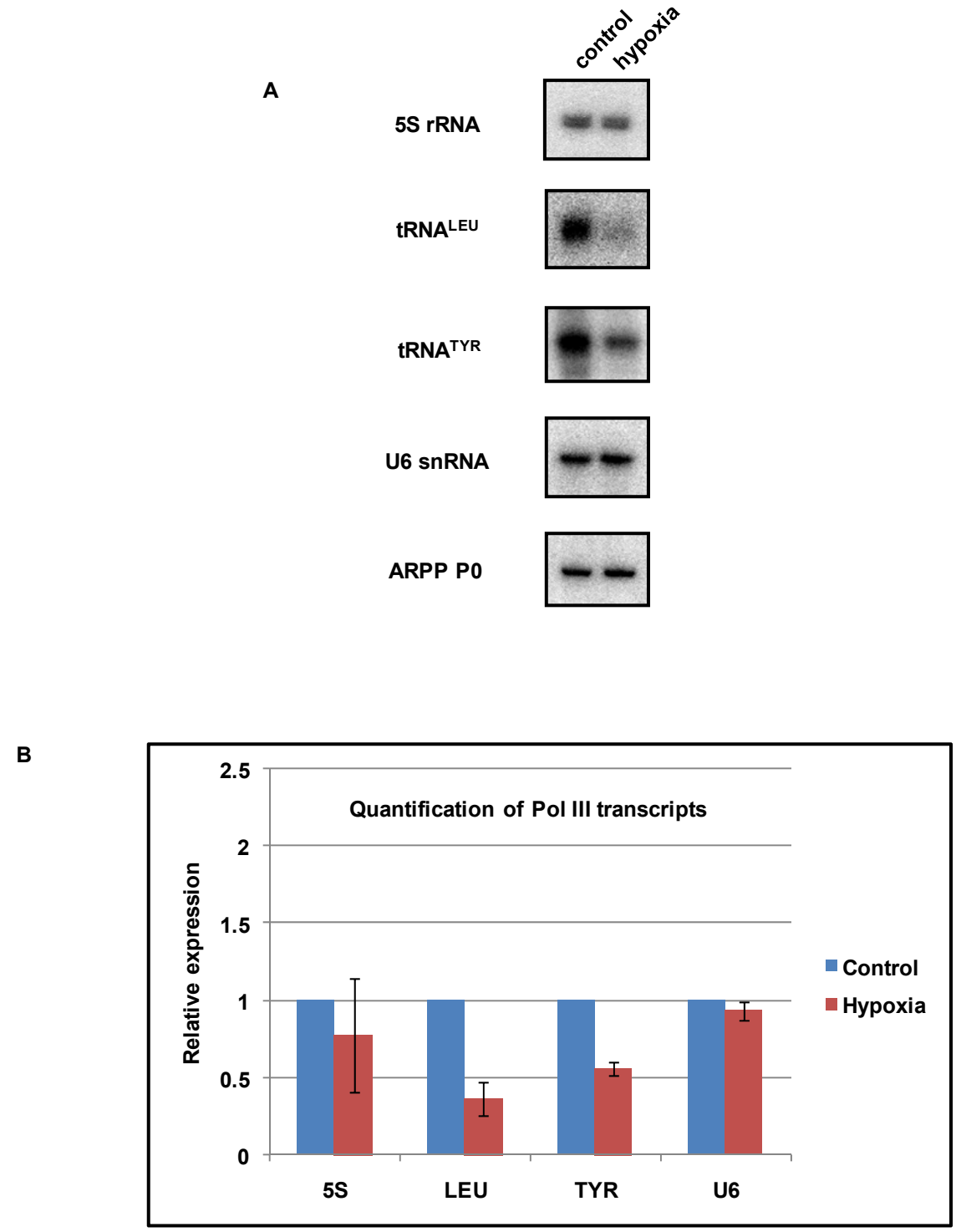


Figure 5.9. tRNA expression levels decrease under hypoxic conditions in HeLa cells. HeLa cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** RT-PCR analysis for Pol III-transcribed genes. ARPP P0 was used as a control. **B.** Quantification of the Pol III transcripts from A. n=3.

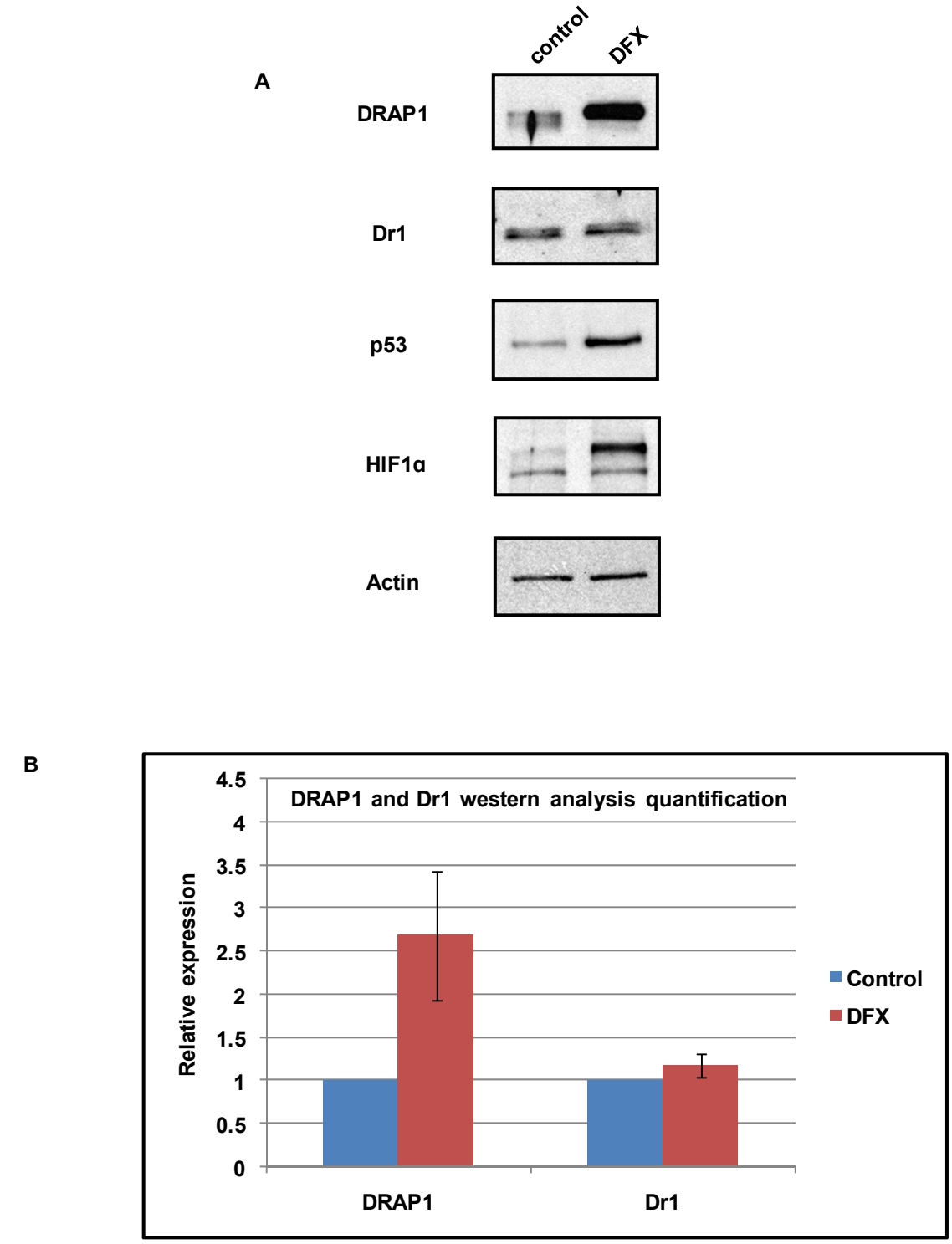


Figure 5.10. DRAP1, but not Dr1, protein levels increase under anoxic conditions induced by deferoxamine (DFX) in HeLa cells. HeLa cells were grown either with or without (control) 150 μM DFX for 24 hours, before being harvested. **A.** Western blotting with whole cell extracts. HIF1α and p53 were used as positive controls and actin as loading control. **B.** Quantification of DRAP1 and Dr1 signals from A. n=2.

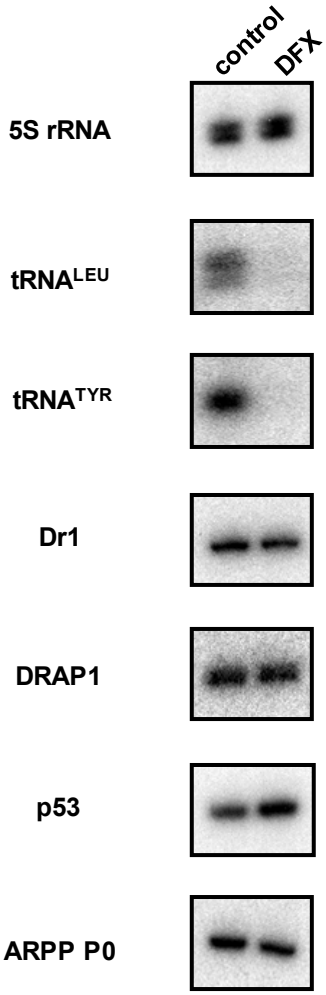


Figure 5.11. tRNA expression levels decrease in DFX-induced anoxia in HeLa cells, while Dr1 and DRAP1 mRNA levels remain stable. HeLa cells were growing either with 150 μM DFX or without (control) for 24 hours, before being harvested. RT-PCR analysis for Pol III-transcribed genes, as well as Dr1, DRAP1 and p53. ARPP P0 was used as a control.

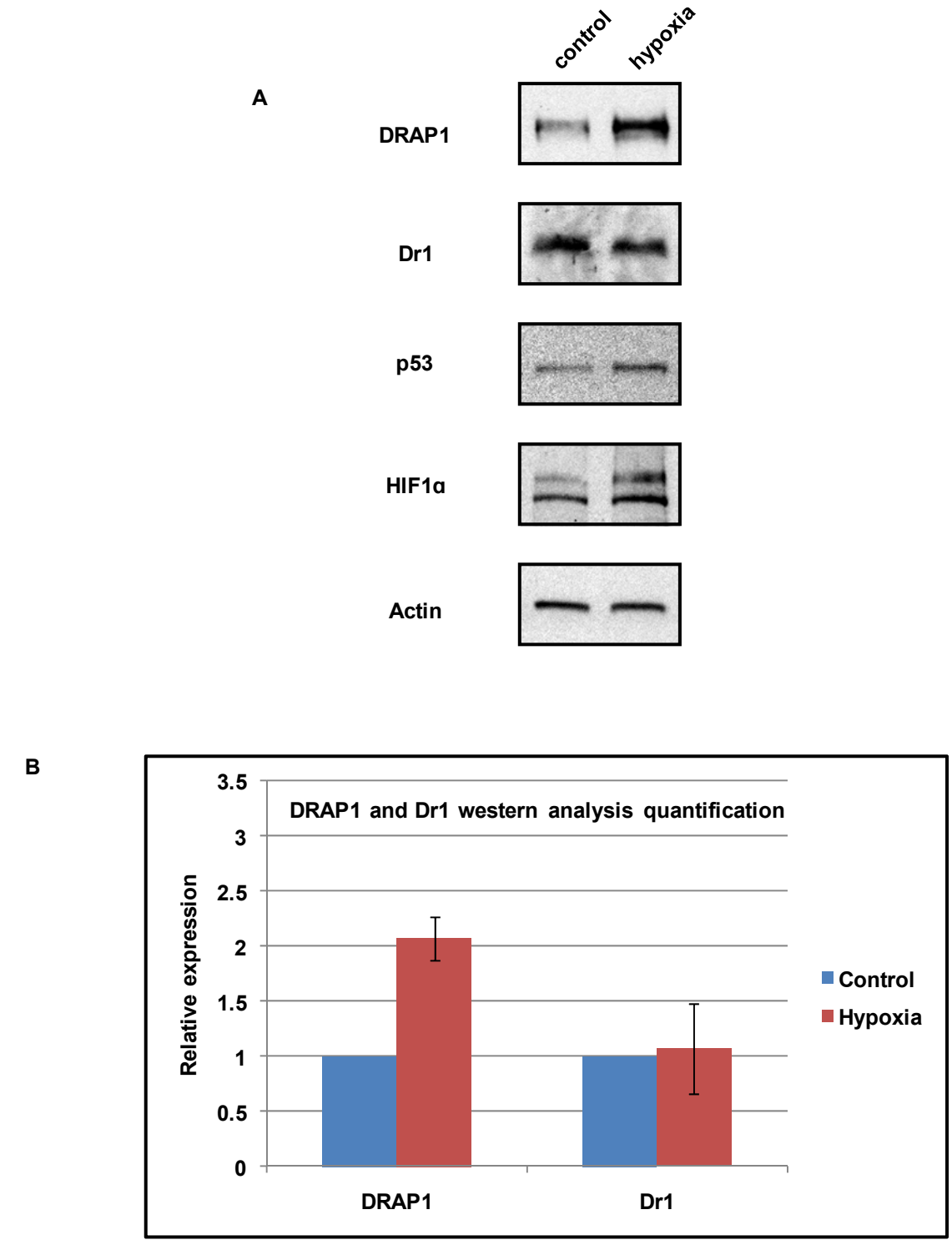


Figure 5.12. Induction of DRAP1, but not Dr1, under hypoxic conditions in U2OS cells. U2OS cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** Western blotting from whole cell extracts. HIF1α and p53 were used as positive controls and actin as loading control. **B.** Quantification of DRAP1 and Dr1 signals from A. n=3.

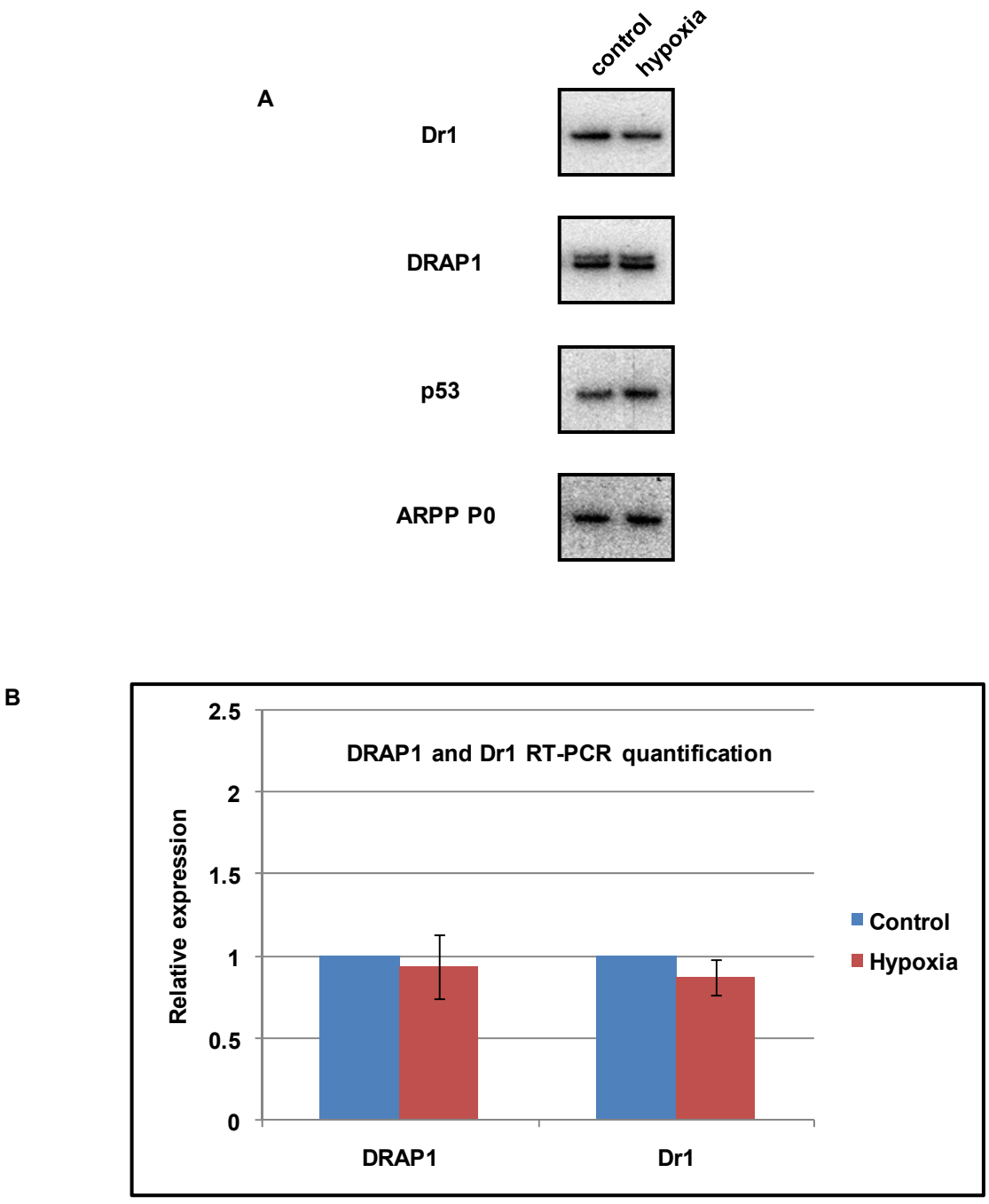


Figure 5.13. Dr1 and DRAP1 mRNA levels remain stable under hypoxic conditions in U2OS cells. U2OS cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** RT-PCR analysis for Dr1, DRAP1, and p53. ARPP P0 was used as a control. **B.** Quantification of DRAP1 and Dr1 signals from A. n=3.

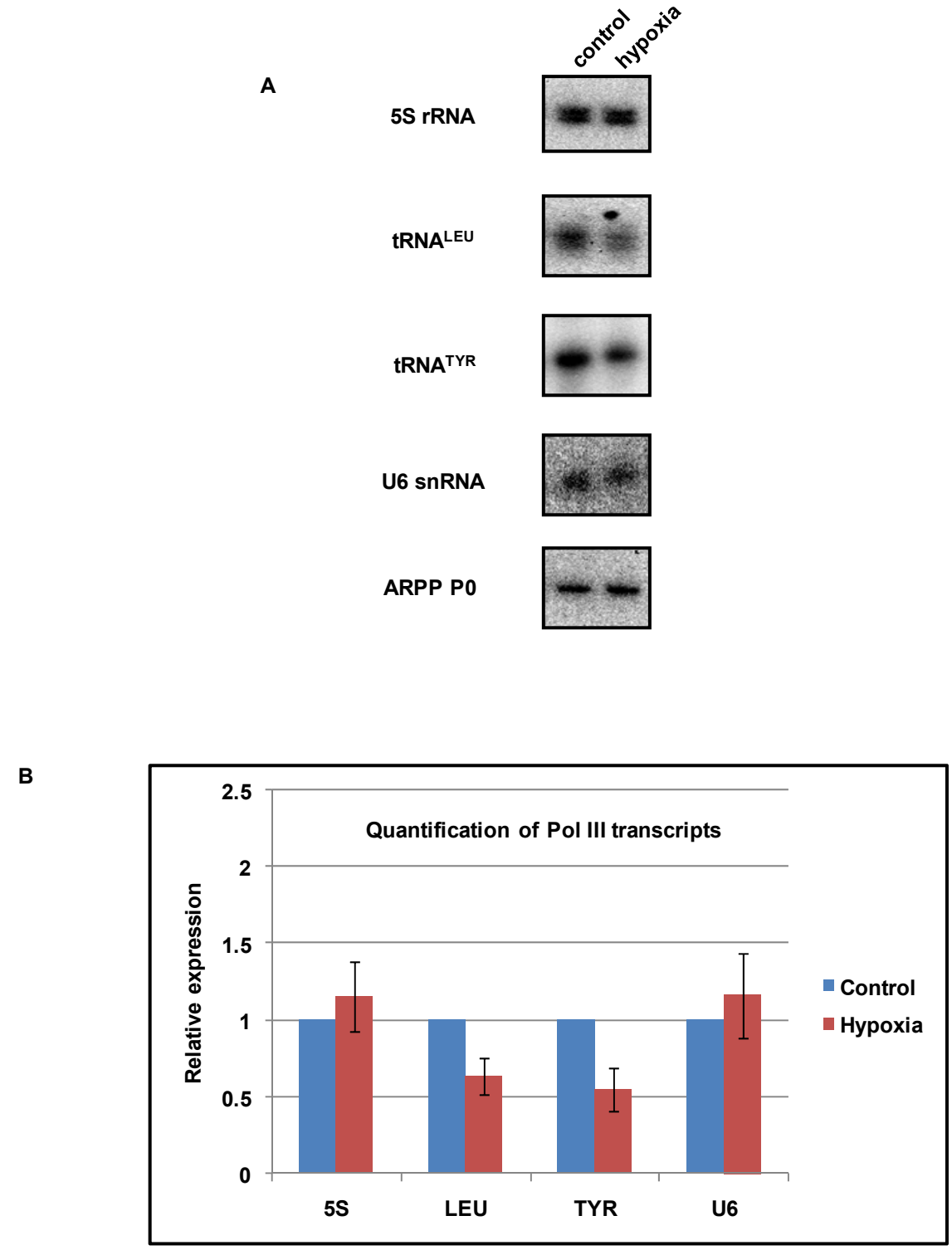


Figure 5.14. tRNA expression levels decrease under hypoxic conditions in U2OS cells. U2OS cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** RT-PCR analysis for Pol III-transcribed genes. ARPP P0 was used as a control. **B.** Quantification of the signals of Pol III transcripts from A. n=3.

Recently, a new p53 database containing p53 family direct targets in human was reported (Sbisa et al, 2007). The database contains p53 target genes, selected by the presence of p53 responsive elements and also by the expression profile of these target genes, as obtained by microarray experiments (Sbisa et al, 2007). A search for DRAP1 and Dr1 revealed that, according to the database, both contain a responsive element at their promoter region (Figure 5.15), while Dr1 has a second one in its intronic region (http://www2.ba.itb.cnr.it/p53FamTaG). Data from the hypoxic and heat shock experiments show no transcriptional effect on mRNA levels of DRAP1 and Dr1 (Figures 5.2, 5.5 and 5.8, 5.11, 5.13), as would be expected if they were p53 targets; however, there might be a correlation between elevated DRAP1 protein and stabilised p53; in both cases when p53 was induced, DRAP1 protein levels were elevated (Figures 5.1, 5.4 and 5.7, 5.10, 5.12).

In order to test if DRAP1 protein stabilisation is related to p53, NARF2-E6 cells were employed. This cell line is a NARF2 derivative, which constitutively expresses the human papillomavirus HPV E6 protein (Rocha et al, 2003); HPV E6 has been shown to bind p53 and target it for degradation via the ubiquitin pathway (Scheffner et al, 1992; Werness et al, 1990). NARF2 itself is a derivative of the human osteosarcoma U2OS cells containing an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible ARF gene (Stott et al, 1998). NARF2-E6 cells were subjected to 1% O_2 hypoxia and it was found that, in contrast to the parental U2OS cells, or HeLa cells, under the same hypoxic conditions, the p53 protein levels in these cells are decreased (Figure 5.16, A and B). HIF1α, however, was induced, indicating that the cells were under hypoxia (Figure 5.16, A). Surprisingly, DRAP1 levels also decreased, while Dr1 remained about the same (Figure 5.16, A and B). At the mRNA level, as tested by RT-PCRs, there seems to be a small decrease in the levels of DRAP1, Dr1 and p53 (Figure 5.17, A and B). Pol III transcription was reduced at the same level as seen previously in U2OS and HeLa cells, indicating that changes in the DRAP1 levels are not responsible for hypoxic repression of the Pol III system (Figure 5.18, A and B).

To further investigate if p53 has a role in stabilising DRAP1 protein levels, shRNA against p53 was employed. NARF2 cells were transfected with a pSUPER-p53 shRNA vector or an empty pSUPER control vector, and left to grow for 48 hours before harvesting. Under these conditions, p53 was knocked-down in the shRNA-treated cells compared to the control (Figure 5.19, A). DRAP1 protein levels also seem to be reduced,

A

Gene Name	EnsEmbl	RefSeq	Chr	REs
DR1, NC2, NC2-BETA	ENSG00000117505	NM_001938	1	2

Start	Size	Strand	Localization	Pattern
93520444	36	→	PROMOTER	
93534424	37	→	INTRON	

B

Gene Name	EnsEmbl	RefSeq	Chr	REs
DRAP1, NC2-alpha	ENSG00000175550	NM_006442	11	1

Start	Size	Strand	Localization	Pattern
65440961	41	→	PROMOTER	

Figure 5.15. Dr1 and DRAP1 contain p53 responsive elements in their promoters. Image adapted from http://www2.ba.itb.cnr.it/p53FamTaG. **A.** Dr1 contains two p53 responsive elements. **B.** DRAP1 contains one p53 responsive element at the promoter region.

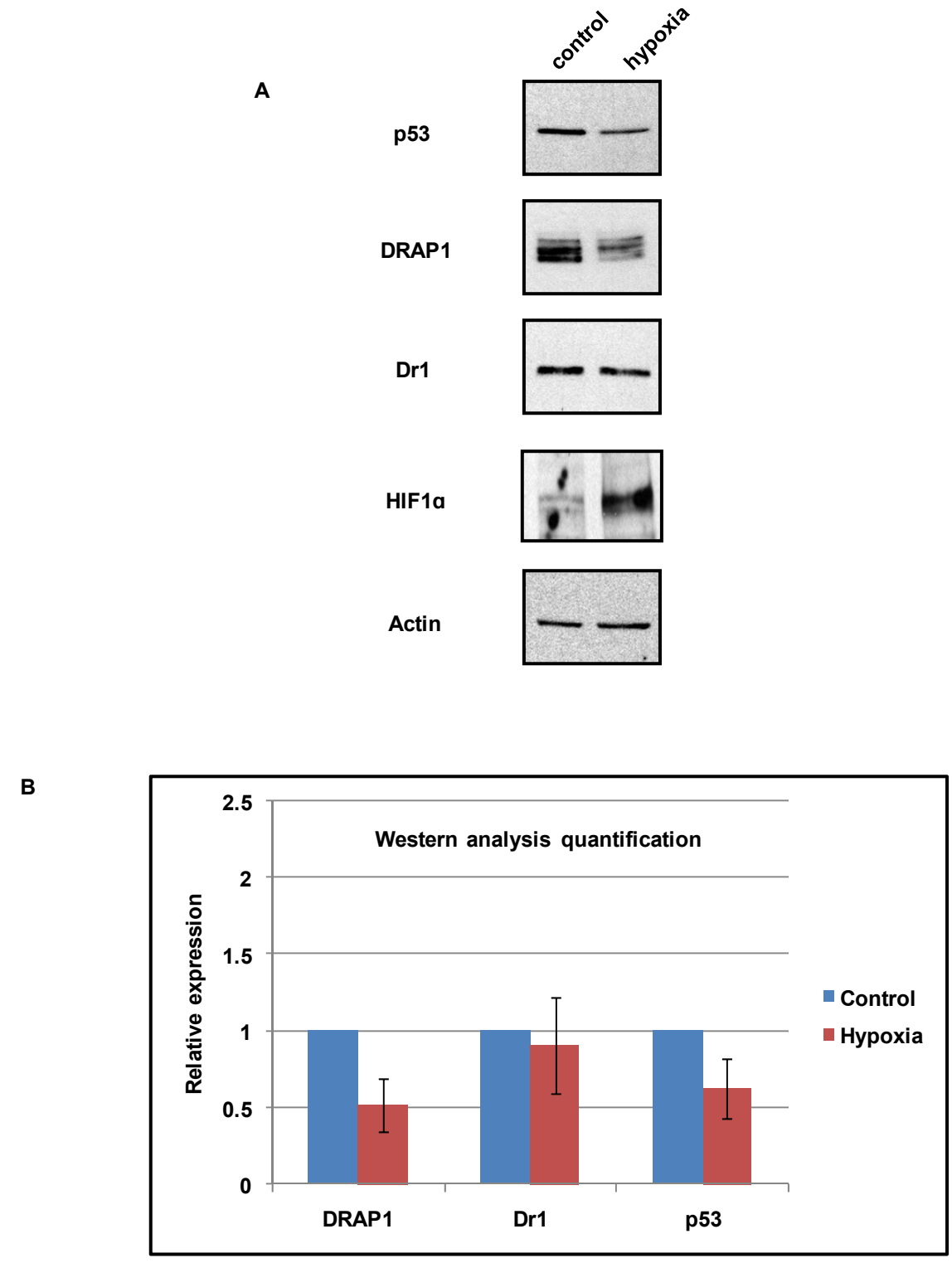


Figure 5.16. DRAP1 is not induced under hypoxic conditions in p53-impaired NARF2-E6 cells. NARF2-E6 cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at normal 20% O_2. **A.** Western blotting from whole cell extracts. HIF1α and p53 were used as positive controls and actin as loading control. **B.** Quantification of DRAP1, Dr1 and p53 signals from A. n=3.

