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Regulation of mammalian SINE transcription

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Abstract

Despite the abundance of the templates, both human and rodent SINEs are normally expressed at a very low level. DNA methylation-mediated silencing has been proposed as a possible cause of their transcriptional repression. The effect of DNA methylation and the effect of DNA methylation-dependent methyl-CpG-binding domain proteins (MBD proteins) on SINE transcription were studied here. It was shown that both human and rodent SINEs are bound by MeCP2, MBD1 and MBD2. Both human and rodent SINEs were also shown to be occupied by HDAC1, HDAC2 and a component of SWI/SNF complex, Brahma. Human Alus were also found to be occupied by components of two corepressor complexes, SIN3 and NuRD. Whether MBD proteins repress SINE transcription in a DNA methylation-dependent manner was further investigated using systems with low or near absent DNA methylation and, in the case of MeCP2 protein, by its direct removal.

MeCP2 was found to have no repressive effect on B1 and B2 expression. RT-PCR analysis showed no increase in B1 and B2 RNA levels in MeCP2 null mice kidneys. ChIP analysis of *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ embryonic fibroblasts, which have less than 5% of the normal DNA methylation level, showed significant reduction in MeCP2 and MBD2 binding, confirming that their presence is DNA methylation-dependant. RT-PCR comparison of *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells, however, detected no increase in B1 or B2 RNA levels. This was consistent with results obtained from MeCP2 null mice, where lack of MeCP2 did not result in increased B1 and B2 expression and with a previous study involving human Alus (Yu et al., 2001). MBD2 also does not seem to repress SINE activity, as its release following loss of DNA methylation did not result in increased SINE RNA levels.

Strikingly, all human and rodent SINEs studied here were found to be bound by transcription factors TFIIIB and TFIIIC at comparable levels with actively transcribed genes. Some RNA polymerase III was also detected, but at levels significantly lower than on active genes, suggesting a defect in RNA polymerase III loading onto SINEs. This occupancy of the transcriptional complex was comparable in cells with normal levels of DNA methylation and in cells with

significantly reduced levels of DNA methylation, suggesting that the occupancy is not affected by methylated DNA or DNA methylation-dependent components of chromatin. Indeed, removal of 50% of histone H1 did not result in increased B1 or B2 expression in this study. The fact that all tested SINEs are occupied by TFIIIB and TFIIIC also brings an unprecedented insight into the number of these transcription factors present in the cell.

Table of contents

List of tables

List of figures

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Author’s declaration

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

Jana Vávrová

List of Abbreviations

Ago2	Argonaute 2
ApoE	Apolipoprotein E precursor
APS	ammonium persulphate
ARPP P0	acidic ribosomal phosphoprotein P0
bp	base pairs
BSA	bovine serum albumin
CAF-1	chromatin assembly factor-1
ChIP	chromatin immunoprecipitation
Co-REST	an associated corepressor of repressor element RE-1 silencing transcription factor
CTCF	CTC-binding factor
DEPC	diethylpyrocarbonate
DKO	double knockout
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
Dnmt	DNA methyltransferase
DTT	dithiolthreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
ES cells	embryonic stem cells
F	forward primer
FBS	foetal bovine serum
FCS	foetal calf serum
flAlu	full length Alu
H3K9	histone 3 lysine 9
HAT	histone acetyltransferase