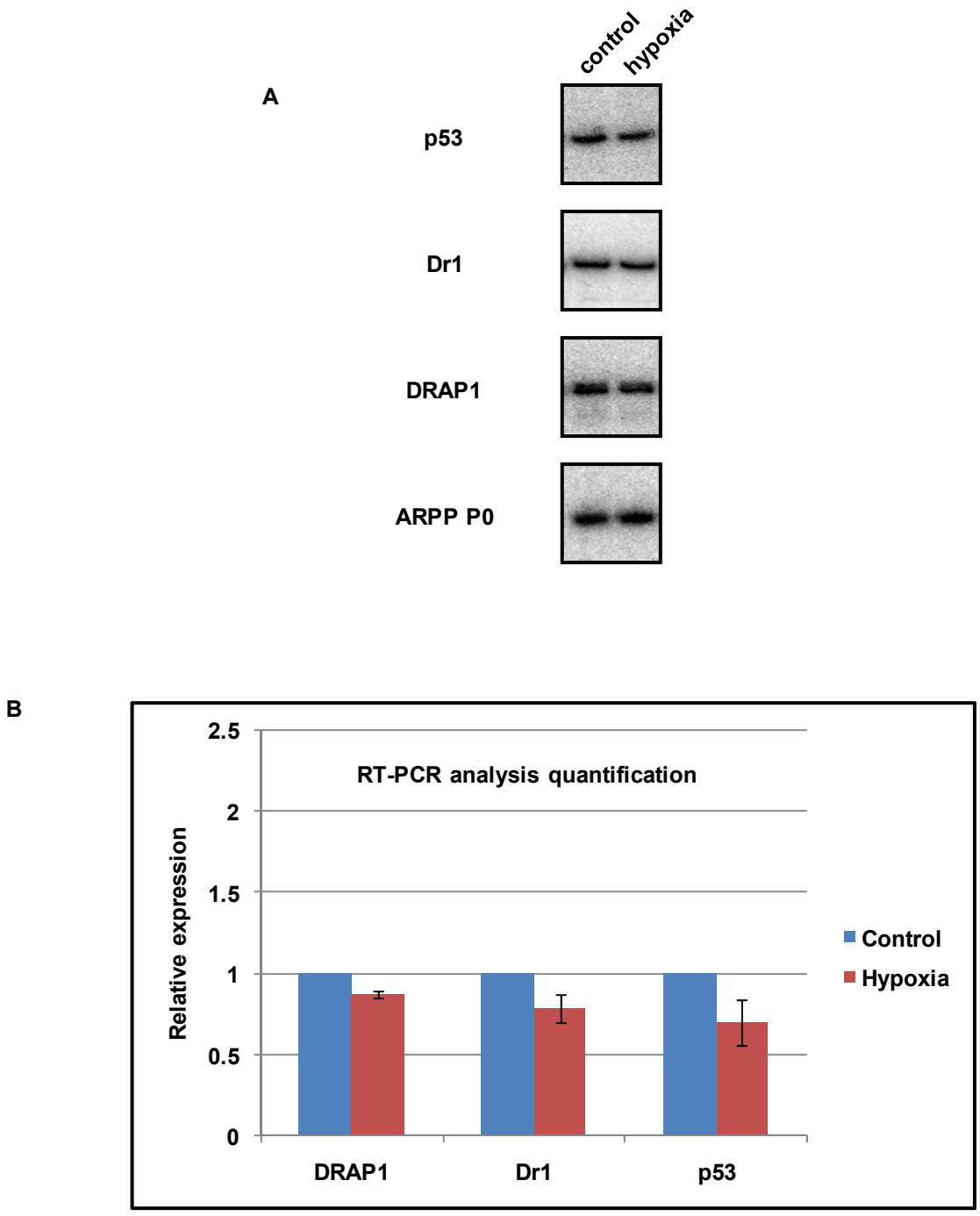


Figure 5.17. Dr1 and DRAP1 mRNA levels are slightly affected under hypoxic conditions in p53-impaired NARF2-E6 cells. NARF2-E6 cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** RT-PCR analysis. ARPP P0 was used as a control. **B.** Quantification of signals from A. n=3.

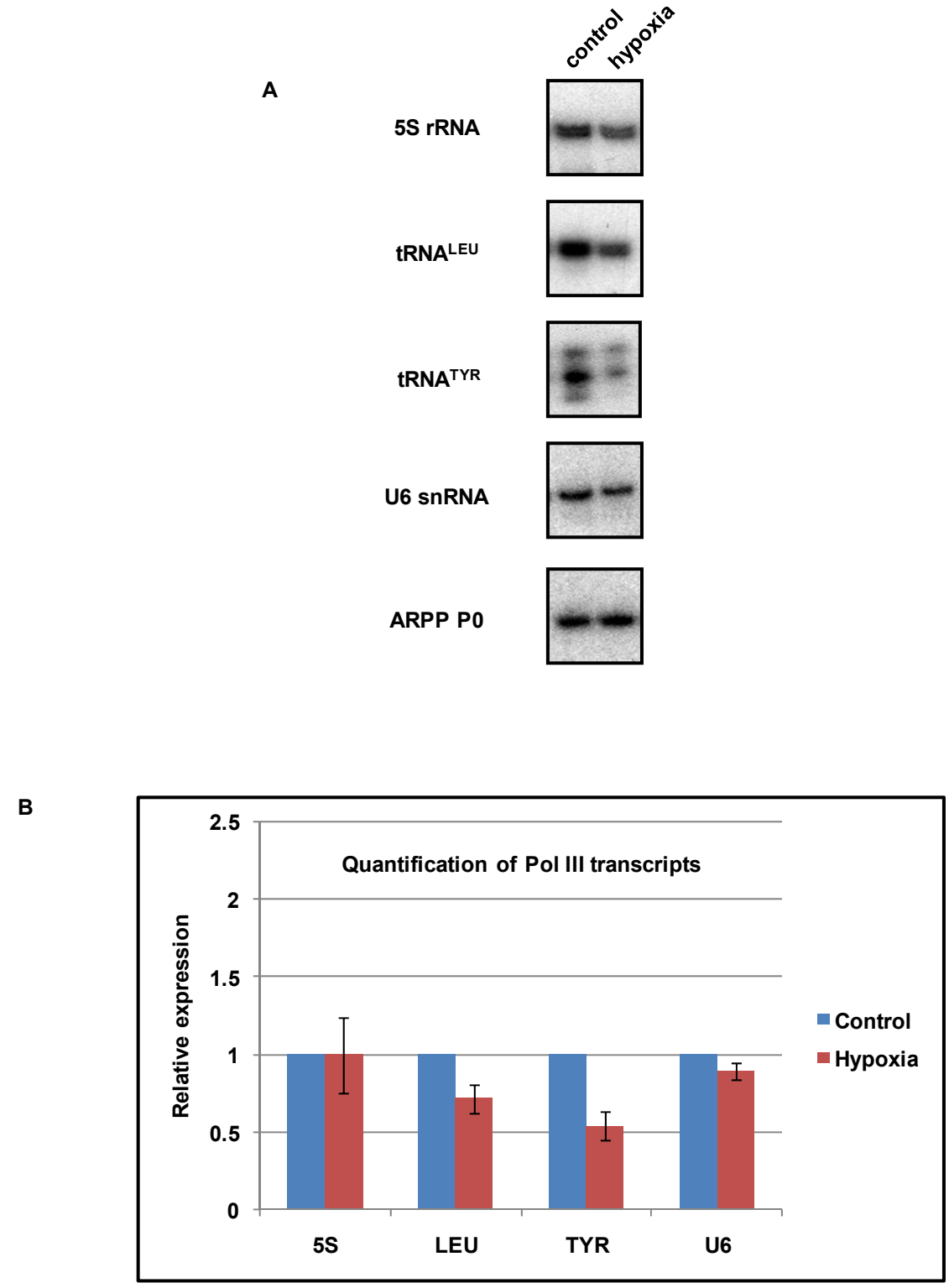


Figure 5.18. tRNA expression levels decrease under hypoxic conditions in NARF2-E6 cells. NARF2-E6 cells were subjected to 1% O_2 hypoxia for 24 hours, before being harvested. Control cells were grown at 20% O_2. **A.** RT-PCR analysis for Pol III-transcribed genes. ARPP P0 was used as a control. **B.** Quantification of the signals of Pol III transcripts from A. n=3.

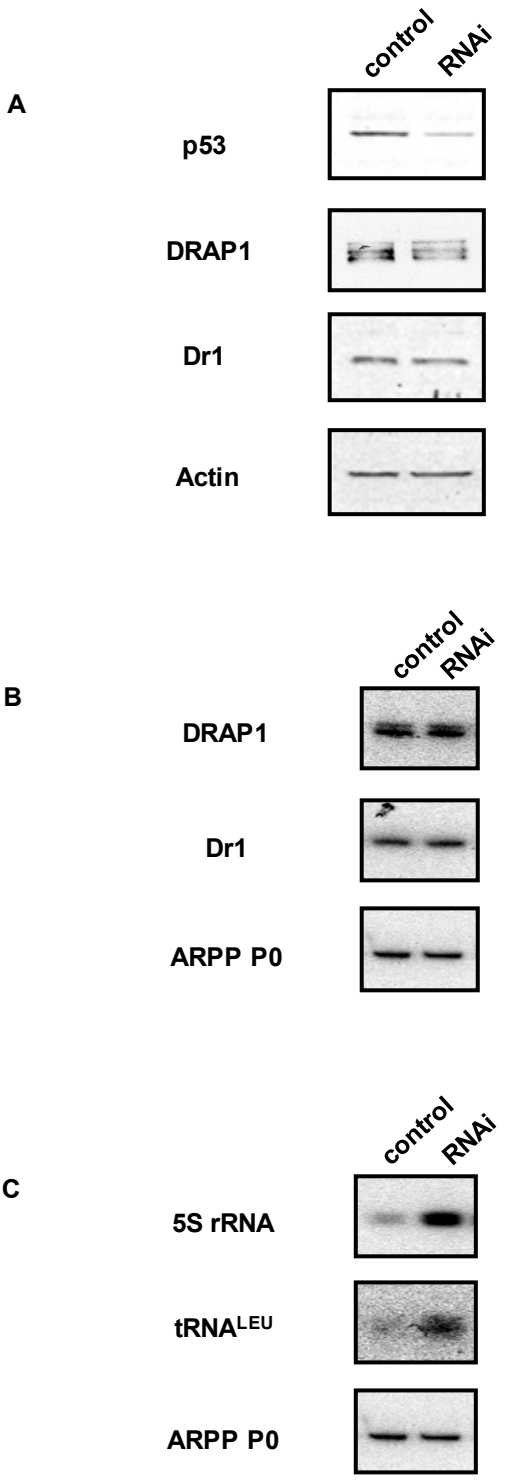


Figure 5.19. p53 depletion by RNAi results in decreased DRAP1 in NARF2 cells. NARF2 cells were transfected by electroporation with either a pSUPER-p53 vector that targets p53 or an empty pSUPER vector control. Cells were left to grow for 48 hours before harvesting. **A.** Western blotting from whole cell extracts. Actin was used as loading control. **B.** RT-PCRs for DRAP1 and Dr1 mRNA. ARPP P0 was used as control. **C.** RT-PCRs for Pol III transcripts. ARPP P0 was used as control.

while Dr1 remained unaffected (Figure 5.19, A). At the mRNA level, as tested by RT-PCRs, both DRAP1 and Dr1 were not affected (Figure 5.19, B), while Pol III transcription was increased (Figure 5.19, C), as would be expected after release of repression by p53 (Cairns & White, 1998).

In summary, the above experiments reveal a differential regulation of the two subunits of the Dr1-DRAP1 complex in hypoxia. DRAP1 is induced about two-fold and this seems to be a posttranscriptional effect, as DRAP1 mRNA levels are not affected. This DRAP1 induction might be related to p53 stabilisation under hypoxic conditions; NARF2-E6 cells with impaired p53 presented decreased DRAP1 levels under the same conditions, while p53 depletion by RNAi also resulted in DRAP1 reduction.

5.2.3 Rapamycin, Pol III transcription and Dr1-DRAP1

Rapamycin is an antifungal agent (Abraham & Wiederrecht, 1996), that has been shown to target the mTOR pathway (Kunz et al, 1993). Rapamycin can inhibit Pol III transcription in mouse cells (Ramsbottom and White, personal communication). To test if rapamycin has the same effect in human cells and if it affects Dr1-DRAP1 levels, HeLa cells were treated either with 100 nM rapamycin or a DMSO control for 4 hours before the cells were harvested. Rapamycin treatment resulted in decreased Pol III transcripts (Figure 5.20, A and B), confirming experiments in mouse (Ramsbottom and White, personal communication). This treatment, did not affect Dr1 or DRAP1, neither at the protein (Figure 5.21, A and B) nor the mRNA level (Figure 5.22, A and B). S6 kinase (S6K), a direct target of mTOR, was not phosphorylated after rapamycin treatment, demonstrating that under the conditions used, the mTOR pathway and subsequently the S6K phosphorylation was inhibited (Figure 5.21, A). Therefore, these results suggest that the effect of rapamycin on Pol III transcription is independent of Dr1-DRAP1.

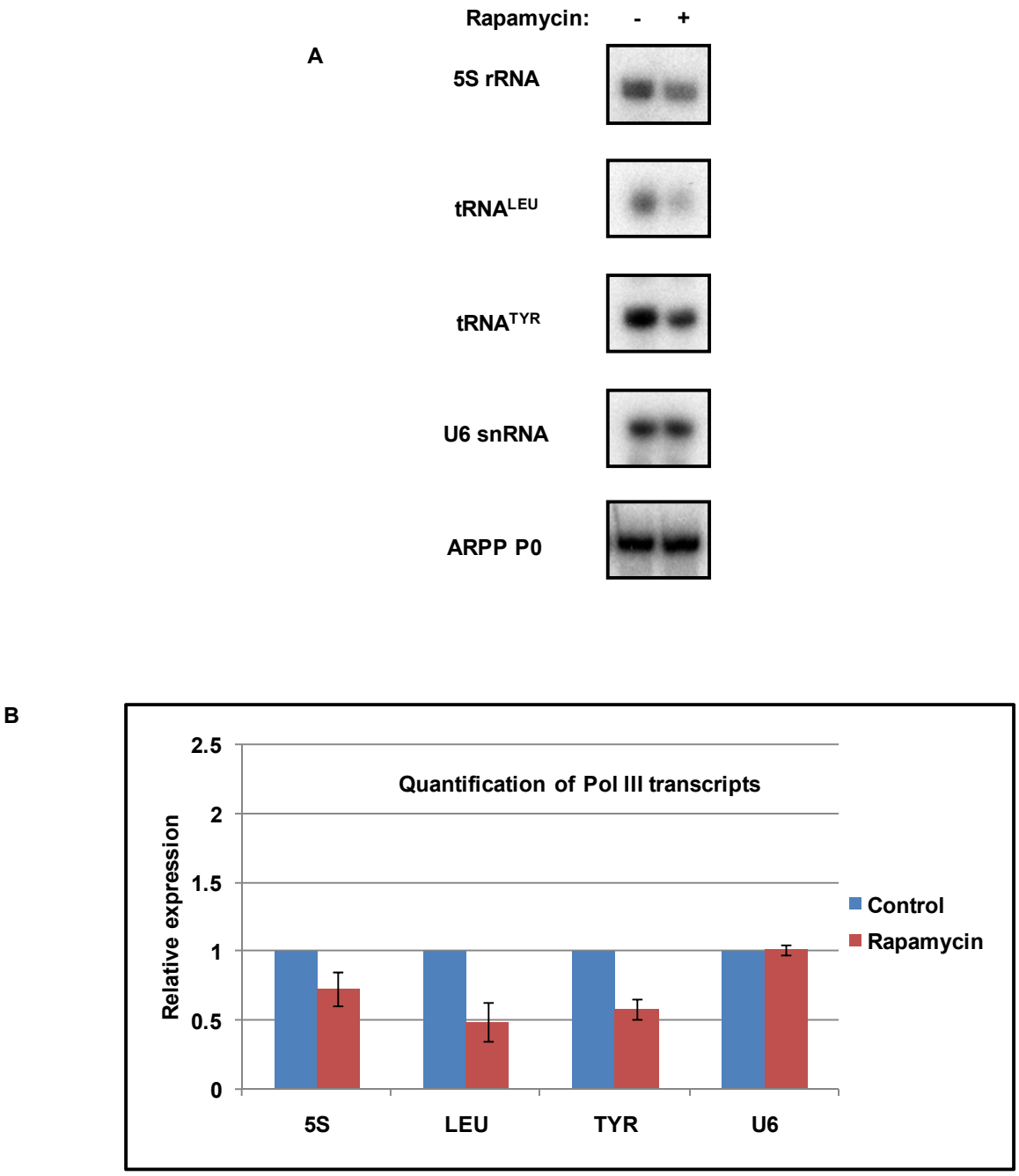
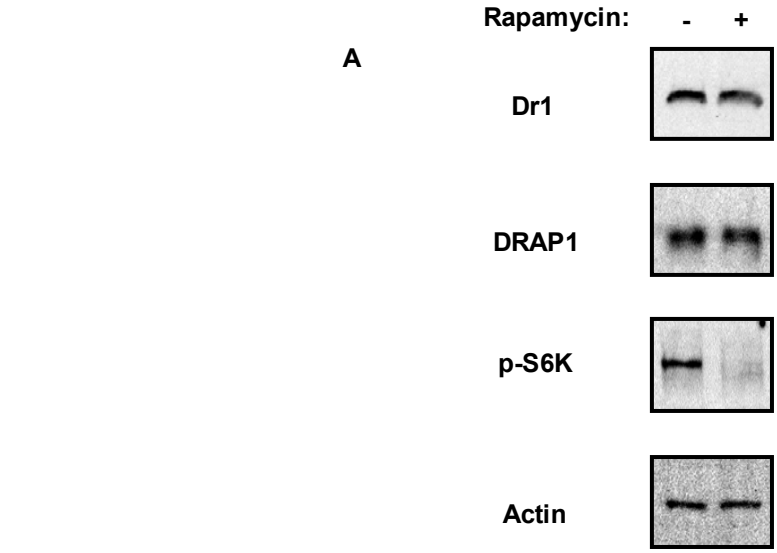


Figure 5.20. Inhibition of the mTOR pathway results in Pol III transcriptional repression in HeLa cells. HeLa cells were grown with 100 nM rapamycin (+) or with DMSO control (-) for 4 hours before harvesting. **A.** Total RNA was isolated, reverse transcribed to cDNA and analysed by PCRs. ARPP P0 was used as control. **B.** Quantification of the signals of Pol III transcripts from A. n=3.



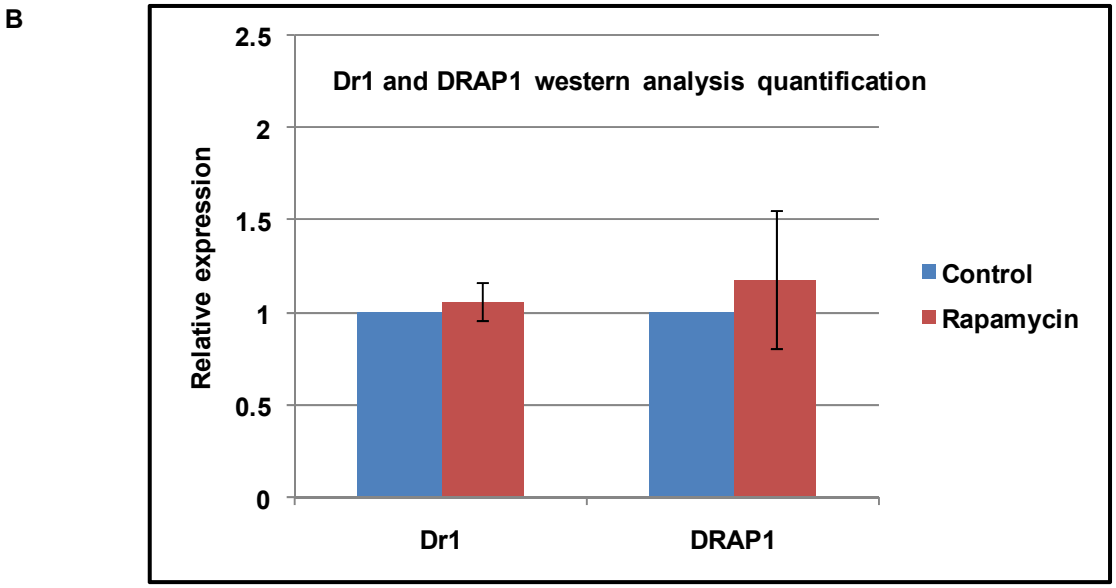


Figure 5.21. Inhibition of the mTOR pathway does not affect Dr1 and DRAP1 proteins in HeLa cells. HeLa cells were grown with 100 nM rapamycin (+) or with DMSO control (-) for 4 hours before harvesting. **A.** Western blotting from whole cell extracts. Actin was used as loading control. **B.** Quantification Dr1 and DRAP1 signals from A. n=3.

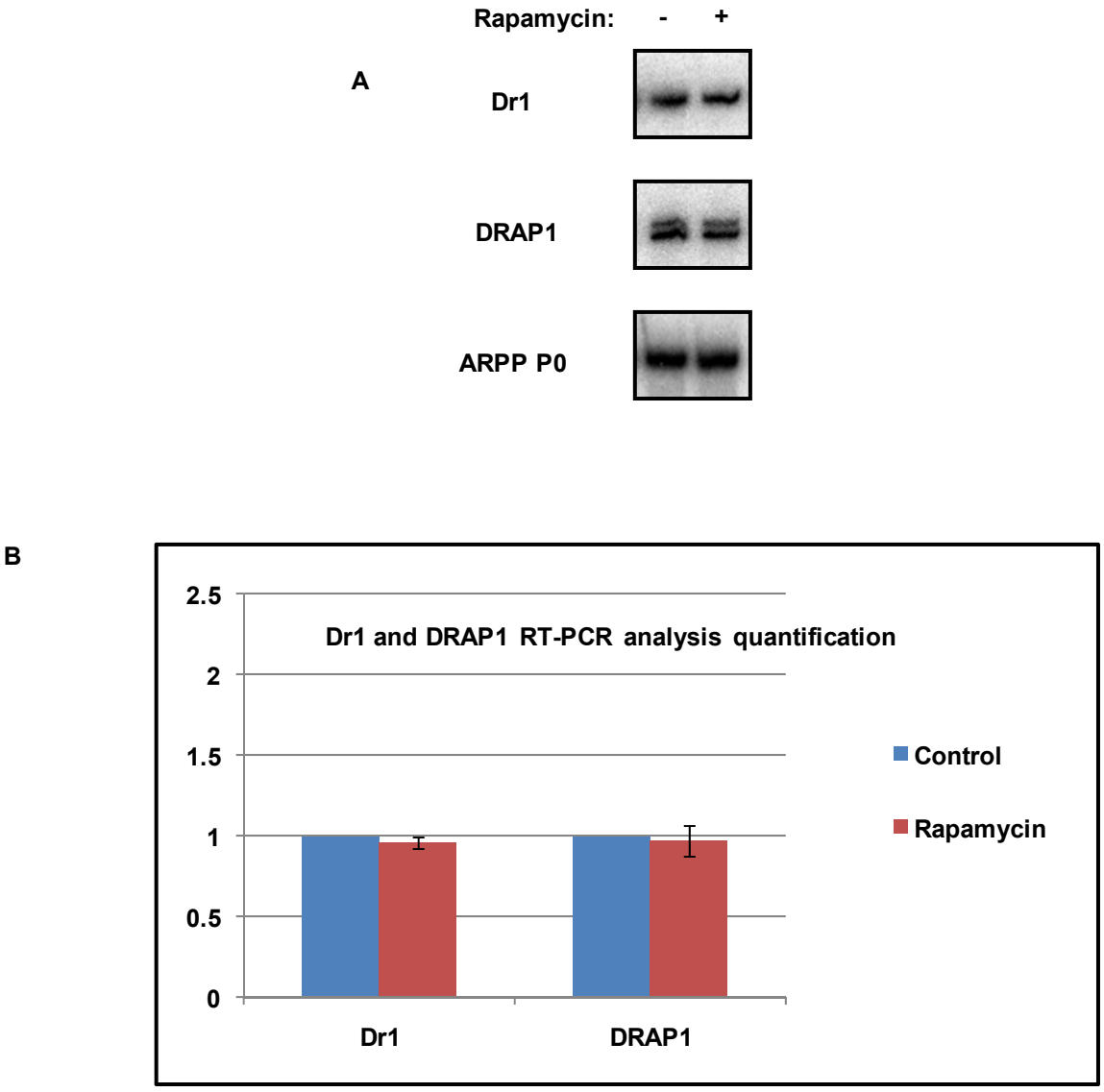


Figure 5.22. Inhibition of the mTOR pathway does not affect Dr1 and DRAP1 mRNA in HeLa cells. HeLa cells were grown with 100 nM rapamycin (+) or with DMSO control (-) for 4 hours before harvesting. **A.** Total RNA was isolated, reverse transcribed to cDNA and analysed by PCRs. ARPP P0 was used as control. **B.** Quantification of Dr1 and DRAP1 signals from A. n=3.

5.2.4 Serum starvation, Pol III transcription and Dr1-DRAP1

Serum starvation has been shown to repress Pol III transcription in mouse A31 cells (Scott et al, 2001). It would be interesting to test if and how Dr1 and/or DRAP1 are regulated under these conditions. A31 cells were grown normally with 10% FBS or serum starved with 0.5% FBS for 24, 48 or 72 hours before harvesting (Scott et al, 2001). Whole cell extracts from Scott et al, (2001) were analysed by western blotting and revealed that under these conditions, Dr1 seems to be reduced, while DRAP1 remains unaffected (Figure 5.23). Further experiments in cells growing with 10% or 0.5% serum for 24 hours, revealed that at the protein level Dr1 was reduced, while DRAP1 remained stable (Figure 5.24). The levels of TFIIIB, TFIIIC or Pol III subunits were also found to be unaffected (Figure 5.24), as previously shown (Scott et al, 2001). At the RNA level, RT-PCRs showed that Pol III transcripts were reduced, while Dr1 and DRAP1 mRNA levels remained stable (Figure 5.25). These results indicate that under these conditions Dr1, but not DRAP1, levels are affected, in contrast to what was observed in hypoxia and heat shock.

Taken together the above results suggest both Dr1 and DRAP1 are subject to posttranscriptional regulation. Interestingly, different stress conditions can affect the two subunits differently, suggesting that they might not always work as a complex.

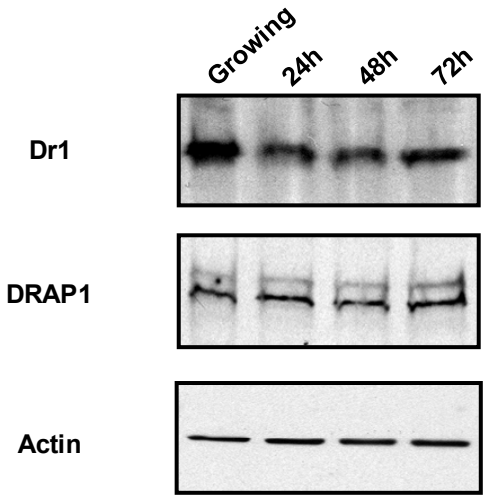


Figure 5.23. Serum starvation results in decreased protein levels of Dr1, but not DRAP1, in mouse A31 cells. Mouse A31 cells were grown either with 10% FBS (Growing) or 0.5% FBS for 24, 48 or 72 hours before harvesting. Western blotting was performed from whole cell extracts. Cell extracts were provided by Scott et al, 2001.

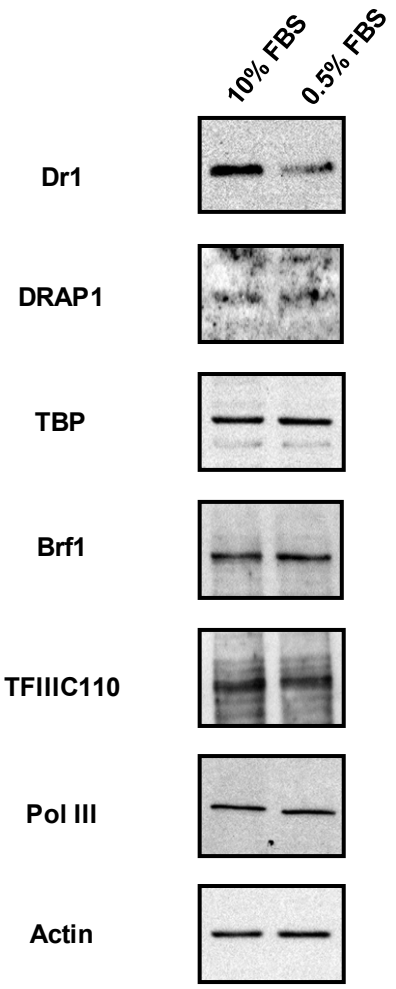


Figure 5.24. Serum starvation downregulates Dr1 protein, but not DRAP1 or TFIIIB, TFIIIC or Pol III subunits, in mouse A31 cells. Mouse A31 cells were grown either with 10% FBS or 0.5% FBS for 24 hours before harvesting. Western blotting was performed from whole cell extracts. Actin was used as a loading control.

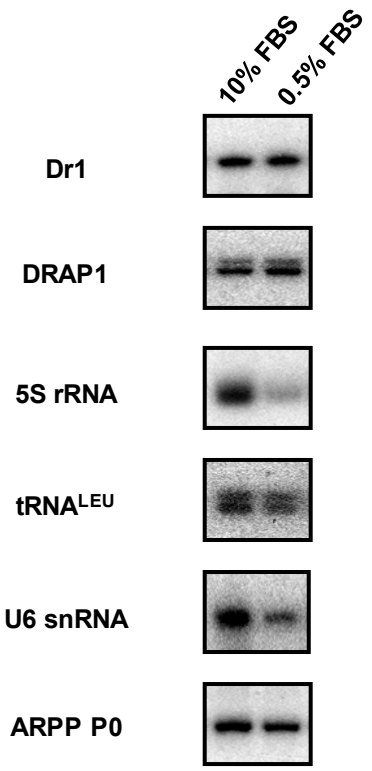


Figure 5.25. Serum starvation results in Pol III transcriptional repression, but does not affect the mRNA levels of Dr1 and DRAP1, in mouse A31 cells. Mouse A31 cells were grown either with 10% FBS or 0.5% FBS for 24 hours before harvesting. Total RNA was isolated, reverse transcribed to cDNA, and analysed by PCRs. ARPP P0 was used as control.

5.3 Discussion

5.3.1 DRAP1 protein induction after heat shock

The heat shock response is a highly conserved in all organisms molecular stress response, that includes induction of gene expression and elevated synthesis of a family of stress-induced proteins called heat shock proteins (Hsps) (Lindquist & Craig, 1988). The Hsps function as molecular chaperones, responsible for 'protein holding' and 'protein folding' (Buchner, 1999; Mayer & Bukau, 2005). The main holding proteins belong to the Hsp70 and Hsp90 families, which bind to unfolded sequences in polypeptides and show preference for hydrophobic regions. These interactions can occur 1) during mRNA translation, when Hsp70 binds to the elongating polypeptide chain to prevent premature self associations in the nascent protein 2) constitutively, when Hsp90 binds to proteins with unstable tertiary structures and 3) during heat shock, when proteins partially unfold and expose hydrophobic sequences (Calderwood et al, 2006; Mayer & Bukau, 2005; Pratt & Toft, 2003).

Heat shock has been shown to affect Pol III transcription. More specifically, after heat shock in human cells, Alu RNA levels were found to be increased, while 5S rRNA, 7SL RNA, 7SK RNA and U6 snRNA remained unaffected (Liu et al, 1995). Similarly in mouse, B1 and B2 RNAs, but not the 5S rRNA, are induced (Allen et al, 2004; Li et al, 1999a). Moreover, the B2 RNAs have been shown to repress mRNA transcription after heat shock, by binding directly to Pol II (Allen et al, 2004; Espinoza et al, 2004).

Experiments in this study have confirmed that the levels of 5S rRNA and U6 snRNA are not affected after heat shock in the human HeLa and U20S cell lines (Figure 5.3 A and B, and Figure 5.6, A). Furthermore, the levels of $tRNA^{LEU}$ and $tRNA^{TYR}$ were investigated. In HeLa cells, $tRNA^{TYR}$ was found to be increased after heat shock; $tRNA^{LEU}$, however, was not significantly altered (Figure 5.3, A and B). Initial experiments in U2OS cells also seemed to indicate enhanced tRNA levels after heat shock (Figure 5.6, A), but further experimental data from replicate experiments failed to established a significant increase (Figure 5.6, B). Thus, it seems that under the conditions tested, tRNA expression, as well as 5S rRNA and U6 snRNA, are not significantly affected by heat shock.

Interestingly, heat shock resulted in elevated levels of DRAP1. DRAP1 protein levels were clearly raised both in HeLa (Figure 5.1, A and B) and in U2OS cells (Figure 5.4, A and B), while Dr1 remained stable. This increase was not due to transcriptional activation of DRAP1, as both DRAP1 and Dr1 mRNA levels remained unaffected in both cell lines (Figure 5.2, A and B and Figure 5.5). Therefore, it appears that DRAP1 induction might be attributed to posttranscriptional regulation, perhaps by enhanced mRNA translation or/and protein stabilisation by decreased degradation.

It has previously been reported that heat shock can lead to DRAP1 recruitment to Hsp gene promoters in yeast (Geisberg et al, 2002). This seems contradictory to the finding that Dr1 release from the human Hsp70 promoter results in alleviation of repression (Kraus et al, 1994). It was suggested that increased presence of DRAP1 relative to Dr1 at the gene promoters might result in transcriptional activation (Creton et al, 2002; Geisberg et al, 2001). It would be interesting to perform ChIP experiments in cells subjected to heat shock and investigate possible changes of the Dr1 and/or DRAP1 at human gene promoters.

Although it is not clear why DRAP1 protein is being induced and what cellular function this might serve, these results are interesting, as it is the first time that differential regulation of the Dr1-DRAP1 subunits is being reported. In contrast to the current model for Dr1-DRAP1 functioning as a complex with 1:1 stoichiometry, DRAP1 induction after heat shock, implies alternative roles for DRAP1 in response to heat shock.

5.3.2 DRAP1 protein induction and Pol III transcriptional repression under hypoxic conditions

Low oxygen supply in cells triggers a coordinated stress response to restore oxygen homeostasis, by enhancing tissue perfusion and anaerobic ATP generation through glycolysis (Cummins & Taylor, 2005). This response includes transcriptional and posttranscriptional events, with the transcription factor HIF1 (hypoxia inducible factor 1) playing a major role in the control of the adaptive responses to hypoxia; under oxygen limiting conditions HIF1 gets stabilised (Schofield & Ratcliffe, 2004), and in turn, rapidly regulates the transcription of a wide range of genes, either positively or negatively (Rocha, 2007; Semenza, 2003). However, gene expression in hypoxia is not only achieved by transcriptional regulation, but also by translational modulation, where translation of certain

mRNAs can be inhibited or enhanced (Koumenis et al, 2002; Liu et al, 2006; Wouters et al, 2005).

It has been recently reported that Pol III transcription is repressed under hypoxia in rat cardiomyocytes (Ernens et al, 2006). It has been shown that at 1% O_2, tRNA transcription is downregulated due to reduced recruitment of the polymerase to promoters (Ernens et al, 2006). Interestingly, Dr1-DRAP1 complex has also been reported to be induced at severe hypoxia (0.01% O_2) and mediate transcriptional repression of Pol II genes in a murine hepatoma cell line (Denko et al, 2003). In an attempt to extend these studies, hypoxic conditions were employed to investigate Pol III transcriptional repression and the role of Dr1-DRAP1 in human cell lines.

DRAP1 protein, but not Dr1, was found to be induced under hypoxic conditions (1% O_2) in HeLa and U2OS cell lines (Figures 5.7 and 5.12). The same conclusion was reached when using chemically-induced anoxia (Figure 5.10). This comes in contrast to the previous report, where both subunits were increased in murine hepatoma cells (Denko et al, 2003). One cannot exclude that this contradiction might be attributed to the different species and cell lines used (human epithelial or osteosarcoma vs murine hepatoma). Another possibility might be the fact that hypoxic conditions in experiments described in this thesis contained 100 times more oxygen (1% O_2 mild hypoxia vs 0.01% O_2 severe hypoxia), even though chemically-induced anoxia reproduced the 1% O_2 hypoxia effect (Figures 5.10 and 5.7, 5.12).

Furthermore, the induction of DRAP1 under hypoxic conditions seems to be a posttranscriptional effect, as the DRAP1 mRNA levels were not altered either in HeLa or U2OS cell lines after hypoxia or in HeLa cells after chemically-induced anoxia (Figures 5.8, 5.11 and 5.13). This result is consistent with the Dr1-DRAP1 changes in 0.01% O_2 in murine hepatoma cells, where the Dr1 and DRAP1 mRNA also remained stable (Denko et al, 2003). Moreover, a number of studies that used genomic approaches to detect gene expression differences between normal and hypoxic conditions, did not detect altered Dr1 and/or DRAP1 expression at the mRNA level, supporting the argument that DRAP1 is posttranscriptionally regulated (Hammond et al, 2006; Ning et al, 2004; Sung et al, 2007). As mentioned previously, this DRAP1 protein induction might be attributed to protein stabilisation by decreased degradation. Since DRAP1, at least *in vitro*, is strongly phosphorylated by CK2, while Dr1 is weakly (Goppelt et al, 1996), it would be interesting

in the future to investigate if DRAP1 stability under hypoxic conditions is related to its phosphorylation status by CK2 and/or other kinases.

Interestingly, DRAP1 was decreased under hypoxic conditions (1% O_2) in NARF2-E6 cells (Figure 5.16). These cells are of U2OS origin and constitutively express the HPV E6 protein, which has been shown to bind p53 and target it for degradation via the ubiquitin pathway (Scheffner et al, 1992; Werness et al, 1990). Therefore, it seems that when p53 is impaired, DRAP1 stability under hypoxic conditions is severely affected. In these cells, the mRNA levels of Dr1 and DRAP1, as well as p53, were found to be relatively reduced in contrast to similar experiments in HeLa and U2OS, where mRNA remained stable (compare Figures 5.17 with 5.8 and 5.13). However, this agrees with the finding that Dr1 and DRAP1 seem to have a p53-responsive element at their promoter region and could therefore potentially be p53 targets (Figure 5.15). Depletion of p53 by RNAi in NARF2 cells also resulted in decreased levels of DRAP1 at the protein, but not mRNA level (Figure 5.19, A and B), further suggesting a relation between p53 and DRAP1 protein stability. It would be interesting to further investigate this in the future, by inducing overexpression of p53 in human cell lines, the prediction being that increased p53 protein levels would result in increased DRAP1 protein, and perhaps also mRNA levels.

Pol III transcription was downregulated under hypoxic conditions in the human HeLa, U2OS and NARF2-E6 cell lines (Figures 5.9, 5.14 and 5.18) and in chemically-induced anoxic conditions in HeLa (Figure 5.11). These results are in accordance with similar experiments in rat cardiomyocytes that showed Pol III transcription downregulation under hypoxic conditions (Ernens et al, 2006). Dr1 seems to have a role in Pol III transcriptional repression, as it was recruited to Pol III-transcribed genes under 1% O_2 hypoxia, although the total protein levels of Dr1 were unaffected (Figures 3.16, A and 5.12).

Interestingly, DRAP1 protein induction during hypoxia does not seem to affect Pol III transcription. Pol III transcription was downregulated when DRAP1 protein levels were physiologically either increased (HeLa and U2OS cells) or decreased (NARF2-E6 cells) due to hypoxia or anoxia (Figures 5.7, 5.9, 5.10, 5.11, 5.12, 5.14, 5.16 and 5.18). Although DRAP1 is expected to be found together with Dr1 at Pol III promoters under hypoxic conditions, the substantial increase at the protein levels does not correspond to any significant changes in Pol III transcription (compare Figures 5.9 and 5.14 with 5.18), and

therefore suggests that under these conditions, DRAP1 does not have a role in the regulation of Pol III transcription. However, this DRAP1 induction might affect the transcription of certain Pol II genes or affect processes other than gene transcription.

As previously mentioned, Pol III transcription is downregulated under hypoxic conditions in rat cardiomyocytes and Pol III is disassociated from its target genes (Ernens et al, 2006). However, in that report TFIIIB was found to be stably associated with Pol III promoters, even when Pol III was displaced in hypoxia. It was suggested that this might be attributed to the nature of cardiomyocytes, as they are post mitotic cells that have withdrawn permanently from cell cycle; it was therefore predicted that in proliferating cells TFIIIB would be displaced under hypoxic conditions (Ernens et al, 2006). Indeed, experiments presented in this thesis revealed that TFIIIB was displaced at Pol III-transcribed genes in U2OS cells under hypoxic conditions (Figure 3.16, A), confirming the work previously done in cardiomyocytes (Ernens et al, 2006).

There are conflicting reports as to whether and to what extent p53 is accumulated under hypoxia, in relation to HIF1α (Schmid et al, 2004). Severe hypoxia has been shown to induce p53 (Graeber et al, 1994) and this was considered to be due to stabilisation of p53 by its association with HIF1α (An et al, 1998). Others found that mild hypoxia (1% O_2) strongly stabilised HIF1α (Triantafyllou et al, 2006; Wenger et al, 1998), but it did not induce p53 and suggested that mechanisms other than HIF1α activation contribute to induction of p53 (Wenger et al, 1998). The latter report was also challenged; it was shown that in HCT116 cells, 1% O_2 hypoxia stabilises HIF1α only after 4 days, while in chemically induced anoxia HIF1α was stabilised after 24 hours and suggested that hypoxia induces a p53-dependent growth arrest without HIF1α stabilization, while anoxia induces significant HIF1α protein stabilisation and p53 activation (Achison & Hupp, 2003). Other reports indicated that hypoxia alone is insufficient to accumulate p53, but cooperates with DNA damage signals (Kaluzova et al, 2004) or glucose deprivation and acidosis (Pan et al, 2004) to provoke p53 activation.

In contrast to the above mentioned reports, in the experiments included in this thesis, hypoxic conditions of 1% O_2 resulted in p53 induction together with HIF1α stabilisation in HeLa and U2OS cells (Figures 5.7 and 5.12). Experiments with chemically-induced anoxia also induced p53, in agreement with previous reports (Achison & Hupp, 2003; Ashcroft et al, 1999) and stabilised HIF1α (Figure 5.10). Since the mechanism of p53 induction in hypoxia is not yet clear and secondary processes seem to affect p53

induction, this contradiction might be attributed to the specific experimental conditions used and also to deviations in the behaviour of different cell lines under these conditions. Interestingly, it seems that expression of HPV E6 (HeLa or NAFR2-E6 cells), which targets p53 for ubiquitination, results in greater stabilisation of HIF1α (compare Figures 5.7 and 5.16 to 5.12). This comes in accordance with the finding that p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of the HIF1α and that, therefore, loss of p53 enhances HIF1α levels (Ravi et al, 2000).

In summary, experiments discussed in this chapter confirmed that Pol III transcription is repressed under mild hypoxia and anoxia in human cell lines. Under these conditions DRAP1 is posttranscriptionally stabilised, while Dr1 is not affected; p53 might have a role in DRAP1 stabilisation, but more experiments are needed to clarify this possibility; interestingly, in heat shock experiments, where DRAP1 was also found to be stabilised, p53 was also induced (Figures 5.1 and 5.4), in agreement with previous reports (Graeber et al, 1994). These results, taken together with the results from heat shock experiments, strongly suggest differential regulation of the two subunits and imply additional roles for DRAP1.

5.3.3 Inhibition of mTOR by rapamycin does not affect Dr1-DRAP1

As previously discussed, Dr1 and DRAP1 are differentially regulated under hypoxic conditions, resulting in elevated DRAP1 levels. It was also mentioned that hypoxia does not only affect gene transcription (Rocha, 2007), but also mRNA translation (Wouters et al, 2005). One of the mechanisms by which hypoxia regulates translation is by inhibiting mTOR; it has been shown that hypoxia results in the inhibition of mTOR and subsequent hypophosphorylation of its substrates 4E-BP1 (4E-binding protein 1) and S6K (ribosomal p70 S6 kinase) (Arsham et al, 2003).

Rapamycin is an antifungal and immunosuppressant drug that forms a complex with FKBP12 (FK506-binding protein); the complex then binds to the kinase domain of mTOR and inhibits its function (Fingar & Blenis, 2004). Transcriptional profiling of rapamycin treatment has shown that the drug can affect about 5% of all the genes in yeast, Drosophila and mammalian cells, indicating that TOR broadly affects cellular functions (Reiling & Sabatini, 2006).

The mTOR is required for the activation of 45S ribosomal gene transcription by Pol I; rapamycin treatment resulted in dephosphorylation of the rDNA transcription factor, UBF, which significantly reduced its ability to associate with the basal rDNA transcription factor SL-1 (Hannan et al, 2003). Inhibition of the mTOR pathway by rapamycin also downregulated Pol III transcription in mouse cells, although the mechanism(s) for activation of Pol III transcription by the mTOR has not yet been described (Ramsbottom and White, personal communication).

Rapamycin was employed to inhibit mTOR in human cells and confirm the observations in mouse cells. Indeed, treatment with rapamycin resulted in repressed Pol III transcription, including reduction in the expression of the stable 5S rRNA, but interestingly, not the U6 snRNA (Figure 5.20, A and B). Since the stable 5S rRNA was affected under these conditions, it is tempting to hypothesize that U6 snRNA is not affected due to an inhibition mechanism relying on Brf1, which is essential for transcription from type 1 and 2 Pol III promoters, but not Brf2, which is needed for type 3 promoters, such that of U6 snRNA genes.

As hypoxia did affect DRAP1 regulation and mTOR inhibition resulted in Pol III downregulation, it was tested if Dr1 and DRAP1 were also regulated under these conditions. Dr1 and DRAP1 were found to be unaltered after rapamycin treatment both at the protein (Figure 5.21, A and B) and mRNA levels (Figure 5.22, A and B). S6K was found to be hypophosphorylated after the rapamycin treatment, confirming that mTOR was inhibited (Figure 5.21, A). It is therefore concluded that Dr1 and DRAP1 are not regulated by components downstream of the mTOR pathway.

5.3.4 Serum starvation affects Dr1, but not DRAP1

Serum starvation results in growth arrest at G1 phase (Pardee, 1974) and is widely used to synchronise mammalian cells and analyse the cell cycle. It has been shown in mouse fibroblasts that serum starvation results in Pol III transcriptional repression of all three types of Pol III promoters and that Rb plays a major role by binding to TFIIIB (Scott et al, 2001). In HeLa cells, Pol III transcription was also shown to be downregulated when cells were growing in low serum (Sinn et al, 1995).

Mouse A31 fibroblasts were used to investigate if serum starvation, and the subsequent mitogen deprivation and growth arrest, would result in regulation of Dr1 and/or DRAP1. It was found that protein levels of Dr1, but not DRAP1, are reduced in serum-starved cells (Figures 5.23 and 5.24). The protein levels of TFIIIB, TFIIIC and Pol III subunits were also tested and found to be unaltered in accordance with previous studies (Scott et al, 2001). At the mRNA level, Dr1 and DRAP1 were not affected (Figure 5.25), while the Pol III transcripts were reduced (Figure 5.25), as expected (Scott et al, 2001). These results indicate that in contrast to the data from heat shock and hypoxia experiments, where DRAP1 was posttranscriptionally regulated, Dr1 can also be, probably by posttranscriptional mechanisms that might involve inhibition of mRNA translation or enhanced degradation of the translated protein.

In summary, a number of experiments employing different stress conditions, such as heat shock, hypoxia, and serum deprivation, reveal that the two subunits of the Dr1-DRAP1 complex can be differentially regulated at the posttranscriptional level. The mechanism of regulation, as well as the role and function of the two subunits under these conditions, are not yet known; it is tempting to hypothesise that under stress Dr1 and DRAP1 might also acquire additional roles, other than forming a dimeric transcriptional regulation complex.

CHAPTER 6

The *in vivo* role of the pocket proteins Rb/p107/p130 in RNA polymerase III transcription

6.1 Introduction

6.1.1 The pocket proteins Rb/p107/p130

The retinoblastoma (Rb) gene family includes three members Rb, p107 and p130, that are commonly referred as the 'pocket proteins' due to their conserved binding pocket region. The pocket region, that mediates interactions with viral oncoproteins and cellular proteins, contains two conserved functional domains separated by a spacer which differs among the members (Figure 6.1) (Classon & Harlow, 2002). The pocket proteins are differentially expressed during the cell cycle; p130 is highly expressed in arrested cells, whereas p107 expression peaks during the S phase and Rb is steadily expressed through the cell cycle (Classon & Harlow, 2002). More than 100 proteins have been reported to interact with the Rb proteins and in most cases the interactions occur through the pocket region (Morris & Dyson, 2001).

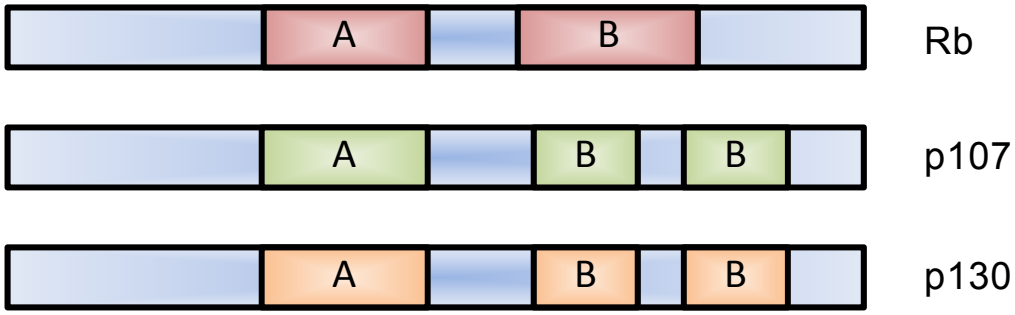


Figure 6.1. The Rb family. Rb, p107 and p130 make up the Rb family proteins. The pocket domain consists of regions A and B, separated by a spacer. Adapted from Classon and Harlow, 2002.

6.1.2 Rb/p107/p130 function and regulation

Major targets of the Rb proteins are the E2F transcription factors. The family of E2F transcription factor consists of at least 9 members that can be transcriptional activators (E2F1, E2F2, E2F3a) or repressors (E2F3b, E2F4, E2F5, E2F6, E2F7, E2F8) (Dimova & Dyson, 2005; Logan et al, 2005). The E2F proteins heterodimerize with DP proteins (DP1 and DP2) to give rise to functional E2F activity that directly regulates the expression of a

large number of genes, which are involved in cell cycle progression, DNA replication and damage repair, apoptosis, development and differentiation (Bracken et al, 2004; Trimarchi & Lees, 2002). Individual E2Fs have distinct mechanisms of action and regulation, and are associated with particular types of biological activities (Dimova & Dyson, 2005); Rb preferentially binds to E2F1-3, while p107 and p130 bind to E2F4 and E2F5 (Cobrinik, 2005). Binding of the E2F/DP dimers by pocket proteins results in blocking their transcriptional domains and, thus, in the inhibition of E2F-dependent transcription (Helin et al, 1993).

The activity of the Rb family members is regulated post-translation by phosphorylation; they can interact with their target proteins when hypophosphorylated, while phosphorylation results in their inactivation (Mittnacht, 1998). This phosphorylation of the pocket proteins is mediated by cyclins and cyclin-dependent kinases (CDKs), with their hyperphosphorylation at the end of G1 being essential for progress to the S phase of the cell cycle (Adams, 2001). More specifically, cyclin D/CDK4,6 and cyclin E/CDK2 can phosphorylate Rb and p130 in G1 (Ewen et al, 1993; Hansen et al, 2001), while p107 seems to be phosphorylated by cyclin D/CDK4 (Beijersbergen et al, 1995). In general, according to the current model (Knudsen & Knudsen, 2006; Mittnacht, 1998), mitogenic signals in G1 activate cyclin D/CDK4,6 and cyclin E/CDK2 complexes that hyperphosphorylate Rb and render it inactive. E2F proteins are thus no longer inhibited and proceed with their transcriptional programmes that facilitate progression through S and G2/M phases; with the exception of G1, Rb is held inactive (hyperphosphorylated) during the rest of the cell cycle (Knudsen & Knudsen, 2006; Mittnacht, 1998).