HCT 116	colorectal cancer cell line
HDAC	histone deacetylase
HMT	histone methyltransferase
HP1	heterochromatin protein 1
IIIB	TFIIIB
IIIC	TFIIIC
IVT	in vitro transcription
Leu	leucine
LINE	long interspersed nuclear element
MBD	methyl-CpG binding domain
MBD proteins	methyl-CpG binding domain proteins
MCAF	MBD1-containing chromatin associated factor
Mi-2/NuRD	nucleosome-stimulated ATPase Mi-2/nucleosome remodelling histone deacetylase
mRNA	messenger RNA
nt	nucleotide
NuRD	nucleosome remodelling histone deacetylase complex
PBS	phosphate-buffered saline
PKR	double-stranded RNA-regulated protein kinase
R	reverse primer
RB	retinoblastoma protein
RbAp48	Rb-associated protein 48
RISC	RNA-induced silencing complex
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
scAlu	small cytoplasmic Alu
SDS	sodium dodecyl sulphate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sec	selenocysteine
SINE	short interspersed nuclear element
snRNA	small nuclear RNA
SUV39H1	suppressor of variegation 39H1
TATA	TATA box
TBP	TATA box-binding protein
TRD	transcriptional repression domain
tRNA	transfer RNA
TSA	trichostatin A
Tyr	tyrosine
UTR	untranslated region
VA1	virus associated 1
WT	wild-type

1 Chapter 1- Introduction

1.1 Class III genes

In all eukaryotes, transcription of nuclear genes is shared by three RNA polymerases and each of them is essential for viability. In addition, two new RNA polymerases have been described recently, RNA polymerase IV in plants (Herr et al., 2005) and single-polypeptide nuclear RNA polymerase (spRNAP-IV) in mammals (Kravchenko et al., 2005). RNA polymerase I is dedicated exclusively to transcribing the rRNA genes, of which there are around 400 copies in the human genome. RNA polymerase II produces mRNAs from the protein-encoding genes, as well as transcribes many genes that encode small nuclear RNA molecules (snRNA) (White, 2005). Transcription of some mRNAs in humans and rodents is mediated by a newly described spRNAP-IV (Kravchenko et al., 2005). RNA polymerase III is responsible for 10-20% of all nuclear transcription (Moss and Stefanovsky, 2002), and synthesizes a variety of small, untranslated RNAs with essential roles in metabolism, including transfer (t)RNAs, 5S rRNA and 7SL RNA and can also transcribe a great number of pseudogenes derived from tRNA and 7SL (White, 2005). Tight regulation of these polymerases, and their respective transcription machineries, dictates gene expression patterns, and hence cell function. Although the proteins encoded by class II genes (genes transcribed by RNA polymerase II) function in a diverse array of processes, the untranslated RNAs produced by RNA polymerase I and RNA polymerase III are more specialised and primarily involved with biosynthesis. This thesis is specifically concerned with RNA polymerase III transcription of short interspersed elements (SINEs) and this introduction emphasizes aspects of RNA polymerase III transcription related to SINEs.

Although greatly out-numbered by RNA polymerase II-transcribed, mRNA encoding genes, RNA polymerase III genes (class III genes) are transcribed at very high frequencies. Confocal and electron microscopy of HeLa cells revealed that RNA polymerase III transcription occurs at around 2000 sites within the nucleus. Each site has a radius of around 20nm and contains, on average, five molecules of active RNA polymerase III (Pombo et al., 1999).

1.1.1 RNA polymerase III-transcribed genes

5S rRNA

Together with approximately 80 proteins (Doudna and Rath, 2002), and the 5.8S, 18S and 28S rRNAs produced by RNA polymerase I, 5S rRNA is an essential component of ribosomes in every eukaryotic organism, and therefore is vital for protein synthesis (Wool, 1979). At approximately 120 nucleotides long, 5S rRNA is the smallest of the ribosomal RNAs and the only one transcribed by RNA polymerase III (White, 2001). Following synthesis in the nucleoplasm, 5S rRNA is transported to the nucleolus where it is processed and integrated into the large ribosomal subunit and has a critical role in translation. Human cells are thought to contain 200 to 300 5S genes present in tandem arrays (Lander et al., 2001). In addition, 5S gene pseudogenes have been described (Doran et al., 1987).