Rb proteins can regulate transcription not only through the inhibition of E2F-mediated gene transcription, but also by changes in chromatin structure. It has been shown that Rb, p107 and p130 can interact with histone deacetylase 1 (HDAC1) and be recruited to E2F complexes (Brehm et al, 1998; Ferreira et al, 1998; Magnaghi-Jaulin et al, 1998). Rb family proteins can also associate with hBRM and BRG1 (Dunaief et al, 1994; Strober et al, 1996; Trouche et al, 1997), homologs of components of the yeast chromatin remodelling SNF2/SWI2 complex, although a physical interaction between Rb and BRG1 is not required for Rb-mediated growth arrest and transcriptional repression of E2F target genes (Kang et al, 2004). Moreover, the pocket proteins can associate with the histone methyl transferase SUV39H1, that specifically methylates K9 of histone H3 (Nicolas et al, 2003; Nielsen et al, 2001; Vandel et al, 2001), and also with the tri-methylating enzymes

Suv4-20h1 and Suv4-20h2 that tri-methylate K20 of histone H4 (Gonzalo et al, 2005), suggesting an important role of the Rb proteins in histone methylation and telomeric/centromeric chromatin. Taken together, the above studies suggest that the Rb family proteins can regulate transcriptional repression at the chromatin level by recruiting HDACs, methylases and other chromatin remodelling enzymes.

6.1.3 The pocket proteins as tumour suppressors

It is believed that in almost all cancers the Rb function is compromised and several mechanisms that can accomplish this have been identified in human tumours (Hanahan & Weinberg, 2000). First, loss or mutation of the Rb locus itself, as occurs in retinoblastoma, can ablate Rb function (Hanahan & Weinberg, 2000). Overexpression of cyclin D or CDK4, as well as loss or mutation of the cyclin-dependent kinase inhibitor $p16^{INK4}$, result in enhanced Rb phosphorylation and its subsequent inactivation (Hanahan & Weinberg, 2000; Knudsen & Knudsen, 2006). Moreover, oncoproteins such as the HPV E7 (Dyson et al, 1989), the SV40 large T antigen (Dyson et al, 1990) or the adenovirus E1A (Whyte et al, 1988) can sequester Rb and inhibit interaction with E2F or corepressor molecules, compromising its function (Hanahan & Weinberg, 2000; Knudsen & Knudsen, 2006).

A number of knock-out mice were generated in order to genetically study the role of the Rb family proteins in tumourigenesis. Mice deficient for Rb ($Rb^{-/-}$) are embryonic lethal (Clarke et al, 1992; Jacks et al, 1992; Lee et al, 1992). Surprisingly, low-passaged cells derived from $Rb^{-/-}$ mice exhibit a cell cycle profile similar to that of Rb proficient cells (Mayhew et al, 2004; Sage et al, 2003); moreover, unlike in human, $Rb^{+/-}$ (or $Rb^{-/-}$) mice do not develop retinoblastoma (Jacks et al, 1992), but instead the inactivation of p107 or p130 simultaneously with Rb is needed (MacPherson et al, 2004; Robanus-Maandag et al, 1998). It is thus believed that there is some redundancy in the function of p107/p130 and Rb and that the lack of Rb is compensated for by p107 and p130 (Donovan et al, 2006; Hurford et al, 1997; Lee et al, 1996); this compensation might be especially important in particular tissues (Vidal et al, 2007). Furthermore, tissue-specific knockouts revealed that, in many cases, Rb inactivation resulted in increased proliferation, but not tumourigenesis, which required additional mutations, often disabling the p53 pathway (Vidal et al, 2007).

Unlike Rb, mutations in p107 and p130 loci are not a common event in cancer (Classon & Dyson, 2001). Knockouts for p107 and p130 showed no predisposition for tumour formation; similarly, $p107^{+/-}$; $p130^{-/-}$ or $p107^{-/-}$; $p130^{+/-}$ mice did not develop tumours (Cobrinik et al, 1996; Lee et al, 1996; Vidal et al, 2007). However, as mentioned before, $Rb^{-/-}$; $p107^{-/-}$ and $Rb^{-/-}$; $p130^{-/-}$ mice, in contrast to $Rb^{-/-}$, do develop retinoblastoma (MacPherson et al, 2004; Robanus-Maandag et al, 1998). Thus, it has been proposed that p107 and p130 may function as tumour suppressors in combination with other mutations, such as those that result in Rb deactivation (Classon & Dyson, 2001); this seems to be the case, as Rb/p107- and Rb/p130- deficient mice are highly cancer-prone and p107 and p130 can suppress tumour development by loss of pRB in a variety of tissues (Dannenberg et al, 2004). Cell culture evidence also supports the role of p107 and p130 as tumour suppressors, as experiments with triple knockout fibroblasts showed that all three Rb family proteins collaborate to maintain control of the cell cycle (Dannenberg et al, 2000; Sage et al, 2000).

6.1.4 The Rb family and RNA polymerase III transcription

Apart from controlling E2F-dependent transcription, Rb can bind to a large number of proteins that participate in various cellular functions (Morris & Dyson, 2001). Rb has been shown to repress Pol III transcription (White et al, 1996) by binding to TFIIIB when it is hypophosphorylated (Chu et al, 1997; Hirsch et al, 2004; Larminie et al, 1997; Scott et al, 2001; Sutcliffe et al, 2000). As a result, TFIIIB is sequestered in an inactive complex and cannot interact with TFIIIC or Pol III at Pol III promoters, resulting in Pol III transcriptional repression (Hirsch et al, 2004; Sutcliffe et al, 2000). The U6 snRNA genes, which possess type 3 Pol III promoters, are an exception, as Rb can be found at these promoters (Hirsch et al, 2004). p107 and p130 have also been shown to interact with TFIIIB and repress Pol III transcription, although Rb may have a dominant role in controlling Pol III transcription, at least in certain cell types (Sutcliffe et al, 1999).

6.2 Results

A number of *in vitro* and *in vivo* experiments have established control of Pol III transcription by the Rb family proteins in mouse cells (Larminie et al, 1997; Sutcliffe et al, 2000; Sutcliffe et al, 1999; White et al, 1996). However, there are considerable differences between the human and mouse models regarding tumour suppression by the Rb family. For example, humans heterozygous for Rb develop retinoblastoma, while heterozygous mice only develop a mild form of dysplasia (Vidal et al, 2007). Therefore, it would be interesting to investigate if the Rb family members can repress Pol III transcription *in vivo*, in human cells, in a similar manner to that already shown in mouse.

6.2.1 Regulation of Pol III-transcribed gene expression by Rb in IMR90 and HeLa cells

To investigate how loss of Rb would affect Pol III transcripts, an RNAi approach was employed. IMR90 human lung fibroblasts were transfected with siRNA against Rb or Oct-1 (control). Twenty four hours post-transfection the cells were harvested and extracts were prepared for protein or RNA analysis. Western blotting revealed that cells treated with the Rb siRNA had reduced levels of Rb in comparison with the control (Figure 6.2, A). Acting as control, Oct-1 was decreased, confirming the effectiveness of the RNAi depletion under these conditions (Figure 6.2, A). Furthermore, RNA analysis revealed that, as expected, Rb mRNA was also diminished (Figure 6.2, B).

RT-PCR analysis revealed that depletion of Rb resulted in about 2-fold and 4-fold increase of 5S rRNA and tRNA expression levels respectively (Figure 6.3, A and B). These results agree with previous studies in mouse cells, where Rb-null cells presented elevated levels of Pol III transcripts (Larminie et al, 1997; White et al, 1996), and suggest that Rb maintains the same Pol III transcriptional repressive role in normal human cells.

HeLa is a human cervical carcinoma cell line that has the viral HPV18 DNA integrated into the cellular genome and expresses the viral E7 protein (Schwarz et al, 1985). E7 can bind to Rb, p107 and p130 (Dyson et al, 1989; Gonzalez et al, 2001), resulting in the inhibition of the activity of these tumour suppressors and elevated Pol III transcription in this cell line (Larminie et al, 1999). Since a fraction of Rb is thought to be

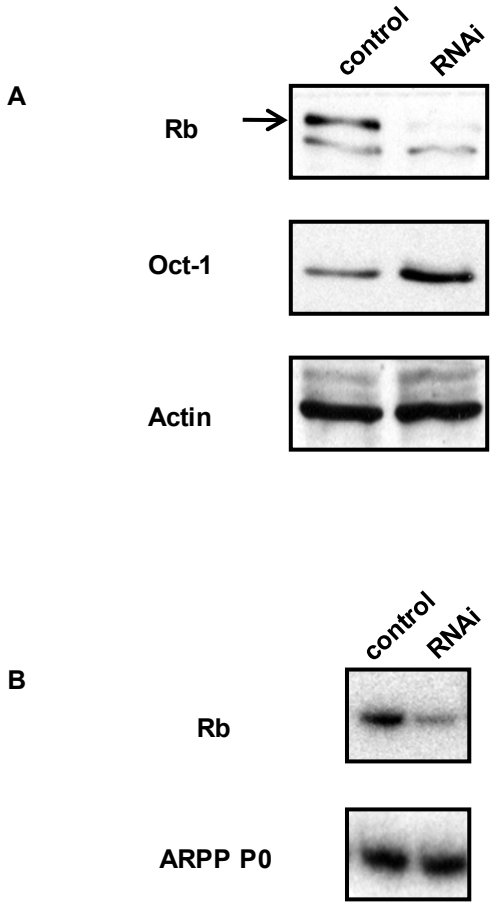


Figure 6.2. Rb RNAi in IMR90 cells. IMR90 human lung fibroblasts were transfected with siRNA (100 nM) against Rb or Oct-1 (control). Cells were harvested 24 hours after transfection. **A.** Western blotting for Rb and Oct-1. Actin was used as loading control. The arrow indicates the Rb protein (total). **B.** RT-PCR analysis to confirm the Rb knock-down at the mRNA level.

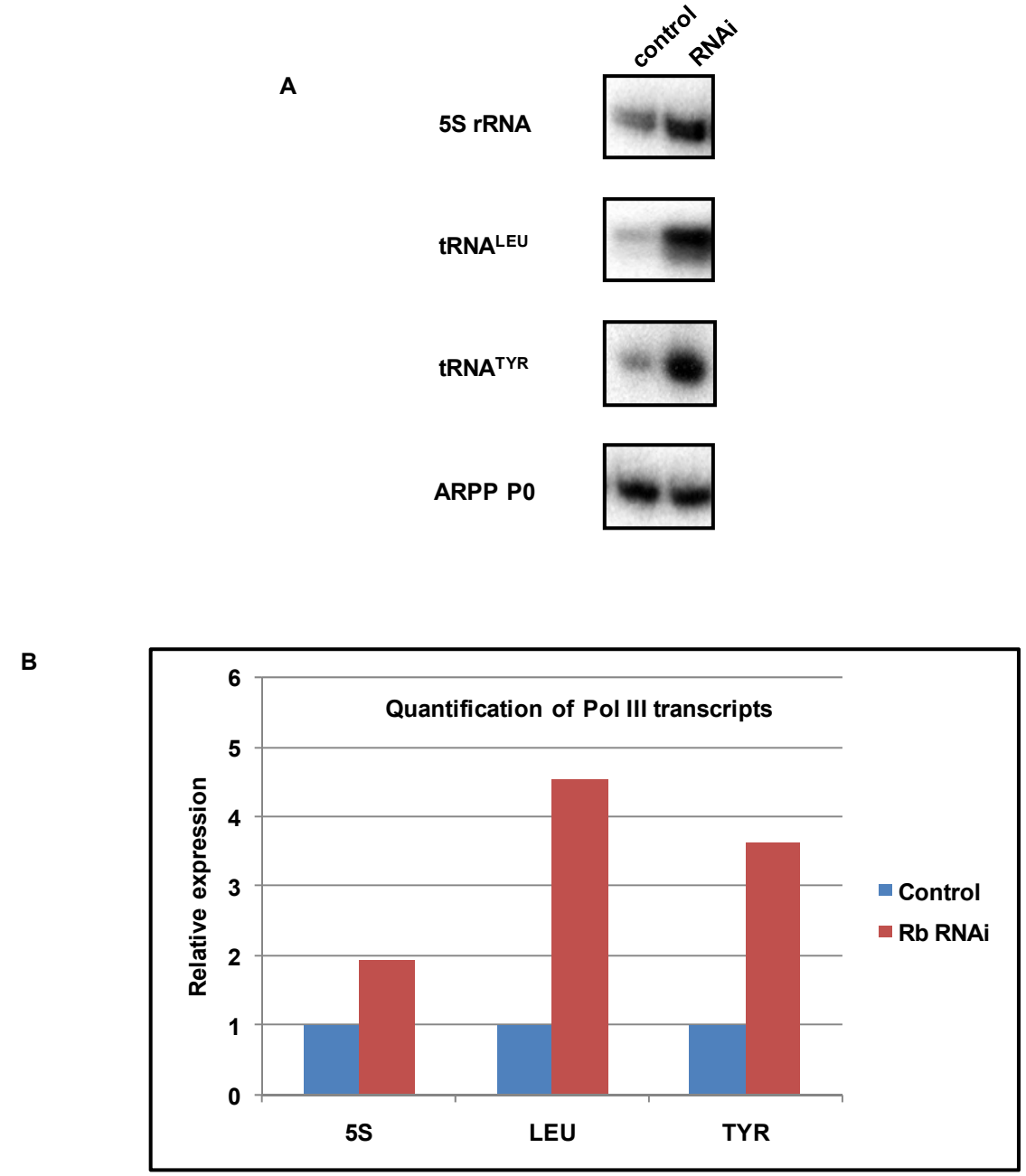


Figure 6.3. Expression of Pol III transcripts is elevated after Rb knock-down in IMR90 cells. IMR90 human lung fibroblasts were transfected with siRNA (100 nM) against Rb or Oct-1 (control). Cells were harvested 24 hours after transfection, total RNA was isolated, reverse transcribed to cDNA and analysed by PCR. **A.** RT-PCR analysis for Pol III templates. **B.** Quantification of the signals of Pol III transcripts from A.

active in HeLa cells and it still binds to a percentage of TFIIIB (Larminie et al, 1997), further depletion of Rb should result in higher levels of Pol III transcription.

To test this hypothesis, HeLa cells were transfected with siRNA targeting Rb or Oct-1 (control) and harvested after 24 hours. Western blotting revealed that Rb protein levels were decreased in the RNAi treated cells compared to the control, while the Oct-1 levels were also decreased as expected (Figure 6.4, A). Furthermore, Rb mRNA was also diminished (Figure 6.4, B). However, depletion of Rb did not result in upregulation of Pol III transcription as anticipated (Figure 6.5). More specifically, 5S rRNA and $tRNA^{LEU}$ levels were about 40-50% reduced after depletion of Rb (Figure 6.5, A and B), in contrast to the results obtained from the untransformed IMR90 cells under the same experimental conditions (Figure 6.3).

6.2.2 Regulation of Pol III-transcribed gene expression by p107 in IMR90 and HeLa cells

To further extend these studies and investigate if the other two family members can affect Pol III transcription in human cell lines, p107 was depleted by RNAi. Knock-down of p107 in IMR90 cells after RNAi treatment was confirmed by reduced p107 protein and mRNA levels in the treated cells compared to the control (Figure 6.6, A and B). Interestingly, p107 depletion resulted in an about 2.5-fold upregulation of tRNA, but about 50% downregulation of 5S rRNA expression (Figure 6.7, A and B).

In order to test if depletion of p107 would result in a similar effect in HeLa cells, p107 RNAi was employed under the same conditions as in the IMR90 cells. The knock-down of p107 in HeLa cells was confirmed by p107 reduction at the protein and mRNA level (Figure 6.8, A and B). In this case, the 5S rRNA expression was found to be significantly decreased, as was also observed in the IMR90 cells, while the tRNA expression was slightly downregulated too (Figure 6.9, A and B).

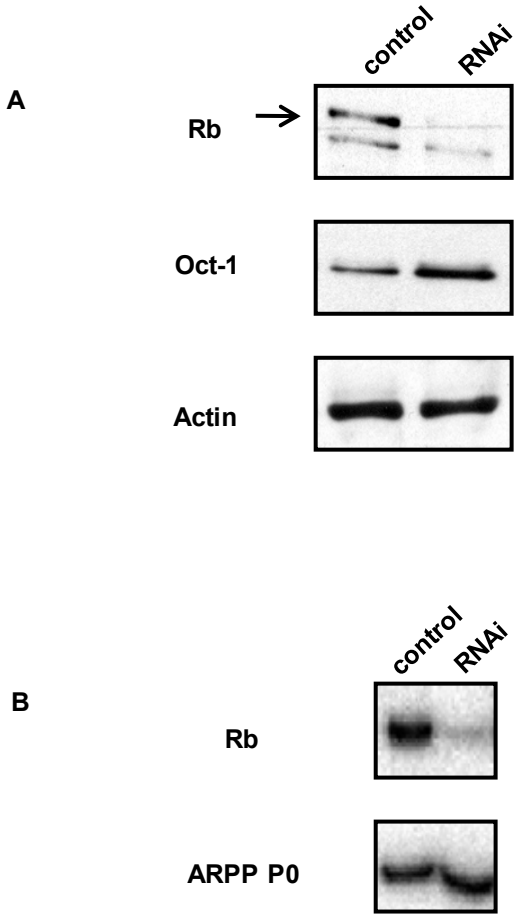


Figure 6.4. Rb RNAi in HeLa cells. HeLa cells were transfected with siRNA (100 nM) against Rb or Oct-1 (control). Cells were harvested 24 hours after transfection. **A.** Western blotting for Rb and Oct-1. Actin was used as loading control. The arrow indicates the Rb protein (total). **B.** RT-PCR analysis to confirm the Rb knock-down at the mRNA level.

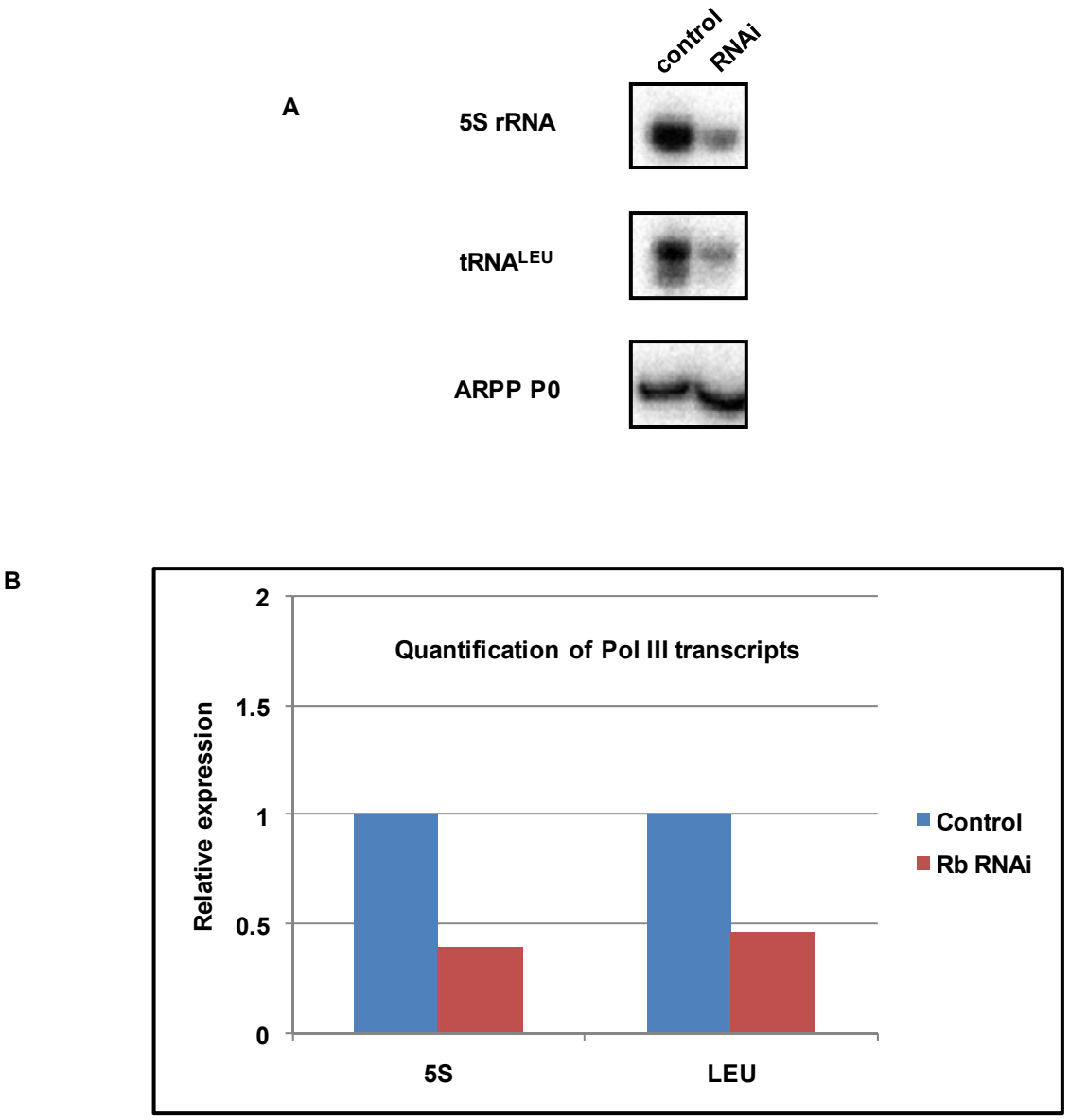


Figure 6.5. Expression of Pol III transcripts is decreased after Rb knock-down in HeLa cells. HeLa cells were transfected with siRNA (100 nM) against Rb or Oct-1 (control). Cells were harvested 24 hours after transfection, total RNA was isolated, reverse transcribed to cDNA and analysed by PCR. **A.** RT-PCR analysis for Pol III templates. **B.** Quantification of the signals of Pol III transcripts from A.

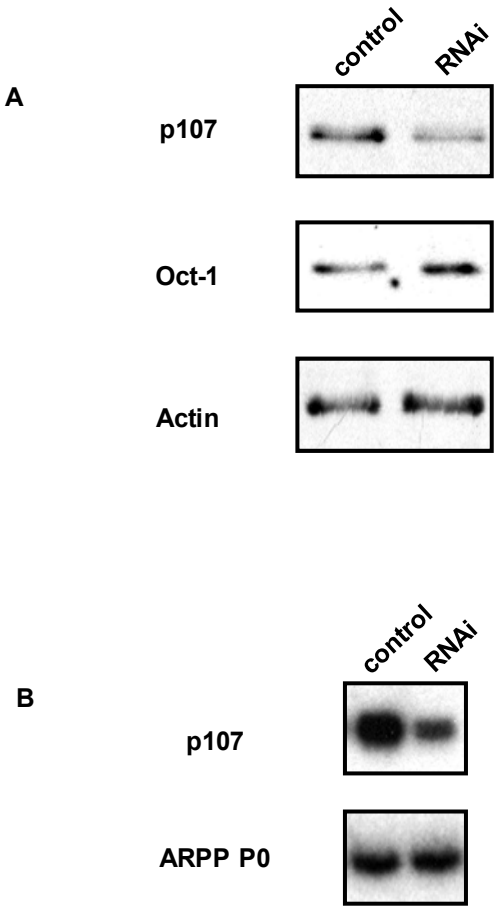


Figure 6.6. p107 RNAi in IMR90 cells. IMR90 fibroblasts were transfected with siRNA (100 nM) against p107 or Oct-1 (control). Cells were harvested 24 hours after transfection. **A.** Western blotting for p107 and Oct-1. Actin was used as loading control. **B.** RT-PCR analysis to confirm the p107 knock-down at the mRNA level.

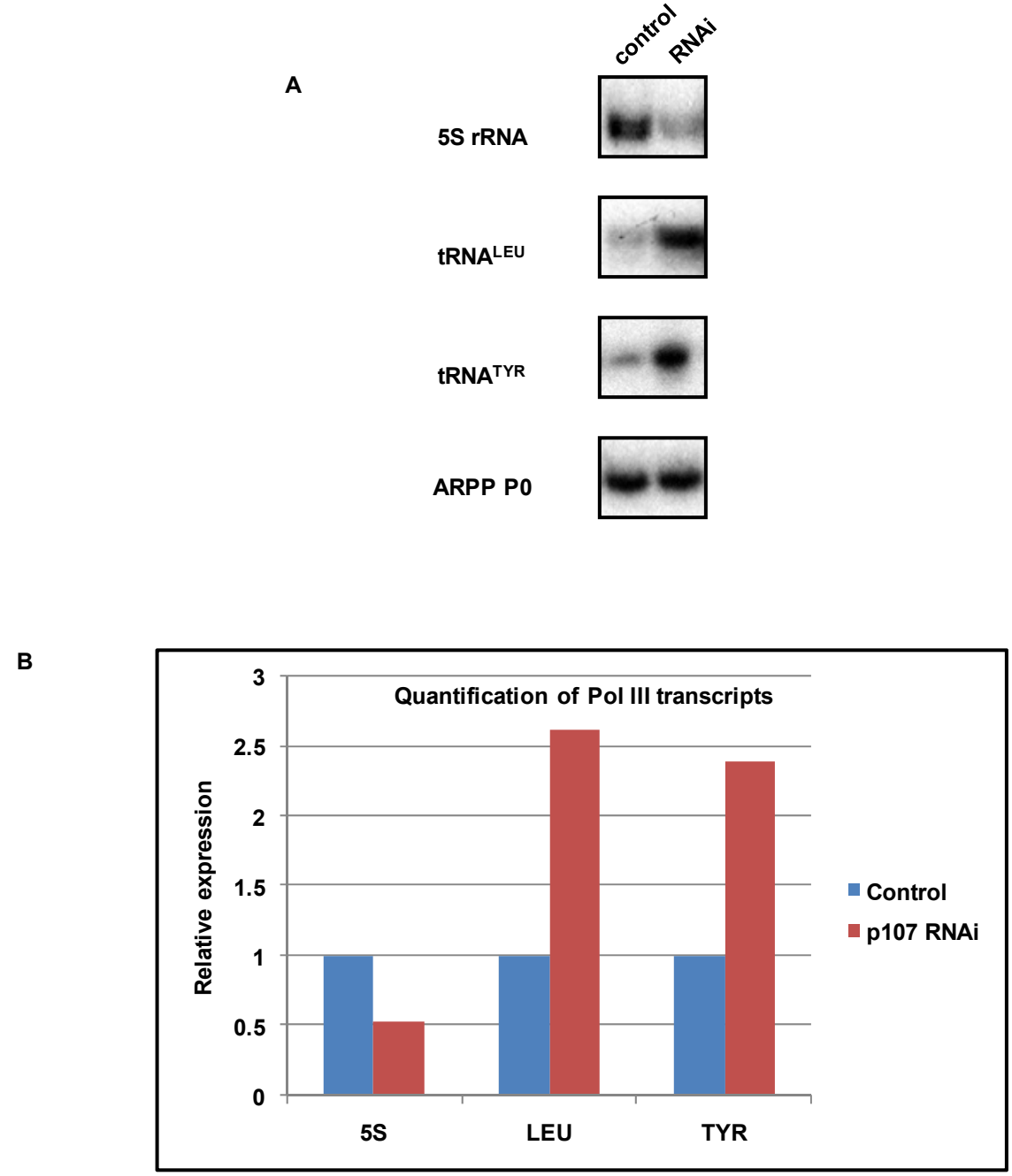


Figure 6.7. Expression of Pol III transcripts after p107 knock-down in IMR90 cells. IMR90 fibroblasts were transfected with siRNA (100 nM) against p107 or Oct-1 (control). Cells were harvested 24 hours after transfection, total RNA was isolated, reverse transcribed to cDNA and analysed by PCR. **A.** RT-PCR analysis for Pol III templates. **B.** Quantification of the signals of Pol III transcripts from A.

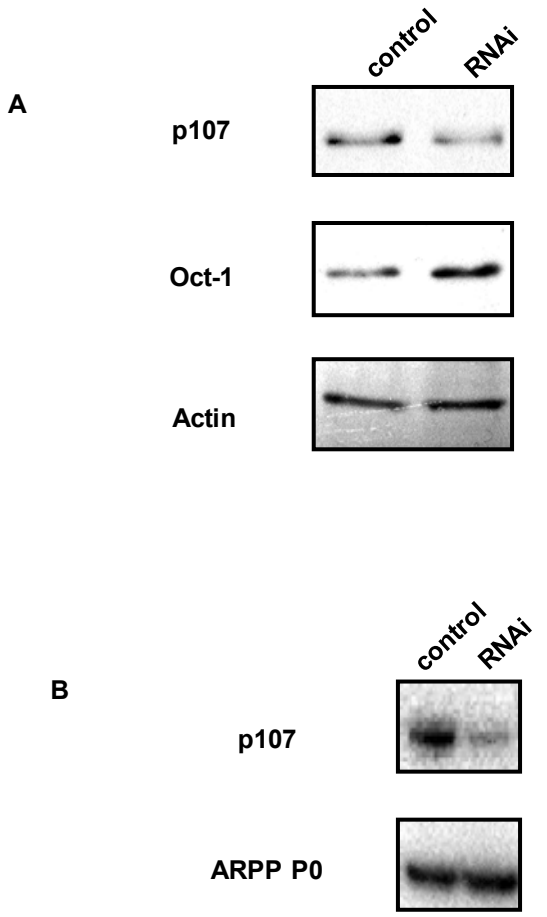


Figure 6.8. p107 RNAi in HeLa cells. HeLa cells were transfected with siRNA (100 nM) against p107 or Oct-1 (control). Cells were harvested 24 hours after transfection. **A.** Western blotting for p107 and Oct-1. Actin was used as loading control. **B.** RT-PCR analysis to confirm the p107 knock-down at the mRNA level.

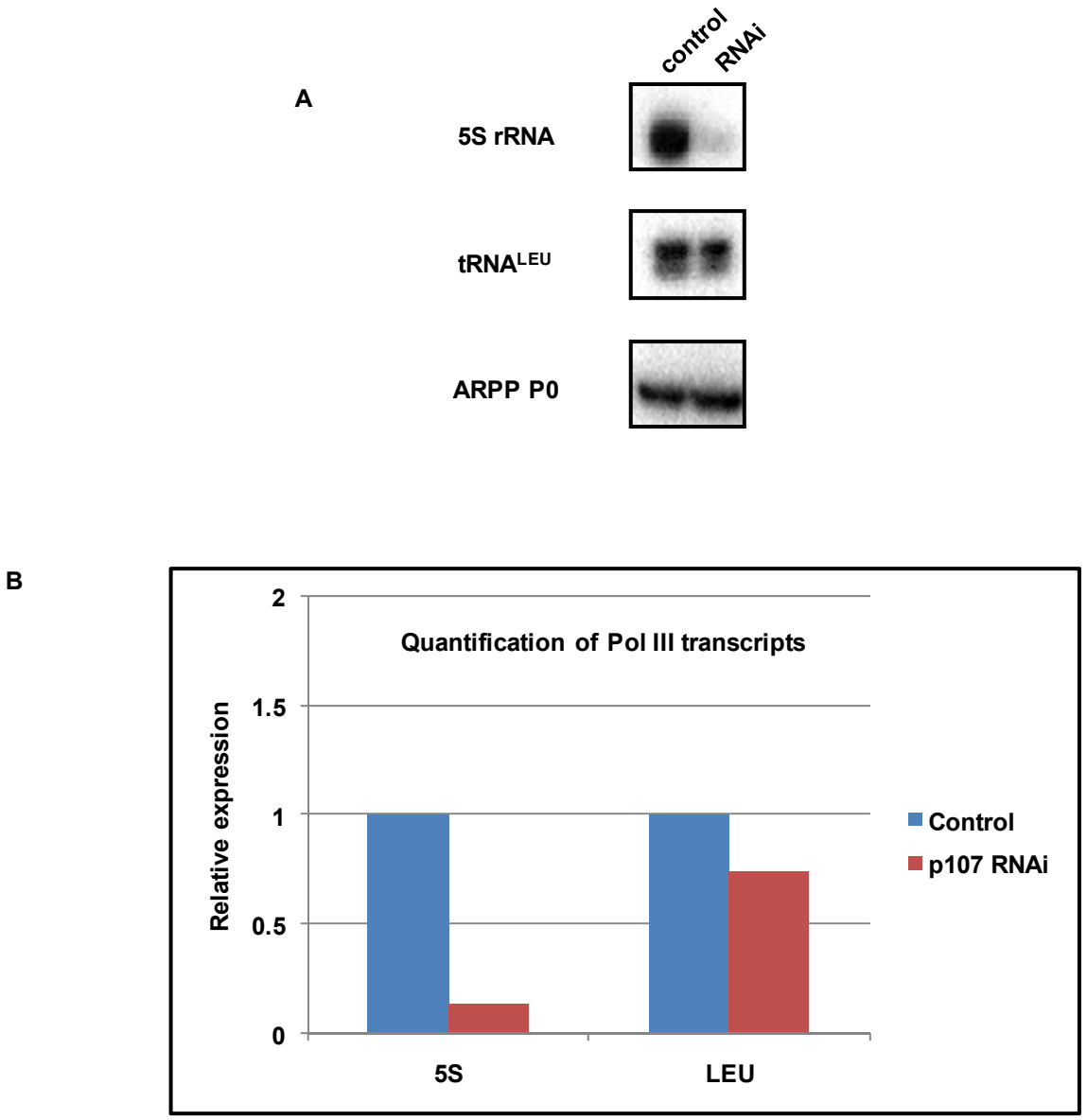


Figure 6.9. Expression of Pol III transcripts after p107 knock-down in HeLa cells. HeLa cells were transfected with siRNA (100 nM) against p107 or Oct-1 (control). Cells were harvested 24 hours after transfection, total RNA was isolated, reverse transcribed to cDNA and analysed by PCR. **A.** RT-PCR analysis for Pol III templates. **B.** Quantification of the signals of Pol III transcripts from A.

6.2.3 Regulation of Pol III-transcribed gene expression by p130 in IMR90 and HeLa cells

Data from Rb and p107 RNAi experiments hint at possible different roles for the two proteins in the regulation of Pol III transcription, that might also vary in different cell lines. To extend this line of investigation to the remaining family member, p130 was knocked-down by RNAi in IMR90 and HeLa cells. p130 was depleted by RNAi in IMR90 under the same conditions used for Rb and p107, and the knock-down was confirmed by western blotting and RT-PCR analysis (Figure 6.10, A and B). p130 depletion resulted in an about 65% increase of tRNA expression and slightly decreased (about 30%) expression of 5S rRNA (Figure 6.11, A and B), in a pattern similar to that observed after p107 depletion in IMR90 cells and in partial contrast to the Rb depletion, which resulted in enhanced 5S rRNA and tRNA expression. RNAi of p130 in HeLa cells resulted in similar decrease of p107 protein and mRNA as in the case of IMR90 cells (Figure 6.12, A and B). Pol III transcription of 5S rRNA and tRNA in this cell line was downregulated by about 40-50% after depletion of p130 (Figure 6.13, A and B), in general accordance with the results obtained after depletion of Rb and p107 in the same cell line.

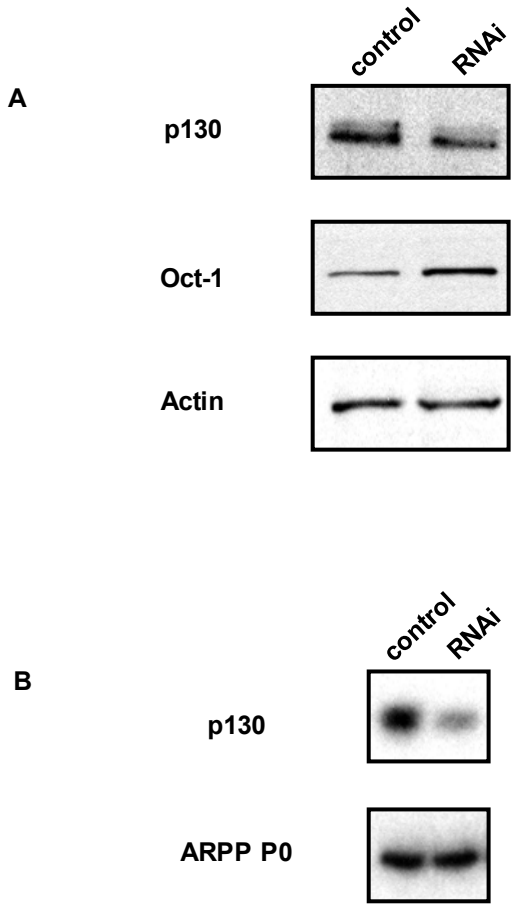
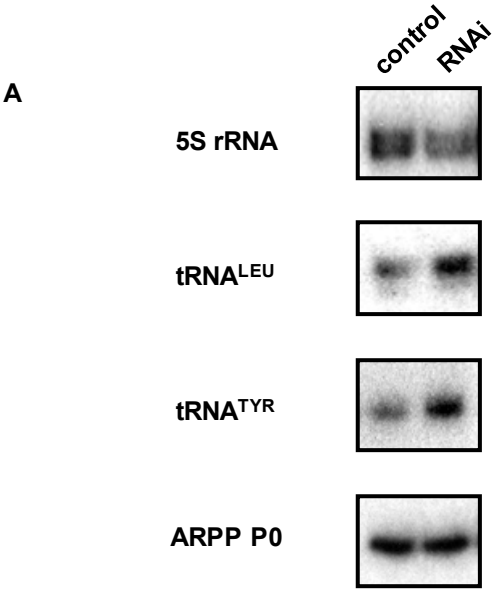


Figure 6.10. p130 RNAi in IMR90 cells. IMR90 fibroblasts were transfected with siRNA (100 nM) against p130 or Oct-1 (control). Cells were harvested 24 hours after transfection. **A.** Western blotting for p130 and Oct-1. Actin was used as loading control. **B.** RT-PCR analysis to confirm the p130 knock-down at the mRNA level.



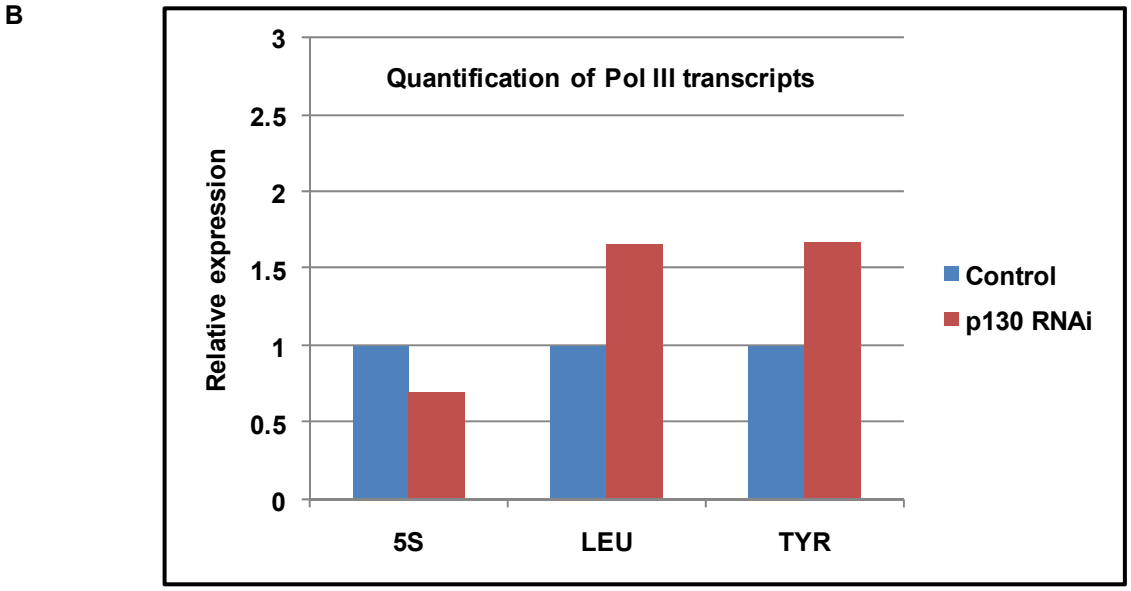


Figure 6.11. Expression of Pol III transcripts after p130 knock-down in IMR90 cells. IMR90 fibroblasts were transfected with siRNA (100 nM) against p130 or Oct-1 (control). Cells were harvested 24 hours after transfection, total RNA was isolated, reverse transcribed to cDNA and analysed by PCR. **A.** RT-PCR analysis for Pol III templates. **B.** Quantification of the signals of Pol III transcripts from A.

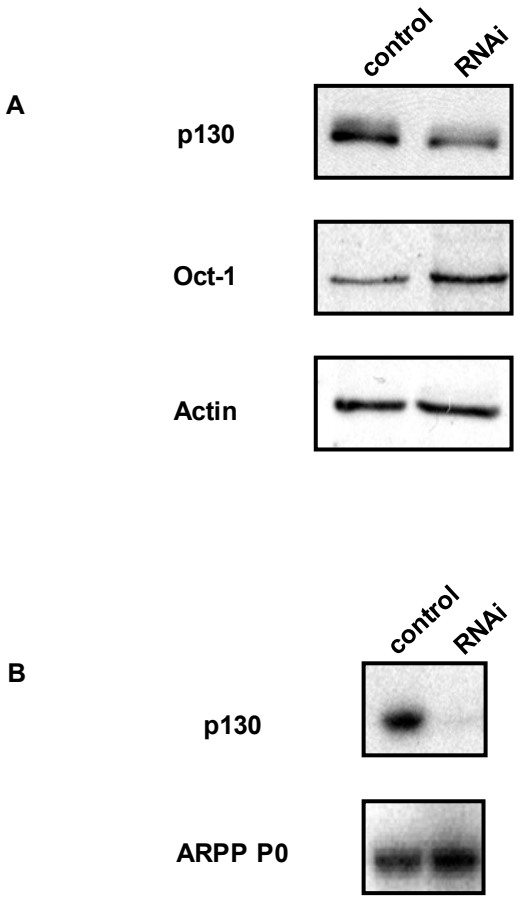


Figure 6.12. p130 RNAi in HeLa cells. HeLa cells were transfected with siRNA (100 nM) against p130 or Oct-1 (control). Cells were harvested 24 hours after transfection. **A.** Western blotting for p130 and Oct-1. Actin was used as loading control. **B.** RT-PCR analysis to confirm the p130 knock-down at the mRNA level.

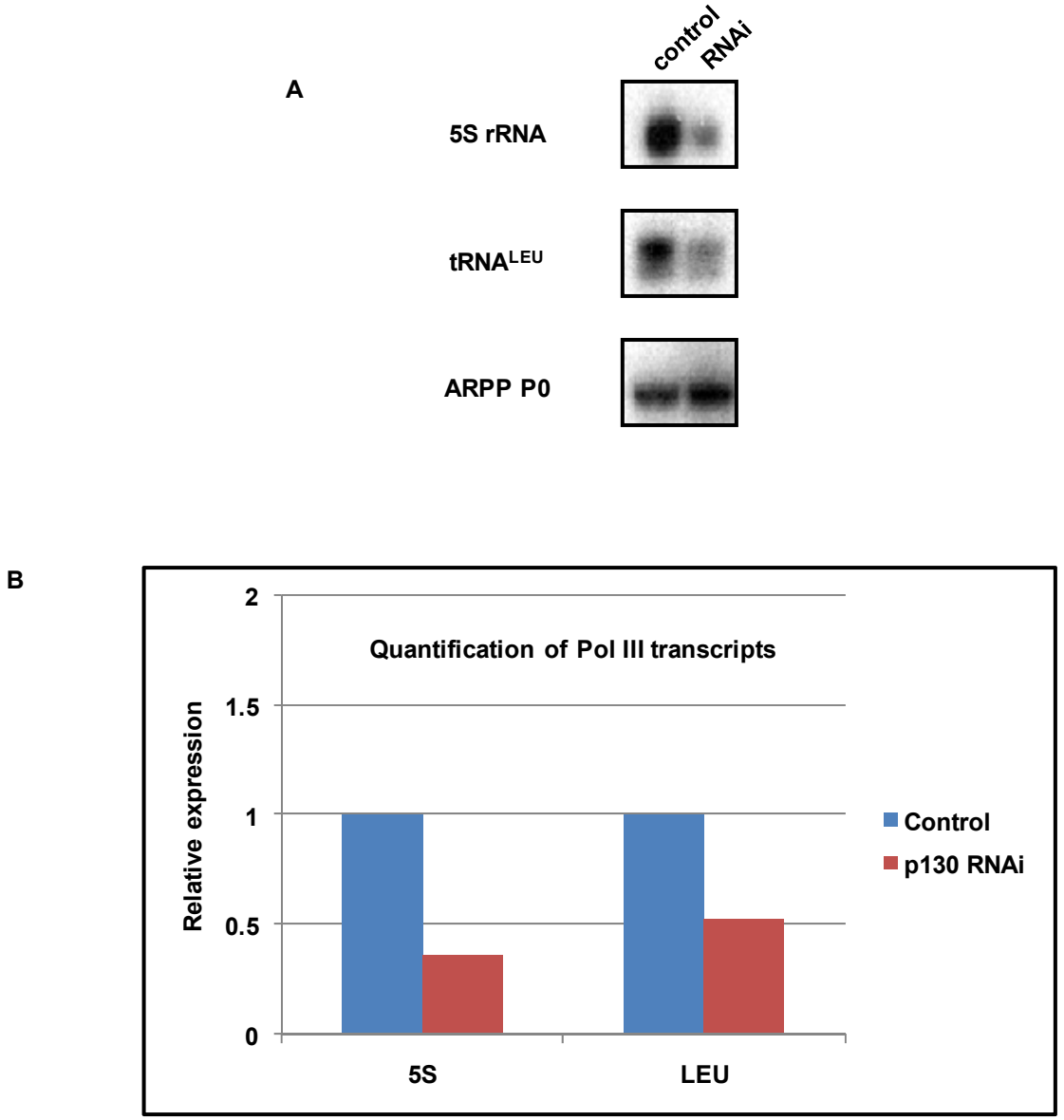


Figure 6.13. Expression of Pol III transcripts after p130 knock-down in HeLa cells. HeLa cells were transfected with siRNA (100 nM) against p130 or Oct-1 (control). Cells were harvested 24 hours after transfection, total RNA was isolated, reverse transcribed to cDNA and analysed by PCR. **A.** RT-PCR analysis for Pol III templates. **B.** Quantification of the signals of Pol III transcripts from A.

6.3 Discussion

6.3.1 The *in vivo* role of Rb family proteins in the regulation of Pol III transcription in human cells

It is believed that almost all cancers need to compromise Rb function in order to develop (Hanahan & Weinberg, 2000). The control of cell growth and proliferation by the Rb tumour suppressor and the other family members is, therefore, of paramount importance. A large number of studies focused on the ability of the Rb family members to bind E2F factors and inhibit E2F-mediated transcription (Bracken et al, 2004; Classon & Harlow, 2002; Dimova & Dyson, 2005). However, the pocket proteins interact with a large number of proteins other than E2Fs (Morris & Dyson, 2001; Mulligan & Jacks, 1998); the 661W mutant Rb is unable to bind E2Fs, but can still suppress cell cycle progression and tumour cell growth (Sellers et al, 1998; Whitaker et al, 1998), indicating important roles for proteins other than E2Fs interacting with the Rb family members.

Growth, as an increase in cell mass, depends on protein synthesis, which in turn is limited by ribosome availability (White, 2005). Consequently, transcription of rRNA and tRNA genes by Pol I and Pol III is essential for maintaining protein synthesis and growth and is therefore tightly regulated (White, 2005). Rb family members can regulate Pol I and Pol III transcription by interactions with the transcriptional factors UBF and TFIIIB, respectively (White, 2005). More specifically for Pol III transcription, interaction of the pocket proteins with TFIIIB seems to inhibit the formation of an active preinitiation complex with TFIIIC and Pol III, sequestering TFIIIB and resulting in transcriptional repression (Larminie et al, 1997; Sutcliffe et al, 2000; Sutcliffe et al, 1999; White et al, 1996).

Since most of the experiments elucidating the role of RB proteins in Pol III transcription were performed in mouse cells, it is important to confirm that the same conclusions stand in human cells. Moreover, studying the effect on Pol III transcription after the loss of each individual protein of the family in the human system could potentially lead to conclusions about the differences within the family members in regulating the expression of different types of Pol III transcripts.

To this end, the *in vivo* role of Rb, p107 and p130 in Pol III transcription was investigated by RNAi in IMR90 fibroblasts. Knock-down of Rb resulted in increased expression of rRNA and tRNA transcripts (Figure 6.3). These data are in accordance with previous reports that showed Rb to repress Pol III transcription in mouse cells (Larminie et al, 1997; White et al, 1996). Interestingly, partial depletion of p107 or p130 also resulted in increased tRNA, but decreased levels of 5S rRNA (Figures 6.8 and 6.12). These results might suggest that p107 and/or p130 can regulate 5S rRNA and tRNA promoters in diverse ways and, in the case of 5S rRNA, distinctly from Rb.

It has been shown that although all three Rb family members can bind TFIIIB and repress Pol III transcription (Larminie et al, 1997; Sutcliffe et al, 1999), Rb and p130, but not p107, can bind to UBF and repress Pol I transcription (Ciarmatori et al, 2001; Hannan et al, 2000). As mentioned previously, the expression pattern of p107 and p130 during the cell cycle is different, with p107 mainly expressed in the S phase and p130 in quiescent cells. Co-ordination of Pol I and Pol III transcription would require equimolar expression of the rRNA produced by the two polymerases and thus, it seems plausible that p107 might not repress transcription of 5S rRNA by Pol III. p107 might compete with Rb for binding with TFIIIB and might not inhibit recruitment of the complex at 5S rRNA promoters. If this is the case, then depletion of p107 could result in greater sequestering of TFIIIB by Rb, resulting in decreased levels of 5S rRNA, as observed in p107 RNAi (Figure 6.7). p130 seems to act similarly to p107, since p130 RNAi did not result in increased expression of 5S rRNA, although the effect was not as strong as that observed with p107 RNAi (Figure 6.11).

The finding that knock-down of Rb resulted in increased Pol III-transcribed gene expression, comes in agreement with previous studies in mouse and human cells (Larminie et al, 1997; Sutcliffe et al, 2000; White et al, 1996). These studies identified Rb as a repressor of Pol III transcription and the RNAi experiments confirm that that this is indeed the case in IMR90 human cells, *in vivo*. p107 and p130 also repress transcription of tRNA genes *in vivo*, extending previous studies in human SAOS cells overexpressing p107 or p130 (Sutcliffe et al, 1999), but they do not repress 5S rRNA transcription (Figure 6.7 and 6.12). This contrasts with previous experiments, where addition of GST-p107 or GST-p130 in human extracts resulted in decreased transcription of 5S rRNA genes (Sutcliffe et al, 1999). However, it is not clear that the effect on 5S rRNA after p107 or p130 knock-down

is a transcriptional one; it might also be attributed to post-transcriptional effects on rRNA stability.

The overall growth characteristics of $Rb^{-/-}$, $p107^{-/-}$, $p130^{-/-}$ and $p107^{-/-}$; $p130^{-/-}$ mouse embryonic fibroblasts (MEF) did not deviate from those of wild-type MEFs (Cobrinik et al, 1996; Herrera et al, 1996; Lee et al, 1996), while triple ($Rb^{-/-}$; $p107^{-/-}$; $p130^{-/-}$) knockouts resulted in cells unable to arrest in G0/G1 (Dannenberg et al, 2000; Sage et al, 2000), suggesting functional redundancy and compensation within the pocket-protein family. There might also be redundancy in the regulation of Pol III transcription by the three family members in human cells; after partially depleting one, the other two might be able to compensate for the loss. However, this was not observed in the RNAi experiments. Knock-down of each individual member resulted in enhanced tRNA levels, while 5S rRNA was increased after knock-down of Rb, but decreased when p107 or p130 were partially depleted. The 5S rRNA expression was less affected after loss of p130 than loss of p107, hinting that p107 might have a dominant role in controlling 5S rRNA transcription compared to p130, if that is a transcriptional effect. Alternatively, however, it could also be attributed to a less efficient p130 knock-down. It is possible that in humans there is a much lower degree of functional redundancy within the family members than in mice; the fact that $Rb^{+/-}$ humans develop retinoblastoma, while $Rb^{+/-}$ mice are unaffected might constitute an example of different functional overlap of the Rb proteins in the two species.

Interestingly, knock-down of the individual Rb family members by RNAi in HeLa cells resulted in decreased expression of Pol III-transcribed genes (Figures 6.6, 6.10 and 6.14), in contrast to the findings in IMR90 cells (Figures 6.4, 6.8 and 6.12). Since Rb, p107 and p130 bind to TFIIIB (Larminie et al, 1997; Sutcliffe et al, 1999) and are partially active in HeLa cells (Goodwin & DiMaio, 2000; Scheffner et al, 1991), their partial depletion should result in release of TFIIIB and stimulation of Pol III transcription. A possible explanation for the diverse effect after partial depletion of Rb proteins in the two cell lines might lie in the physiology of HeLa cells.

HeLa is a human cervical carcinoma cell line that expresses wild type p53 and Rb (Scheffner et al, 1991), but also has the viral HPV18 DNA integrated into the cellular genome and expresses the viral E6 and E7 proteins (Schwarz et al, 1985). E7 can bind to the Rb family proteins and mediate their proteosomal degradation (Dyson et al, 1989;

Gonzalez et al, 2001), resulting in inhibition of the activity of these tumour suppressors. Overexpression of HPV16 E7 has been shown to stimulate Pol III transcription in mouse and human cells, probably due to release of TFIIIB from repression by Rb, p107 and p130 (Larminie et al, 1999; Sutcliffe et al, 1999). Due to E7 expression in HeLa cells and the consequent degradation of the Rb proteins, it would be anticipated that a higher percentage of TFIIIB would be available, resulting in stimulated Pol III transcription; indeed, Pol III transcription is elevated in HeLa cells and it is estimated that only 5-10% of TFIIIB is associated with Rb in extracts made from asynchronous HeLa cells (Larminie et al, 1997).