tRNAs

tRNAs play an essential role in mRNA translation. These molecules serve as adaptors, allowing the genetic information carried in a particular nucleotide sequence to be translated by the ribosome into the appropriate amino acid sequence. The three residue anticodon sequence of a given tRNA is specific for a particular amino acid. Consequently, base-pairing of the tRNA anticodon with the complementary codon of the mRNA ensures the accurate synthesis of the polypeptide chain encoded by the mRNA nucleotide sequence. Following their initial synthesis, tRNA transcripts are processed, and in some cases covalently modified, resulting in mature tRNAs which range in length from 70 to 90 nucleotides, and adopt a conformation with complex secondary structure (Creighton, 1997; Hopper and Phizicky, 2003). The human haploid genome contains 821 tRNA-related loci, 497 of which are tRNA genes, the other 324 are tRNA-derived putative pseudogenes (Lander et al., 2001). The considerable redundancy displayed among tRNA genes results in an average copy number of around 10 genes for each amino acid tRNA adaptor.

U6 snRNA

U6 is the smallest of five snRNA species contained in a spliceosome. Four other snRNAs are produced by RNA polymerase II (Hastings and Krainer, 2001); however, the smallest (106 nucleotides), most highly conserved snRNA U6 is manufactured by RNA polymerase III (Reddy et al., 1987). Spliceosomes function in post-transcriptional processing of pre-mRNA (Maniatis and Reed, 1987). Following their initial synthesis by RNA polymerase II, pre-mRNAs are extensively processed prior to translation. For example, 5' and 3' end modifications and deadenylation (Yamashita et al., 2005) take place and, in addition, splicing is required to remove non-coding intron regions, thus producing a continuous coding sequence compatible with the translation machinery. Pre-mRNA splicing occurs in the nuclei of all eukaryotic cells and is performed by spliceosomes.

H1 and MRP

H1 is a 369 nucleotide RNA which forms part of RNase P, an endoribonuclease involved in processing the 5'-termini of pre-tRNA and which exhibits several blocks of sequence homology to MRP RNA (Bartkiewicz et al., 1989). MRP is a 265 nucleotide RNA forming part of RNase MRP, another endoribonuclease, which serves an important role in the endonucleolytic processing of pre-rRNA (Morrissey and Tollervey, 1995).

7SL RNA

In the human genome, there are four 7SL genes encoding a highly conserved 300 nucleotide transcript and a number of pseudogenes (Ullu and Tschudi, 1984; Ullu and Weiner, 1984). The class III gene 7SL encodes the RNA component of the signal recognition particle. The signal recognition particle (SRP) also contains six polypeptides and is responsible for the appropriate targeting of ribosomes engaged in translation to the endoplasmic reticulum, thus delivering nascent polypeptide chains to this organelle, where they are modified, correctly folded and then further directed to their final destinations (see later (Walter and Blobel, 1982a; Walter and Blobel, 1982b).

7SK

The 7SK gene encodes a snRNA transcript of 330 nucleotides in length (Murphy et al., 1986). It associates with eight proteins to form a 12S RNP with an unknown role. It also acts as a negative regulator of the RNA polymerase II elongation factor P-TEFb; a factor responsible for the phosphorylation of RNA polymerase II's carboxyl-terminal domain (Nguyen et al., 2001; Yang et al., 2001).

Viral genes transcribed by RNA polymerase III

Some viral genomes contain class III genes which are necessary for viral replication (White, 2004). Adenovirus is one such example and encodes two RNA polymerase III products, VA1 and VA2 (Soderlund et al., 1976; Weinmann et al., 1976). These short ~ 160 bp RNAs are expressed at very high levels late in infection (Soderlund et al., 1976) and contribute to manipulation of the host cell's translational apparatus, ensuring the synthesis of viral proteins (Thimmappaya et al., 1982). The genome of Epstein-Barr virus (EBV) also contains two small adjacent genes; EBER1 and EBER2 that are transcribed by RNA polymerase III and share homologous regions. During adenovirus infection EBERs can functionally substitute for VA1 (Bhat and Thimmappaya, 1985). Although EBER RNA is only ~ 170 bp, it has been shown to be sufficient to induce growth in soft agar and tumorigenicity in mice (Ruf et al., 2000; Yamamoto et al., 2000). Clones with the highest EBER expression are the most tumorigenic (Ruf et al., 2000). EBER RNA has the ability to bind and inhibit PKR (the double-stranded RNA activated protein kinase), a key mediator of the antiviral interferon-α response (Nanbo et al., 2002; Yamamoto et al., 2000). EBER RNA provides a first example of an oncogenic RNA and a very important precedent for the possibility that a RNA polymerase III product can transform cells.