The effect observed on Pol III transcription in HeLa cells after knocking-down members of the Rb family might not be a direct effect as expected, i.e. depletion of Rb leading to release of TFIIIB and stimulation of Pol III transcription, but rather indirect, implicating the p53 pathway. According to this scenario, knock-down of Rb would result in release of E7 that could bind to available p107 and p130. Thus, depletion of Rb and further inhibition of p107 and p130 would result in transcriptional activation of E2F targets, including ARF (Lowe & Sherr, 2003). Activation of ARF would inhibit Mdm2, stabilise p53 and repress Pol III transcription (Cairns & White, 1998; Crighton et al, 2003; Morton et al, 2007), explaining the repression of Pol III transcription observed after Rb RNAi. Repression after partial depletion of p107 and p130 by RNAi might be explained similarly. This response would require an intact Rb pathway in HeLa cells, which seems to be the case as repression of E7 in HeLa resulted in reactivation of the Rb and p53 pathways (Goodwin & DiMaio, 2000).

In summary, data included in this chapter suggest that Rb, p107 and p130 can repress tRNA transcription in normal human cells, confirming previous studies in mouse cell lines. The role of the Rb proteins in transformed cells, like HeLa, is more complex as viral proteins, like E7, might tip the balance between different pathways, such as the Rb and p53 pathways, resulting in complicated responses.

CHAPTER 7

The tumour suppressors p53 and ARF can regulate RNA polymerase III transcription in human cells

7.1 Introduction

7.1.1 The p53 tumour suppressor

p53 is a key player in tumour development; approximately half of all malignancies contain p53 mutations, and tumours that do not have these kind of mutations, usually have inactivated p53 by other mechanisms (Vogelstein et al, 2000). Moreover, p53 null mice are born and develop normally, but develop cancer before 6 months (Donehower et al, 1992), while humans that suffer from Li Fraumeni syndrome, and carry a germline mutation in p53, display an abnormally high and early incidence of cancer (Srivastava et al, 1990). Therefore, p53 is considered to be an important tumour suppressor. Moreover, a number of other roles have recently been suggested for p53, including the regulation of glycolysis and autophagy, the repair of genotoxic damage, regulation of oxidative stress, angiogenesis, cell differentiation and others (Vousden & Lane, 2007).

The tumour suppression function of p53 is mainly achieved through its ability, as a transcription factor, to positively or negatively regulate a large number of target genes and induce different cellular responses (Figure 7.1), including cell cycle arrest, senescence and apoptosis (Prives & Hall, 1999; Vogelstein et al, 2000). p53 may promote transcription by Pol II via a number of mechanisms. It can bind to its response elements within promoters and recruit histone transacetylases, methyltransferases and chromatin remodelling factors, resulting in histone modifications and subsequently in alterations in chromatin structure and promoter opening (Laptenko & Prives, 2006). Furthermore, p53 can interact with components of the Mediator complex and facilitate the formation of the preiniation complex and also, bind and recruit basal transcription factors and consequently stimulate transcription (Laptenko & Prives, 2006). Several mechanisms have also been documented for the transcriptional repression of Pol II-transcribed genes by p53. It can directly interact with transcriptional activators and exclude them from the promoter or displace them from their binding sites within promoter complexes, resulting in the inhibition of preinitiation complex formation. Moreover, p53 might repress transcription through novel DNA binding elements or by recruiting histone deacetylases and other chromatin modifying factors (Laptenko & Prives, 2006), while p53-mediated repression can also be achieved by activation of p53 targets, such as p21 (Lohr et al, 2003).

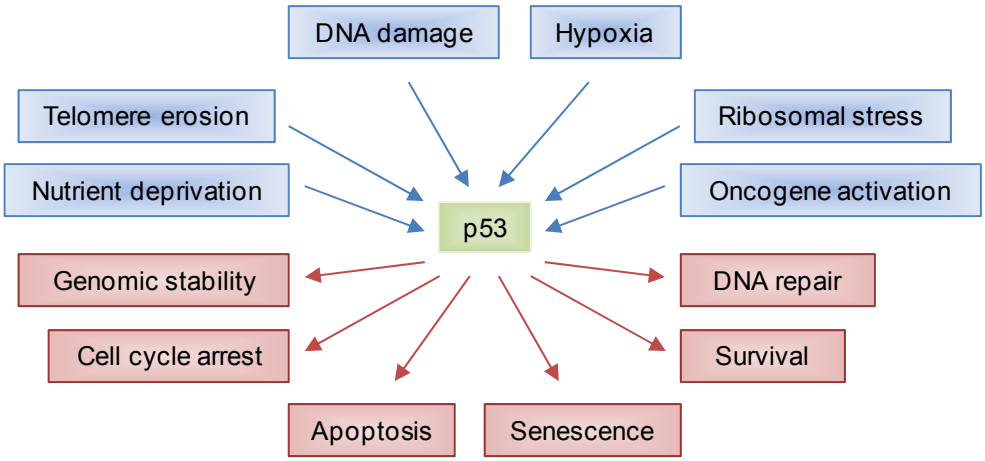


Figure 7.1. Activation and functions of p53. p53 can be induced by a number different stress conditions (blue boxes) and its activation can result in different cellular responses (pink boxes). Adapted from Vousden and Lane, 2007.

The anti-proliferative, such as cell cycle arrest and senescence, and apoptotic activities of p53 would require efficient control of p53 expression to allow normal cell growth and development. Indeed, p53 protein stability is tightly regulated. p53 is degraded by the proteasome after being ubiquitinated and a number of ubiquitin ligases can promote the degradation of p53 (Horn & Vousden, 2007), Mdm2 (also known as Hdm2 in human) being the best studied. Mdm2 functions as an ubiquitin ligase that can ubiquinate and target p53 for degradation (Fang et al, 2000). Interestingly, Mdm2 is also a target of p53, creating a feedback loop, through which p53 levels are kept low (Zauberman et al, 1993). A number of other proteins, like Yin Yang 1 (Sui et al, 2004), gankyrin (Higashitsuji et al, 2005) and p300 (Grossman et al, 2003), interact with Mdm2 and contribute to p53 poly-ubiquitination and degradation.

Apart from the regulation of p53 stability, other processes can also contribute to the control of p53 function. MdmX (also known as Mdm4) is a relative of Mdm2 that does not show E3 ligase activity (Jackson & Berberich, 2000), but rather functions by binding to p53 and directly inhibiting its transcriptional role (Shvarts et al, 1996). Furthermore, p53 can be modified by the ubiquitin-like proteins NEDD8 and SUMO-1 (Watson & Irwin, 2006), resulting in inhibition or activation of p53, respectively (Bischof et al, 2006; Xirodimas et al, 2004), while p53 modifications in a ubiquitin-ligase-dependent manner by Mdm2 (Li et al, 2003), Cullin 7 (Andrews et al, 2006), WWP1 (Laine & Ronai, 2007) and Ubc13 (Laine et al, 2006) can result in accumulation of transcriptionally inactive p53 in

the cytoplasm. Furthermore, p53 is also subject to several post-translational modifications, such as phosphorylation, acetylation, methylation, glycosylation and ribosylation that play important roles in controlling p53 interactions and cellular responses (Bode & Dong, 2004).

Tight regulation of p53 is essential for normal cell growth, but p53 must be stabilised and activated to function as a tumour suppressor. This can be achieved by a number of stress signals (Figure 7.1), including DNA damage and oncogene activation (Horn & Vousden, 2007). DNA damage results in p53 phosphorylation by a cascade of Ser/Thr kinases that includes ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related), Chk1 (Checkpoint kinase1) and Chk2 (Checkpoint kinase 2) (Appella & Anderson, 2001), and probably other kinases, such as JNK (c-Jun N-terminal kinase) and p38 (Bulavin et al, 1999; Efeyan & Serrano, 2007; Fuchs et al, 1998). Oncogenic signalling e.g. from Ras (Palmero et al, 1998), E1A (de Stanchina et al, 1998) or Myc (Zindy et al, 1998), activates p53 via the ARF tumour suppressor. ARF (also known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice) responds to hyperproliferative signals originating from oncogenic stimuli by interacting directly with Mdm2 and inhibiting p53 ubiquitination and degradation (Pomerantz et al, 1998; Zhang et al, 1998); this results in p53 activation and consequently, the re-routing of cells that endured oncogenic damage to growth arrest or apoptosis (Sherr, 2001). As expected, mice deficient in ARF present a tumour-prone phenotype, but have a normal DNA damage response (Kamijo et al, 1997); moreover, recent experiments showed that p53 is unable to suppress tumourigenesis in the absence of ARF, despite the ability to respond to DNA damage effectively, suggesting that ARF might be responsible for almost all tumour suppressor activity of p53 (Christophorou et al, 2006; Efeyan et al, 2006). This, however, contrasts with other studies that indicated DNA damage can be induced by oncogenes and activate p53 in an ARF-independent manner (Bartkova et al, 2005; Bartkova et al, 2006; Di Micco et al, 2006; Gorgoulis et al, 2005).

p53 has a profound effect on Pol III transcription. It can inhibit the synthesis of a range of Pol III targets from all types of Pol III promoters, such as 5S rRNA, tRNA, U6 snRNA, Alu elements and others, while certain promoters like U6 snRNA and Alu might be more sensitive to p53-mediated repression than others (Cairns & White, 1998; Chesnokov et al, 1996). Mechanistically, this is achieved by the ability of p53 to interact with TBP and prevent the association of TFIIIB with TFIIIC and Pol III, inhibiting the

formation of a functional transcription preinitiation complex and resulting in transcriptional repression (Cairns & White, 1998; Crighton et al, 2003). Several regions of p53 are implicated in the repression and p53 mutations commonly found in tumours, as well as mutant p53 from Li-Fraumeni patients, contribute to the upregulation of Pol III transcription (Stein et al, 2002a; Stein et al, 2002b). Interestingly, in UV-treated MCF7 cells, p53 could be found at U6 snRNA promoters (type III), although the p53 induction caused little or no repression of Pol III transcription (Gridasova & Henry, 2005). This finding contrasts with the mode of action of p53 at 5S RNA and tRNA promoters (type I and II), where it inhibits recruitment of TFIIIB (Crighton et al, 2003), suggesting a complex effect of p53 at Pol III transcription, that might depend on different factors, such as the promoter type, the cell type and the conditions under which the p53 was induced. Since most of the above experiments in human cells were based on induction of p53, either under stress or by plasmid overexpression, it would be interesting to confirm the effect of p53 on Pol III transcription by decreasing, rather than increasing, the endogenous p53.

Pol I transcription is also repressed by p53, and this is attributed to direct interaction between p53 and SL1, resulting in the inhibition of recruitment of SL1 at rRNA promoters and interfering with the assembly of a productive transcriptional machinery (Budde & Grummt, 1999; Zhai & Comai, 2000). p53 can thus regulate both Pol I and Pol III transcription and contribute to the co-ordination of rRNA production (White, 2005). As ARF can interact with Mdm2 and stabilise p53, it might indirectly regulate Pol I and Pol III transcription through p53. Interestingly, ARF has been found to affect rRNA, independently of p53, by inhibiting the processing of the primary rRNA transcript (Sugimoto et al, 2003), by interacting with the nucleolar endoribonuclease nucleophosmin/B23 and inhibiting rRNA maturation (Bertwistle et al, 2004) or by repressing transcription by Pol I (Ayrault et al, 2006). It would be interesting to investigate if ARF can regulate Pol III transcription and if this is a direct or indirect effect, through p53.

7.2 Results

7.2.1 p53 can regulate RNA polymerase III transcription in human cells

Previous studies have demonstrated that induction of p53 in human cells results in repression of Pol III transcription (Crighton et al, 2003). In an attempt to extend these studies and investigate the effect of p53 on Pol III transcriptional repression under more physiological conditions, an RNAi approach was employed. U2OS cells were transiently transfected either with pSUPER-p53 plasmid, which encodes a shRNA against p53 or the empty pSUPER vector as control (Brummelkamp et al, 2002). The cells were harvested 24 hours later, whole cell extracts were prepared and RNA was isolated. Western blotting confirmed that p53 was knocked-down at the protein level (Figure 7.2, A), which was consistent with p53 reduction at the mRNA level, as determined by RT-PCRs (Figure 7.2, B). The knock-down of p53 resulted in 2-3 fold elevated levels of 5S rRNA and $tRNA^{LEU}$ (Figure 7.3, A and B), suggesting an *in vivo* repressive role for p53 on Pol III transcription in U2OS cells, in accordance with the p53 induction experiments previously reported in different human cell lines (Crighton et al, 2003; Stein et al, 2002b).

It has been previously shown that induction of p53 following treatment with the DNA damaging agent methane methylsulfonate (MMS) results in repression of tRNA expression in HeLa cells (Crighton et al, 2003). However, induction of p53 after exposure to UV light or overexpression of p53 in the same cell line did not result in repression of Pol III transcription (Gridasova & Henry, 2005). To further investigate if p53 can affect Pol III transcription in HeLa cells under more physiological conditions, the RNAi methodology previously used in U2OS cells was followed. Western blotting and RT-PCRs confirmed that the protein (Figure 7.4, A) and mRNA (Figure 7.4, B) levels of p53 were depleted after RNAi application. RT-PCR analysis revealed that, in contrast to the observations in U2OS cells, the expression of Pol III transcripts was not upregulated (Figure 7.5, A and B); tRNA transcription was not affected, in contrast to results obtained after MMS treatment (Crighton et al, 2003), while the expression of 5S rRNA presented a slight, but repeatable reduction, in agreement with results previously observed in overexpression experiments in the same cell line (Gridasova & Henry, 2005). It must be noted that the lack of Pol III transcriptional repression in this cell line cannot be attributed to inactive or non-functional

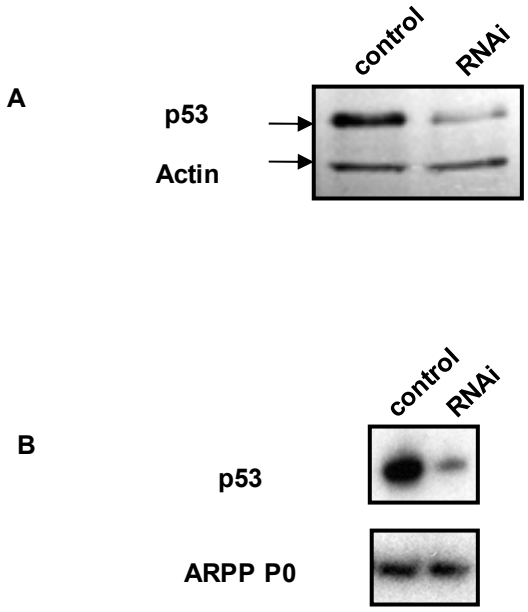


Figure 7.2. p53 shRNA knock-down in U2OS cells. U2OS cells were transfected with the pSUPER-p53 vector by electroporation and harvested 24 hours later. An empty pSUPER vector was used as control. **A.** Western analysis for p53 from whole cell extracts of control and p53 targeted cells. Actin was used as loading control. **B.** RT-PCRs for p53 mRNA. The Pol II-transcribed gene ARPP P0 was used as control.

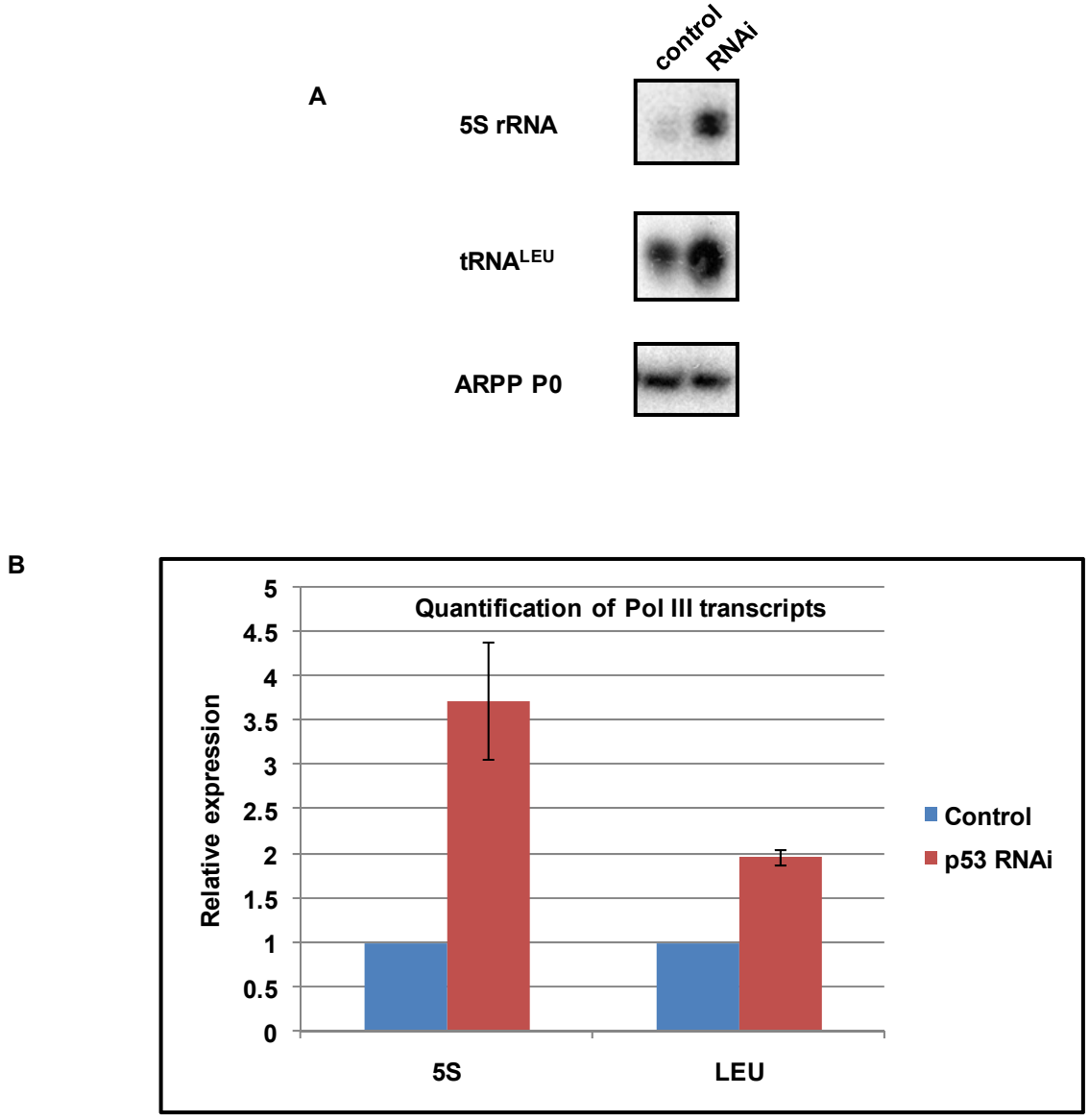


Figure 7.3. Knock-down of p53 by shRNA results in elevated levels of Pol III transcription in U2OS cells. U2OS cells were transfected with the pSUPER-p53 vector by electroporation and harvested 24 hours later. An empty pSUPER vector was used as control. **A.** RT-PCRs revealed increased levels of Pol III transcripts. The Pol II-transcribed gene ARPP P0 was used as control. **B.** Quantification of the signals of Pol III transcripts from A. n=2.

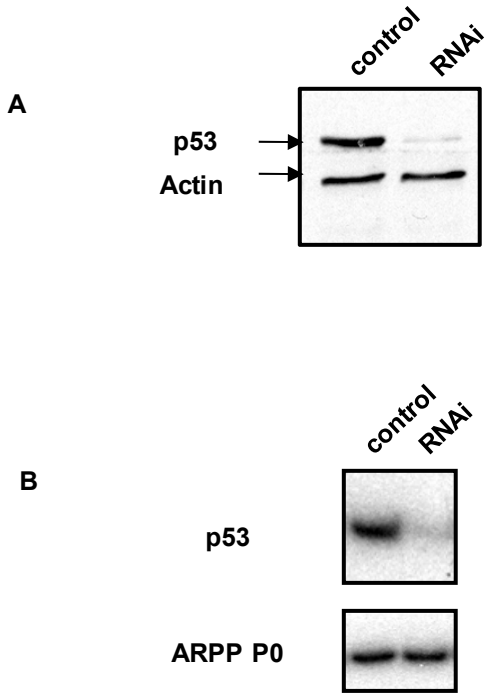


Figure 7.4. p53 shRNA knock-down in HeLa cells. HeLa cells were transfected with the pSUPER-p53 vector by electroporation and harvested 24 hours later. An empty pSUPER vector was used as control. **A.** Western analysis for p53 from whole cell extracts of control and p53 targeted cells. Actin was used as loading control. **B.** RT-PCRs for p53 mRNA. The Pol II-transcribed gene ARPP P0 was used as control.

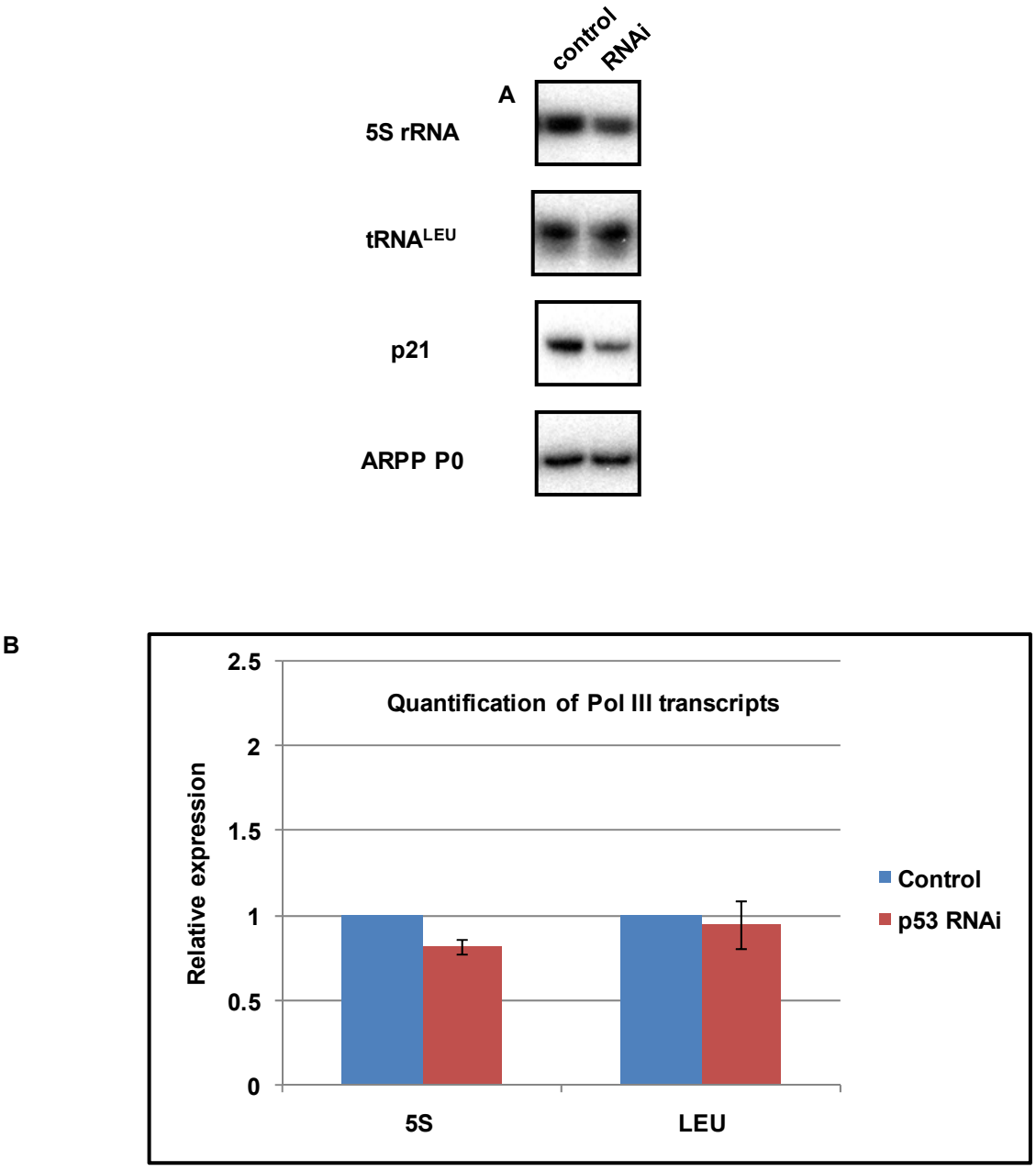


Figure 7.5. Knock-down of p53 by shRNA does not upregulate Pol III transcription in HeLa cells. HeLa cells were transfected with the pSUPER-p53 vector by electroporation and harvested 24 hours later. An empty pSUPER vector was used as control. **A.** RT-PCRs did not reveal elevated Pol III transcripts. The Pol II-transcribed genes p21 and ARPP P0 were used as controls. **B.** Quantification of the signals of Pol III transcripts from A. n=2.

p53 protein, as p21, a direct downstream target of p53, was accordingly reduced (Figure 7.5, A).

7.2.2 ARF can regulate RNA polymerase III transcription

As previously discussed, it has been shown that p53 can repress Pol III and Pol I transcription by interfering with the formation of the preinitiation complexes on the promoters (Budde & Grummt, 1999; Cairns & White, 1998; Crighton et al, 2003; Zhai & Comai, 2000). Furthermore, Pol I transcription can be directly regulated by ARF (Ayrault et al, 2006). In order to test if ARF can affect Pol III transcription as well, the NARF2 cell line, a derivative of U2OS osteosarcoma cells carrying an IPTG-inducible ARF gene (Stott et al, 1998), was employed. Addition of IPTG resulted in a substantial increase in the expression of ARF after 24 hours, accompanied by elevated p53 levels, as expected from its stabilisation due to ARF (Figure 7.6, A) (Stott et al, 1998). RT-PCR analysis revealed that under these conditions the expression of tRNAs was downregulated by about 50% (Figure 7.6, B and C), indicating that ARF can regulate Pol III transcription. This effect was due to the induction of ARF, as addition of IPTG in the parental U2OS cell line did not affect tRNA expression (Figure 7.7).

To confirm that p53 affects Pol III transcription in the NARF2 cell line in a similar manner to the U2OS cells, p53 was knocked-down by shRNA, as previously described, and the depletion of p53 at the protein and mRNA level was confirmed by western blotting and RT-PCR analysis (Figure 7.8, A and B respectively). Indeed, the p53 knock-down in the NARF2 cells resulted in about 2-fold elevated levels of Pol III transcripts (Figure 7.9, A and B), as was the case in the parental U2OS cells. To test whether depletion of p53 by RNAi would affect Pol III transcription when the cells are expressing ARF, the experiment was repeated with NARF2 cells induced to express ARF. The p53 knock-down was confirmed by western blotting and RT-PCR analysis (Figure 7.10, A and B respectively). RT-PCR analysis revealed about 2-fold elevated expression levels of Pol III transcripts when p53 was partially depleted by RNAi (Figure 7.11, A and B), suggesting that the ARF repression of Pol III transcription is mediated by p53. To further confirm that ARF can repress Pol III transcription through p53 and be able to compare directly the effects of ARF

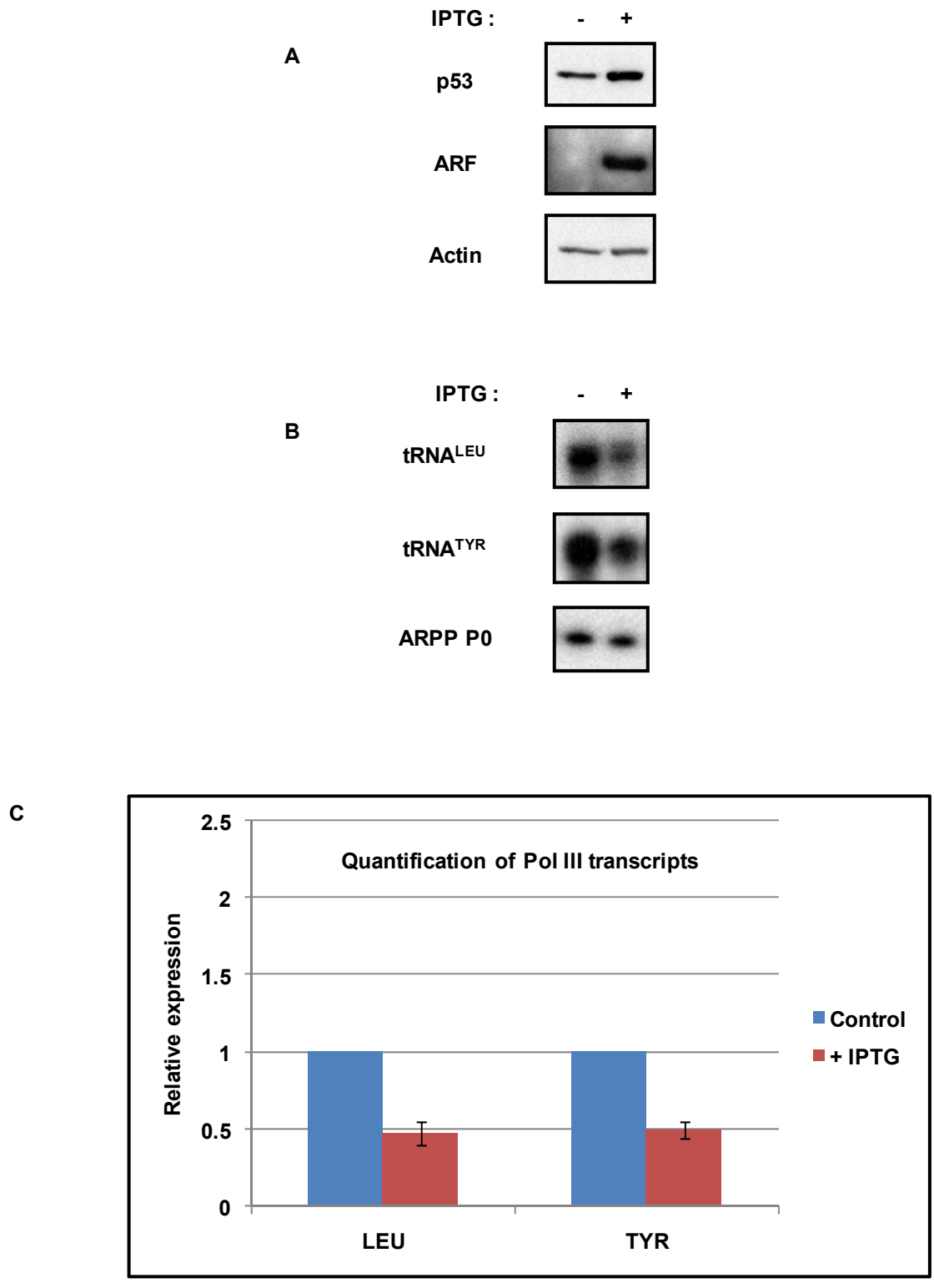


Figure 7.6. ARF induction in NARF2 cells results in decreased expression of tRNAs. NARF2 cells were induced to express ARF after addition of 1 mM IPTG. The cells were harvested after 24 hours, cells extracts were prepared and RNA was isolated. **A.** Western blotting confirms induction of ARF. Actin was used as loading control. **B.** RT-PCRs reveal decreased expression of tRNAs. The Pol II-transcribed ARPP P0 was used as control. **C.** Quantification of tRNA signals from B. n=2.

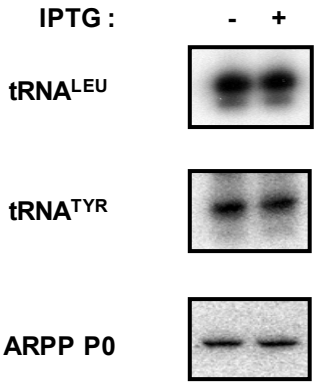


Figure 7.7. IPTG does not affect tRNA expression in U2OS cells. 1 mM IPTG was added in U2OS cells. The cells were harvested after 24 hours and RNA was isolated. RT-PCR analysis revealed no effect on the expression of tRNAs. The Pol II-transcribed ARPP P0 was used as control.

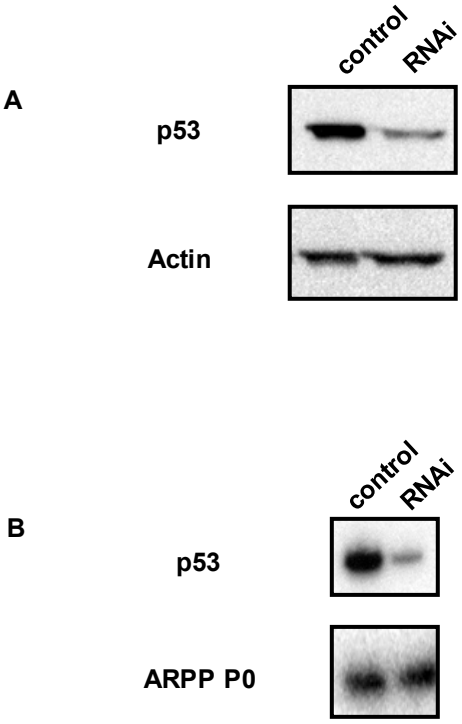


Figure 7.8. p53 shRNA knock-down in NARF2 cells. NARF2 cells were transfected with the pSUPER-p53 vector by electroporation and harvested 24 hours later. An empty pSUPER vector was used as control. **A.** Western analysis for p53 from whole cell extracts of control and p53 targeted cells. Actin was used as loading control. **B.** RT-PCRs for p53 mRNA. The Pol II-transcribed gene ARPP P0 was used as control.

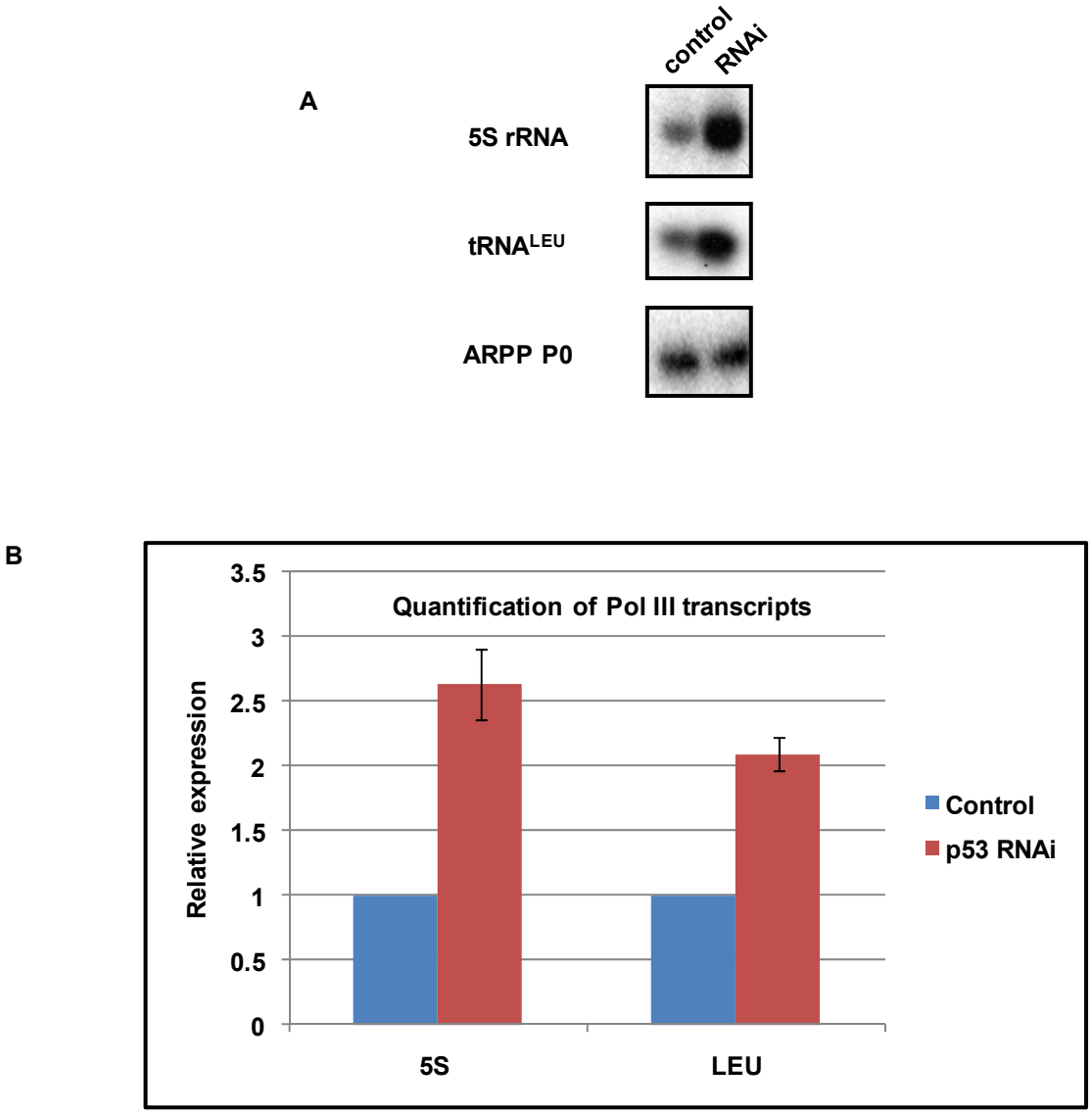


Figure 7.9. Knock-down of p53 by shRNA results in elevated levels of Pol III transcription in NARF2 cells. NARF2 cells were transfected with the pSUPER-p53 vector by electroporation and harvested 24 hours later. An empty pSUPER vector was used as control. **A.** RT-PCR analysis reveals increased levels of Pol III transcripts in the RNAi treated cells. The Pol II-transcribed gene ARPP P0 was used as control. **B.** Quantification of the signals of Pol III transcripts from A. n=2.

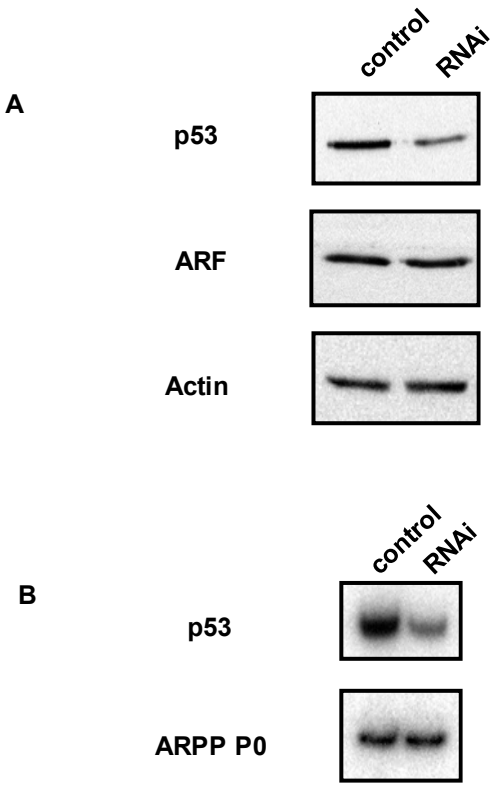


Figure 7.10. p53 shRNA in NARF2 cells induced to express ARF. NARF2 cells were transfected with the pSUPER-p53 vector by electroporation and grown in medium containing 1 mM IPTG for 24 hours. An empty pSUPER vector was used as control. **A.** Western analysis for p53 from whole cell extracts of control and p53 targeted cells. Actin was used as loading control. **B.** RT-PCRs for p53 mRNA. The Pol II-transcribed gene ARPP P0 was used as control.

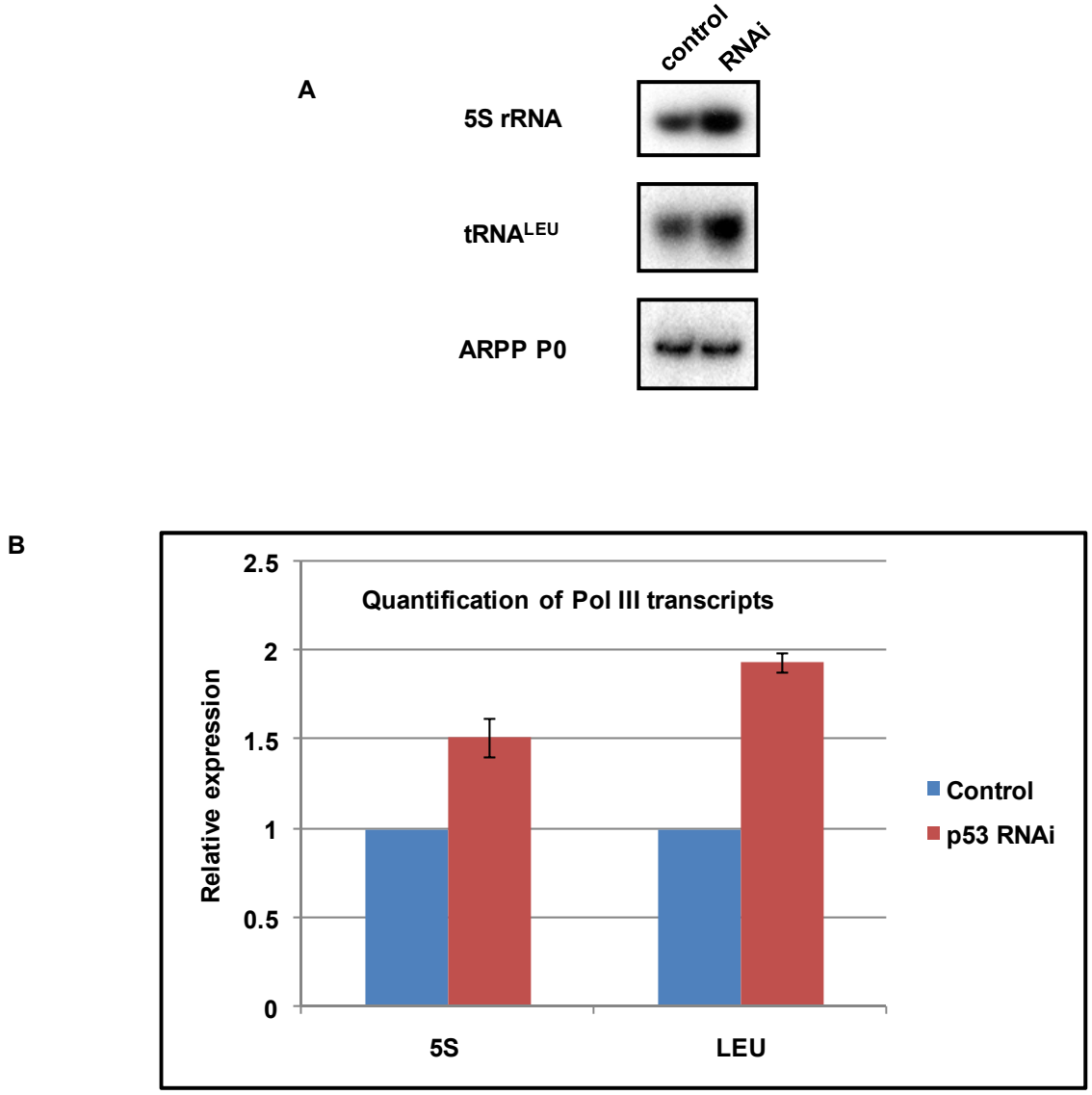


Figure 7.11. Knock-down of p53 by shRNA results in elevated levels of Pol III transcription in NARF2 cells induced to express ARF. NARF2 cells were transfected with the pSUPER-p53 vector by electroporation and grown in medium containing 1mM IPTG for 24 hours. An empty pSUPER vector was used as control. **A.** RT-PCR analysis reveals increased levels of Pol III transcripts in the RNAi treated cells. The Pol II-transcribed gene ARPP P0 was used as control. **B.** Quantification of the signals of Pol III transcripts from A. n=2.

induction and p53 RNAi, the experiments were repeated in parallel with uninduced NARF2 cells (lane 1), uninduced NARF2 cells subjected to p53 RNAi (lane 2), ARF-induced NARF2 cells (lane 3) and ARF-induced NARF2 cells subjected to p53 RNAi (lane 4) (Figure 7.12). Western blotting confirmed the induction of ARF and the knock-down of p53 (Figure 7.12). RT-PCR analysis (Figure 7.13, A and B) showed that the partial knock-down of p53 caused elevation of tRNA expression by about 2-fold (compare lanes 1 and 2), consistent with the role of p53 in Pol III repression. Furthermore, it prevented the repression of tRNA expression (lane 4) that otherwise accompanies the ARF induction in NARF2 cells (about 2-fold, compare lanes 1 and 3). Since the tRNA levels are no longer diminished in response to ARF when p53 has been depleted (compare lanes 2 and 4), these data suggest that the repression of Pol III transcription by ARF occurs indirectly, in a p53-dependent manner.

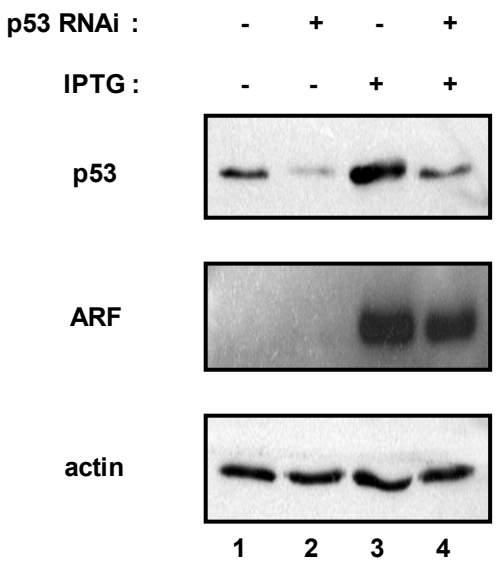


Figure 7.12. p53 shRNA knock-down and ARF induction in NARF2 cells. NARF2 cells were transfected with pSUPER-p53 (lanes 2 and 4) or an empty pSUPER vector (lanes 1 and 3). IPTG (1 mM) was added 24 hours later (lanes 3 and 4) and cells were harvested after a further 24 hours. Western analysis confirmed the induction of ARF and the knock-down of p53. Actin was used as loading control.

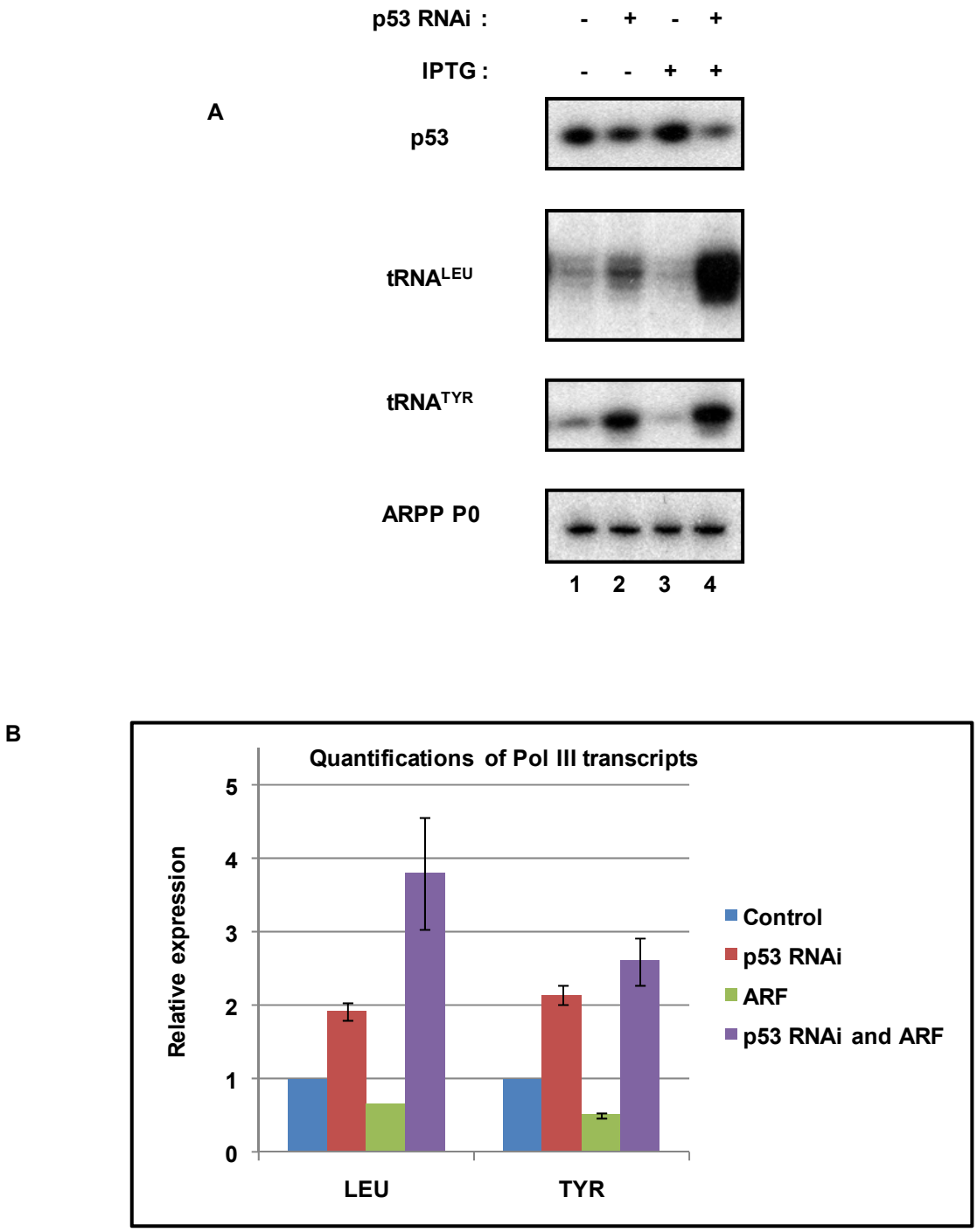


Figure 7.13. ARF-mediated repression of Pol III transcripts is blocked by RNAi of p53. NARF2 cells were transfected with pSUPER-p53 (lanes 2 and 4) or an empty pSUPER vector (lanes 1 and 3). IPTG (1 mM) was added 24 hours later (lanes 3 and 4) and cells were harvested after a further 24 hours. **A.** RT-PCR analysis revealed that ARF cannot repress Pol III transcription when p53 is knocked-down. The Pol II-transcribed ARPP P0 was used as control. **B.** Quantification of tRNA signals from A. The numbers correspond to the lanes. n=2.

7.3 Discussion

7.3.1 p53 can regulate RNA polymerase III transcription in human cells

Several studies investigated the effect of p53 in Pol III transcription regulation in mouse and human cells (Cairns & White, 1998; Chesnokov et al, 1996; Crighton et al, 2003; Gridasova & Henry, 2005; Stein et al, 2002a; Stein et al, 2002b). Experiments *in vitro* and *in vivo* have shown that overexpression of p53 or its activation under stress conditions, results in transcriptional repression of Pol III transcription from all types of Pol III promoters (Cairns & White, 1998; Chesnokov et al, 1996; Crighton et al, 2003; Gridasova & Henry, 2005). Furthermore, p53-null mouse fibroblasts present higher levels of Pol III transcripts due to relief of repression from p53 (Cairns & White, 1998). These studies were extended by depleting endogenous p53 in human cells, rather than inducing exogenous or endogenous p53, by employing a tested RNAi methodology to specifically reduce the protein levels of p53 (Brummelkamp et al, 2002). Knock-down of p53 (Figures 7.2 and 7.8) resulted in upregulation of Pol III transcription in U2OS and NARF2 cells (Figures 7.3 and 7.9), which is consistent with the previous reports. The fact that depletion of endogenous p53 had an effect on the expression of Pol III products suggests that Pol III transcription is restrained by p53 not only as a response to stress signals, but also under physiological conditions in human cells, in accordance with the results obtained by experiments in mouse p53-null fibroblasts (Cairns & White, 1998).

Interestingly, the effect of p53 on Pol III transcription seems to differ between different cell types. For example, p53 activation by UV radiation repressed the transcription of U6 snRNA in MCF-7 cells, but not in HeLa cells (Gridasova & Henry, 2005). However, treatment of HeLa cells with MMS clearly repressed tRNA expression (Crighton et al, 2003), while overexpression of p53 in HeLa cells also did not have an effect on U6 snRNA transcription, but seemed to slightly increase transcription of 5S rRNA (Gridasova & Henry, 2005). To further extend these studies in HeLa cells, RNAi was employed to deplete endogenous p53. Knock-down of p53 (Figure 7.4) did not affect the expression of tRNA in HeLa cells (Figure 7.5). Moreover, the expression of 5S rRNA was slightly reduced (Figure 7.5), in agreement with the previous overexpression experiments in the same cell line (Gridasova & Henry, 2005). The latter finding could

suggest that under physiological conditions in HeLa cells, the two types of Pol III promoters for 5S rRNA and tRNA might be regulated differently; for example, U6 snRNA and Alu elements have been shown to be more sensitive to p53-mediated Pol III repression (Cairns & White, 1998; Chesnokov et al, 1996). However, under the same experimental conditions both 5S rRNA and tRNA transcripts were upregulated in U20S cell and therefore, although one cannot exclude that this is due to promoters differences only in HeLa cells, it seems unlikely that this is the case.

The findings in HeLa cells clearly contrast with observations in several other cell lines, where p53 does have a repressive effect on Pol III transcription (Crighton et al, 2003; Gridasova & Henry, 2005; Stein et al, 2002a). A possible explanation might lie in the physiology of this cell type. HeLa is a human cervical carcinoma cell line that has the viral HPV18 DNA integrated into the cellular genome and expresses the viral E6 protein (Schwarz et al, 1985). HPV E6 can bind to p53 (Werness et al, 1990) and promote its degradation via the ubiquitin pathway (Scheffner et al, 1990). It is possible that in HeLa cells the interference of HPV E6 protein with p53 can affect the regulation of Pol III transcription by the latter. Indeed, overexpression of HPV E6 in the HPV-negative SAOS2 cells resulted in relief of Pol III repression by p53 (Stein et al, 2002a), providing evidence that Pol III transcription can be affected by E6-mediated p53 degradation. Although depletion of p53 might have been expected to upregulate Pol III transcription, it might be that in HeLa cells, due to the low levels of functional p53, Pol III transcription might no longer be regulated by p53 under physiological conditions; instead, stress conditions such as DNA damage that induce p53 might be needed to repress Pol III transcription, as observed after treatment of HeLa cells with MMS (Crighton et al, 2003).

7.3.2 ARF can regulate RNA polymerase III transcription in human cells

As previously mentioned, ARF can bind to Mdm2 (also known as Hdm2 in human) and inhibit the ubiquitination and degradation of p53, resulting in its stabilisation (Pomerantz et al, 1998; Zhang et al, 1998). By doing so, ARF can indirectly, via p53, repress Pol III and Pol I transcription. Furthermore, ARF can repress Pol I transcription (Ayrault et al, 2006) and disrupt the process of rRNA maturation (Bertwistle et al, 2004; Sugimoto et al, 2003) independently of p53. Since in most situations Pol I transcription is

co-ordinated with Pol III transcription (White, 2005), the effect of ARF on Pol III transcription was investigated. NARF2 cells, an ARF-inducible cell line which originates from the ARF-negative U2OS cells and thus provide a null background to study the effects of ARF induction, were employed (Stott et al, 1998). Expression of ARF resulted in elevated p53 and decreased levels of tRNA expression (Figure 7.6), indicating that ARF can regulate Pol III transcription.

To further investigate if the regulation of Pol III transcription by ARF is mediated by p53, RNAi was used to knock-down p53 in NARF2 cells. Partial depletion of p53 in NARF2 cells that were induced (Figures 7.8 and 7.12) or not (Figures 7.10 and 7.12) to express ARF resulted in elevated expression of tRNAs (Figures 7.9, 7.11 and 7.13). Partial depletion of p53 by shRNA in NARF2 cells resulted in increased tRNA levels (Figure 7.13, lane 2), while induction of ARF reduced tRNA transcripts (Figure 7.13, lane 3); more importantly, induction of ARF and partial depletion of p53 together did not result in decreased tRNA levels, but rather elevated (lane 4, compare lane 4 with 1 and 3). This finding clearly suggests that regulation of Pol III transcription by ARF is mediated through p53, at least in U2OS cells. In support of this conclusion, induction of ARF in NARF2-E6 cells, a NARF2 derivative cell line that constitutively expresses HPV E6 protein (Rocha et al, 2003), resulted in elevated levels of tRNA expression and, therefore, the expression of ARF did not repress Pol III transcription, as was the case in NARF2 cells (Morton et al, 2007). These data are also consistent with the fact that although p53-null mouse fibroblasts display high levels of ARF (Qi et al, 2004), they transcribe tRNA and 5S rRNA genes much more actively than wild type fibroblasts (Cairns & White, 1998).

When p53 was knocked-down in the presence of ARF rather in its absence, the effect on tRNA transcription was substantially higher (Figure 7.13, compare lane 4 to lane 2). This finding might suggest that the ability of p53 to regulate Pol III transcription is strengthened when cells induce ARF. In support of this argument, time-course experiments revealed robust repression of Pol III transcription after the induction of ARF, which was faster and more potent than the induction of p21, a direct downstream target of p53 (Morton et al, 2007). In this light, the lack of response after depletion of the endogenous p53 in HeLa cells might be explained by the low levels of ARF, a result of its weak activation due to the low p53 levels in the cells. Furthermore, it has been argued that p53 is unable to suppress tumourigenesis in the absence of ARF, despite the ability to respond to DNA damage effectively (Christophorou et al, 2006; Efeyan et al, 2006). It might be that

the repression of Pol III transcription by ARF is part of the protective cellular response to hyperproliferative signals originating from oncogenes, which initially activate ARF; although Pol III transcriptional repression is mediated by p53, ARF might be ultimately responsible for its control.

In summary, data included in this chapter extend previous studies of p53 by depleting endogenous p53 and confirming that p53 can regulate Pol III transcription under physiological conditions in human cells. Furthermore, ARF is shown to repress Pol III transcription and this effect is mediated by p53. Interestingly, the effect of p53 on Pol III transcription might differ in different cell lines and might depend on the availability of ARF. By regulating Pol III transcription, ARF adds another layer of transcriptional control in the Pol III system, and combined with its ability to also regulate Pol I transcription, ARF can potentially co-ordinate the production of rRNA and, through the control of ribosome availability, control cellular growth.

CHAPTER 8

Conclusion

8.1 Regulation of Pol III transcription by Dr1 and DRAP1

8.1.1 Dr1 and DRAP1 RNAi

Dr1 has been shown to repress Pol II and Pol III transcription *in vitro* in human cells (Inostroza et al, 1992; White et al, 1994). In this study it was investigated if partial depletion of endogenous Dr1 would affect Pol III transcription. It was found that knock-down of Dr1 by RNAi resulted in a two-fold upregulation of tRNA expression in human cells. This finding therefore, in accordance with the previous studies in human (White et al, 1994) and yeast cells (Kim et al, 1997), indicates a repressing role for Dr1 in Pol III transcription in human cells.

It is not clear why only the expression of tRNAs was affected, while the expression of other Pol III transcripts was not. This might be attributed to the partial nature of the Dr1 knock-down resulting in little or no change on the steady-state levels of Pol III transcripts; the effect on tRNA, thus, might have been detected due to the use of primers specific for the short-lived, intronic, pre-tRNA transcripts, which represent a better measure of Pol III transcription than the rather stable 5S rRNA and U6 snRNA transcripts. The use of primers specific for the detection of the mature form of tRNAs might provide more information on this issue in the future. Nevertheless, the occupancy of tRNA, but also 5S rRNA and U6 snRNA genes by Dr1, adds credibility to this hypothesis. It must be noted, however, that similar, albeit relatively more effective, knock-downs of other Pol III repressors, such as Rb, p107, p130 and p53, performed by the same methodology and conditions, resulted in altered tRNA and 5S rRNA levels, suggesting that Dr1 might specifically target type 2 promoters and thus only modulate tRNA transcription. If that is the case, this repressive property of Dr1 would be an interesting example of a repressor that only affects one type of Pol III transcripts. This might be a beneficial strategy for the cellular economy and the coordination of Pol I and Pol III transcription for the production of ribosomal RNAs.

Experiments with human cell extracts have shown that although Dr1 can repress transcription from Pol II templates, DRAP1 alone cannot (Mermelstein et al, 1996; Yeung et al, 1997). In an attempt to examine its role in Pol III transcription in human cells, DRAP1 was knocked-down by RNAi. Unfortunately however, shRNA and siRNA

experiments gave opposing results as to the effect of DRAP1 on Pol III transcription; partial depletion of DRAP1 by shRNA resulted in modestly elevated levels of tRNAs, but siRNA gave modestly decreased levels. In both cases, the experimental errors do not allow for safe interpretation of these results. It would be interesting in the future to continue these experiments by employing different siRNAs, the hypothesis being that depletion of DRAP1 would result in impaired repression by Dr1.

8.1.2 Promoter occupancy by Dr1 and DRAP1

Dr1 and DRAP1 have been previously found to occupy a variety of Pol II genes in yeast and human (Albert et al, 2007; Christova & Oelgeschlager, 2002; Creton et al, 2002; Geisberg et al, 2001; Gilfillan et al, 2005). Regarding Pol III genes, a study in yeast reported that the yeast DRAP1 (BUR6) was not found on tRNA genes (Geisberg et al, 2001). However, ChIP experiments in this study revealed that Dr1 and DRAP1 are found at Pol III-transcribed genes of promoter types 1, 2 and 3 in human cells. Geisberg *et al* (2001) concluded that BUR6 was not found at tRNA genes due to the low ratio of BUR6/TBP at Pol III promoters compared to the ratio at Pol II promoters. This does not seem to be the case in human cells, as the DRAP1/TBP occupancy ratio was very similar between Pol II and Pol III templates for both Dr1 and DRAP1. These results might suggest differences in the regulation of Pol III transcription between yeast and human, but it cannot be excluded that they are due to the different methodologies used. For example, ChIP assays were performed with antibodies against the endogenous Dr1 and DRAP1 in this study, while Geisberg et al (2001) employed anti-HA antibodies for use with a yeast strain expressing HA-tagged BUR6.

The finding of Dr1-DRAP1 occupancy at Pol II and Pol III genes is a surprising one. According to the conventional view on Dr1 function, Dr1 binds to TBP and inhibits recruitment of TFIIA and TFIIB at the promoters, resulting in an incomplete preinitiation complex and transcriptional repression (Goppelt et al, 1996; Inostroza et al, 1992). Therefore, Dr1 would not be expected to be found at active promoters; however, it seems to be present (Creton et al, 2002; Geisberg et al, 2001). In the current study, sequential ChIP assays showed co-occupancy of Dr1 with Pol III, indicating that Dr1 might also be found at active Pol III-transcribed genes.

It was suggested that the Dr1-DRAP1 complex can be found at active Pol II promoters because it can play a direct positive role at certain promoters *in vivo* (Geisberg et al, 2001). However, it has also been argued that the presence of the complex at promoters does not necessarily indicate a positive role, as it might be possible for the complex to still bind TBP, but not affect the recruitment of TFIIA or TFIIB (Kamada et al, 2001). The knock-down of Dr1 and the subsequent upregulation of Pol III transcription suggest that although Dr1 is found at Pol III promoters, it negatively affects Pol III transcription.

To explain the presence of Dr1-DRAP1 at active genes (Geisberg et al, 2001) and its ability to stimulate transcription from specific promoters in yeast (Cang & Prelich, 2002; Lemaire et al, 2000; Prelich, 1997), it was proposed that whereas Dr1-DRAP1 can repress transcription by inhibiting TFIIA and TFIIB as originally described, it can also activate transcription by stimulating TBP binding (Cang & Prelich, 2002; Gilfillan et al, 2005). According to this model, the Dr1-DRAP1-TBP-DNA complex can be considered as an intermediate state which can result in negative or positive effects, depending on the absence or presence of an appropriate activator (Cang & Prelich, 2002). In human cells, ChIP-chip experiments (Albert et al, 2007) showed an overall positive correlation of mRNA levels with DRAP1 occupancy in accordance with the Cang and Prelich (2002) model.

A number of observations on the regulation of Pol III transcription by Dr1 in human cells also seem to fit in that model. Dr1 RNAi resulted in the upregulation of tRNA expression suggesting that Dr1 can repress tRNA transcription by Pol III. Moreover, under hypoxic conditions, where Pol III transcription is downregulated, Dr1 occupancy at Pol III-transcribed genes was increased, consistent with a repressive role for Dr1 in Pol III transcription, as was previously suggested for Pol II transcription in anoxic conditions (Denko et al, 2003). Interestingly, however, sequential ChIP experiments showed Dr1 to co-occupy Pol III-transcribed genes together with Pol III, suggesting that Dr1 can be found at active Pol III-transcribed genes, although it cannot be excluded that these genes were negatively regulated by posttranscription initiation mechanisms. Moreover, induction of Brf1 resulted in enhanced occupancy of Pol III-transcribed genes by TFIIIB and Dr1, and stimulated Pol III transcription, suggesting that Dr1 can occupy Pol III-transcribed genes without, always, repressing their transcription. However, it cannot be excluded that the observed increase in transcription is due to genes not bound by Dr1.

Since the Dr1-DRAP1 complex is found at Pol III-transcribed genes it must be recruited to them by a specific mechanism. Co-IP experiments showed that, in contrast to what was expected from the *in vitro* experiments (White et al, 1994), Dr1 can interact with TFIIIB and TFIIIC *in vivo*, an observation consistent with the ChIP findings. Interestingly, induction of the Brf1 subunit of TFIIIB resulted in enhanced occupancy of Pol III-transcribed genes by Dr1, suggesting that TFIIIB has a role in recruiting Dr1-DRAP1 to Pol III promoters. Moreover, in support of this argument, promoter mapping experiments indicated that Dr1-DRAP1 localises with TFIIIB at Pol III promoters, with the Dr1-DRAP1 being placed preferentially at the beginning of the gene, rather than the end with TFIIIC or Pol III. These findings indicate that TFIIIB, and more specifically Brf1, have a role in recruiting Dr1 to Pol III-transcribed genes.

It has been known for some time that genes like the ones coding for heat shock proteins (Gilmour & Lis, 1986; Rasmussen & Lis, 1993; Rougvie & Lis, 1988), and some oncogenes, like c-Myc and junB (Aida et al, 2006; Krumm et al, 1992), and other genes in human, such as ERalpha and Igkappa (Aiyar et al, 2004; Raschke et al, 1999), have their expression attenuated by stalling of polymerase elongation within the promoter-proximal region (Saunders et al, 2006). Interestingly, however, recent genome-wide studies in *Drosophila* reported that this is a much more widespread phenomenon, with a large numbers of genes being regulated this way (Muse et al, 2007; Zeitlinger et al, 2007). Since Dr1 has been found to regulate Hsp70 (Christova & Oelgeschlager, 2002; Kraus et al, 1994) in human cells, one might speculate that the Dr1-DRAP1 complex might have a role in this phenomenon. However, this hypothesis remains to be tested. Furthermore, it would be interesting to investigate if Pol III transcription can be regulated in a similar way.

Dr1 has been shown to interact with the elongating form of Pol II, suggesting that Dr1 might have a positive role in transcription elongation (Castano et al, 2000). Regarding the Pol III system, however, there is no evidence for an interaction between Dr1 and different subunits of Pol III (data not shown). Furthermore, promoter localisation experiments indicated that Dr1 is localised, together with TFIIIB, at the beginning of Pol III promoters rather than near the end of the gene, offering no evidence that Dr1 might be involved in transcript elongation by Pol III.

8.2 Differential regulation of Dr1 and DRAP1 under stress conditions

8.2.1 Heat shock

Heat shock in yeast cells resulted in a rapid increase of BUR6 (Geisberg et al, 2001; Masson et al, 2007) and Dr1 (Masson et al, 2007) occupancy at heat shock protein (Hsp) promoters. Heat shock in human cells has been shown to affect Pol III transcription by increasing the levels of Alu RNA; the levels of Pol III transcripts like 5S rRNA and U6 snRNA were not affected (Liu et al, 1995). Furthermore, Dr1 can be found at Hsp70 promoters (Christova & Oelgeschlager, 2002) and affect Hsp70 transcription (Kraus et al, 1994). Since the Dr1-DRAP1 complex can affect the transcription of tRNA genes and the effect of heat shock on tRNA gene expression was not reported previously (Liu et al, 1995), it was investigated if the heat shock stress in human cells affects tRNA expression.

Heat shock stress did not result in a convincing upregulation of tRNA expression in human cell lines. Interestingly, however, it resulted in striking induction of DRAP1, but not Dr1, protein levels. This was a posttranscriptional effect, as the mRNA levels of Dr1 and DRAP1 remained stable before and after the heat shock. This is an interesting result, as it is the first time that differential regulation of the endogenous levels of the two subunits is reported.

A very recent study reported that heat shock in yeast cells resulted in increased occupancy of the heat shock promoter Hsp12 by both Dr1 and DRAP1 and induction of the Hsp12 protein (Masson et al, 2007). This finding agrees with that previously published (Geisberg et al, 2001) and suggests that the Dr1-DRAP1 complex stimulates transcription from heat shock promoters under stress conditions. However, when the yeast cells expressed a mutant Dr1, the Hsp12 gene transcription was derepressed compared to wild type cells prior to heat shock, but was increased after, although to a lesser degree (Masson et al, 2007). This finding suggests that Dr1 can act as a repressor or an activator for the same gene under different conditions. In the Pol III system, the transcription of tRNA genes was negatively regulated by Dr1, but tRNA promoter occupancy by Dr1 was increased, both when Pol III transcription was upregulated due to induction of Brf1 or

downregulated in hypoxia, suggesting that promoter occupancy by Dr1 does not necessarily reflect activation or repression by it.

Interestingly, the same study reported that Dr1 and DRAP1 can control the association of TFIIB at promoters both negatively and positively (Masson et al, 2007). Thus, it was proposed that Dr1 and DRAP1 can regulate the promoter association of TFIIB in a highly gene specific and dual manner (Masson et al, 2007). These results are in accordance with the sequential ChIP experiments that showed Dr1 and TFIIB co-occupancy at Pol II promoters and also do not support the model that Dr1 functions by inhibiting TFIIB recruitment (Goppelt et al, 1996; Inostroza et al, 1992; Mermelstein et al, 1996). Furthermore, they provide support to the argument that Dr1 can be found at repressed and active promoters in Pol II and Pol III transcription systems.

8.2.2 Hypoxia

It has been reported that under severe hypoxic conditions, of 0.01% O_2, the Dr1-DRAP1 complex is induced, resulting in transcriptional repression (Denko et al, 2003). Since it was shown that hypoxia of 1% O_2 can affect Pol III transcription and downregulate the expression of tRNA genes in rat cardiomyocytes (Ernens et al, 2006), these studies were extended by investigating the effects on Pol III templates in human cell lines and the possible role of the Dr1-DRAP complex.

It was found that under mild hypoxia (1% O_2) or chemically induced anoxia the expression of tRNA genes was downregulated in HeLa and U2OS cells, in accordance with the study in rat cardiomyocytes (Ernens et al, 2006). However, the expression of other transcripts like 5S rRNA and U6 snRNA was not affected, in a manner reminiscent of the Dr1 RNAi, where, again, only the expression of tRNA genes was affected. This finding is suggestive of the involvement of the Dr1-DRAP1 complex in the regulation of tRNA transcription. Indeed, ChIP experiments revealed increased occupancy of tRNA promoters by Dr1 under hypoxic conditions, indicating a role for Dr1 in tRNA repression, in broad agreement with the Dr1 RNAi experiments.

Dr1 and DRAP1 protein levels were induced under severe hypoxic/anoxic conditions (0.01% O_2) in a murine hepatoma cell line and this was considered to be a

posttranscriptional effect, as the Dr1-DRAP1 mRNA levels were not affected (Denko et al, 2003). However, when the protein levels of Dr1 and DRAP1 were tested in human cell lines that were subjected to 1% O_2 hypoxia or chemically-induced anoxia, DRAP1, but not Dr1, was strikingly induced. This was also a posttranscriptional effect, as the mRNA levels of both Dr1 and DRAP1 remained unaltered. It is interesting that under two different stress conditions, heat shock and hypoxia, DRAP1 is induced, as this finding indicates that the two subunits are differentially regulated under stress.

The stabilisation of DRAP1 protein under both heat shock and hypoxic conditions might be related to the induction of p53, as p53 was induced under the experimental conditions employed for both heat shock and hypoxia. Interestingly, when the p53 impaired NARF2-E6 cells were subjected to hypoxia, DRAP1 instead of being 2-fold or more induced, was markedly decreased as was p53, while Dr1 remained stable. The mRNA levels of Dr1, DRAP1 and p53 were not affected, while the expression of tRNA genes, but not other Pol III templates, was downregulated as previously observed and expected. This striking result strongly argues for a connection between the observed DRAP1 stabilisation after stress and the presence of p53. Partial depletion of p53 by RNAi in NARF2 cells also resulted in a small decrease in DRAP1 protein levels without affecting the mRNA levels of Dr1 and DRAP1, supporting the above argument and suggesting that even under conditions where p53 is not induced, it might still have a role in regulating the protein levels of DRAP1. Future experiments employing p53-inducible cell lines should examine the above observations.

8.2.3 mTOR inhibition

The mTOR pathway integrates multiple environmental signals to regulate translation and cell growth in response to stresses such as nutrient deprivation, DNA damage, osmotic stress, heat shock and hypoxia (Reiling & Sabatini, 2006). Since heat shock and hypoxia can affect mTOR (Hardie, 2005; Ohji et al, 2006) and differentially regulate Dr1 and DRAP1 in human cells, but mTOR can also stimulate Pol III transcription in mouse cells (Ramsbottom and White, personal communication), it was tested if inhibition of mTOR by rapamycin has the same effect in human cells and if Dr1 and DRAP1 are affected.

As was the case in mouse cells, inhibition of the mTOR pathway by rapamycin in HeLa cells resulted in downregulation of 5S rRNA and tRNA gene expression, while U6 snRNA remained unaffected. At the promoter level, ChIP experiments indicated that treatment with rapamycin resulted in the displacement of Dr1, Brf1, TFIIIC220 and Pol III from 5S rRNA and tRNA promoters, though TBP seemed not to be displaced. Thus, these data collectively suggest that the mechanism of Pol III repression by rapamycin might rely on the interaction of Brf1 with TBP, explaining why type 3 promoter templates (U6 snRNA) are not affected by rapamycin treatment. Inhibition of mTOR by rapamycin did not affect Dr1 and DRAP1, as their protein and mRNA levels were not affected, suggesting that they are not regulated by components downstream of mTOR and that downregulation of Pol III transcription by Dr1-DRAP1 is a specific event, not correlating with all cases of repression of Pol III transcription.

8.2.4 Serum starvation

Pol III transcription is regulated through the cell cycle (White et al, 1995a; White et al, 1995b). It is repressed in mitosis (White et al, 1995b) and early G1, and gradually increases to reach maximal levels during S and G2 phases (White et al, 1995a). During mitosis, Brf1 is hyperphosphorylated and associated, along with TBP, with promoters in condensed chromosomes, while Bdp1 is selectively released; it was proposed that hyperphosphorylation disrupts the TFIIIB complex, compromising Pol III transcription (Fairley et al, 2003). Interestingly, it has also been shown that Dr1 can remain associated with some, but not all of its target genes in mitotic cells (Christova & Oelgeschlager, 2002). It is tempting to speculate that since Dr1 can bind with Brf1 and TBP, it might be present at Pol III templates in mitosis and might also have a role in their transcriptional repression.

Protein extracts from serum starved cells were analysed with regards to levels of Dr1 and DRAP1 and it was found that in arrested cells, DRAP1 protein levels remain stable, while Dr1 levels are reduced. The mRNA levels of both proteins remained unaffected, indicating that this is a posttranscriptional effect. Since both Dr1 and DRAP1 are phosphoproteins (Goppelt et al, 1996; Inostroza et al, 1992; Mermelstein et al, 1996), it is possible their stability might depend on site-specific phosphorylation and that they might

be regulated during the cell cycle. This finding is interesting, because combined with those after heat shock, hypoxia or rapamycin treatment, it indicates that not only DRAP1, but also Dr1 can be posttranscriptionally regulated under specific conditions. These data show that the two subunits of the Dr1-DRAP1 complex are differentially regulated under specific conditions and suggest that they might not always act together as a complex.

8.3 Regulation of Pol III transcription by Rb, p107 and p130 in human cells

The pocket proteins have been shown to regulate Pol III transcription by binding to TFIIIB and inhibiting recruitment of TFIIIB to TFIIIC (Chu et al, 1997; Hirsch et al, 2004; Larminie et al, 1997; Sutcliffe et al, 2000; Sutcliffe et al, 1999; White et al, 1996). Since most of the above studies investigated the role of Rb and family members in mouse cells, it was investigated if Rb, p107 and p130 can also repress Pol III transcription in human cells. Partial depletion of Rb, p107 and p130 results in elevated expression of tRNAs in IMR90 cells, in accordance with their transcription repression role in mouse cells. 5S rRNA transcripts were increased after knock-down of Rb, but decreased in the case of p107 and p130 knock-down. Although this result might suggest a potential positive role of p107 and p130 in the regulation of 5S rRNA in human, it might also be attributed to posttranscriptional effects on 5S rRNA stability.

Similar experiments in HeLa cells resulted in decreased, rather than increased Pol III transcripts, after partial depletion of Rb, p107 or p130. It is not clear why HeLa cells respond differently to IMR90 and mouse cells, but it might be due to their transformed nature and the expression of the viral proteins, like HPV E7 (Schwarz et al, 1985), which can bind to the pocket proteins and mediate their proteasomal degradation (Dyson et al, 1989; Gonzalez et al, 2001).

8.4 Regulation of Pol III transcription by p53 and ARF in human cells

p53 has been shown to negatively regulate Pol III transcription by interacting with TFIIIB and block its association with TFIIIC and Pol III (Cairns & White, 1998; Crighton et al, 2003). Induction of the tumour suppressor ARF results in stabilisation of p53, due to interaction with Mdm2 and inhibition of p53 ubiquitination and degradation (Pomerantz et al, 1998; Zhang et al, 1998).

The induction of ARF, and the subsequent stabilisation of p53, results in repression of Pol I transcription, but ARF can also affect rRNA independently of p53; ARF can inhibit primary rRNA transcript processing (Sugimoto et al, 2003), interact with nucleophosmin/B23 and inhibit rRNA maturation (Bertwistle et al, 2004), and also directly repress transcription by Pol I (Ayrault et al, 2006). As p53 directly regulates Pol III transcription, it was hypothesised that ARF induction might also have an effect on Pol III transcription, at least indirectly through p53.

Indeed, experiments presented in this thesis revealed that induction of ARF resulted in downregulation of Pol III transcription. Partial depletion of endogenous p53 by RNAi enhanced transcription by Pol III as expected, while partial depletion of p53 combined with induction of ARF did not result in downregulated tRNA expression, suggesting that ARF represses Pol III transcription indirectly via p53. In support of this, induction of ARF in cells expressing the HPV E6 protein did not repress Pol III transcription (Morton et al, 2007), while p53-null mouse fibroblasts, which display high levels of ARF (Qi et al, 2004), presented higher levels of Pol III transcription than wild type fibroblasts (Cairns & White, 1998).

Oncogenes, like Myc, can induce expression of ARF (Zindy et al, 1998) and might therefore be expected to downregulate Pol III transcription. On the contrary, Myc has been shown to stimulate Pol III output (Gomez-Roman et al, 2003). There is also evidence that ARF can bind to Myc and block its ability to activate transcription and induce hyperproliferation and transformation (Cleveland & Sherr, 2004; Qi et al, 2004). It has been suggested that Myc might be able to activate Pol III transcription in cells that are compromised in the function of p53 and/or ARF (Morton et al, 2007). Indeed, loss of ARF or p53 is essential for cell immortalisation by Myc (Eischen et al, 1999; Zindy et al, 1998).

Thus, an intact p53-ARF pathway can protect against oncogene activation, while when it is impaired in immortalised cells, Pol III transcription is not restrained anymore by p53-ARF and Myc can activate the system.

It might be that the ability of p53 to regulate Pol III transcription is enhanced when ARF is induced. For example, the effect on Pol III transcription after partial depletion of p53 by RNAi was higher when ARF was induced. Furthermore, the repression of Pol III transcription was much more robust after the induction of ARF than before (Morton et al, 2007). Moreover, it has been argued that p53 is unable to suppress tumourigenesis in the absence of ARF, despite the ability to respond to DNA damage effectively (Christophorou et al, 2006; Efeyan et al, 2006). Therefore, it might be that the repression of Pol III transcription by ARF is part of the protective cellular response to hyperproliferative signals originating from oncogenes and although Pol III transcriptional repression is mediated by p53, ARF might be responsible for its control.

Hyperproliferative signals that originate from oncogene activation induce ARF expression, which consequently stabilises p53, initiating a downstream series of events that include inhibition of the cell cycle, proliferation and cellular growth. The ability of ARF and p53 to regulate Pol I transcription and rRNA production, and thus ribosome availability, provides a potent means to control mass accumulation and therefore growth. By also controlling transcription by Pol III, ARF and p53 contribute to the coordination of 5S rRNA production with that of the other rRNA transcripts from Pol I, with important benefits in terms of cellular metabolic economy.

CHAPTER 9

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