1.1.2 RNA polymerase III-transcribed pseudogenes - SINEs

Almost all short interspersed elements (SINEs) reported from eukaryotic genomes are derived from tRNA, with the exception of Alu and B1 families (Okada, 1991a; Okada, 1991b; Ullu and Tschudi, 1984). The tRNA-derived SINEs are not simple pseudogenes for tRNAs, but have a composite structure, with a tRNA-like RNA polymerase III promoter and a C-terminal region homologous to a tRNA, a middle

tRNA-unrelated region, and a terminal AT-rich region (Okada, 1991a; Okada, 1991b). MIR elements are transcriptionally inactive interspersed repeats of tRNA origin and can be found in all mammalian orders (Smit and Riggs, 1995). They are about 260 bp long and contain an RNA polymerase III promoter. With approximately 120,000 copies still detectable in the human genome (0.2-0.3% DNA), MIRs represent a 'fossilized' record of a major genetic event preceding the radiation of placental orders (Jurka et al., 1995).

B2 and B1 are the most abundant rodent SINE families (Kramerov et al., 1979). The B2 family is a highly conserved rodent tRNA-derived family (Daniels and Deininger, 1985). It is usually about 190 bp long composed of a 5'-tRNA related region containing an RNA polymerase III promoter, a tRNA –unrelated region, and a 3' AT-rich region (Krayev et al., 1982). It is highly abundant in the mouse genome with about 348 000 members (Waterston et al., 2002). It is also found at low abundance in humans (Mayorov et al., 2000). The B1 family, with about 564 000 members (Waterston et al., 2002), originated from 7SL RNA (Maraia, 1991; Quentin, 1989). It is usually about 140 bp long with a short A-rich region (Krayev et al., 1980; Krayev et al., 1982). It shares ~78% sequence homology with both 7SL and Alu over their first ~75 and last ~30 bases, while containing a central region of ~30 bases not found in 7SL (Maraia, 1991). As opposed to the majority of Alu sequences, B1 is a monomer. B1 elements also contain an RNA polymerase III promoter and both B2 and B1 are transcribed by RNA polymerase III (Carey et al., 1986; Krayev et al., 1982; Singh et al., 1985). They typically lack RNA polymerase III terminator sequences (see later (Maraia, 1991; Singh et al., 1985).

Alu elements are the most abundant and the most studied primate-specific family of SINEs. With over a million-copies in the human genome, it makes them the most abundant of all mobile elements in the human genome (Batzer and Deininger, 2002; Deininger and Batzer, 1999; Deininger and Batzer, 2002; Mighell et al., 1997). Alu elements are about 300 nucleotides long and were shown to be derived from the 7SL gene (Ullu and Tschudi, 1984) and it is thought that they evolved through internal deletion of the S domain, acquisition of a 3' poly-A tail and subsequent tandem duplication (Figure 1.1)(Quentin, 1992; Ullu et al., 1982). They originated approximately 65 million years ago and their propagation resulted in the generation of a series of Alu subfamilies of different evolutionary

age (Shen et al., 1991). A typical Alu element has a dimeric structure, consisting of two similar but distinct monomers (Batzer and Deininger, 2002; Deininger and Batzer, 2002). The right Alu monomer contains a 31 bp insert absent from the left monomer. The left monomer contains a functional A- and B-block RNA polymerase III type 2 promoter (see later), which is changed by various mutations and is inactive in the right monomer. The elements also contain a central A-rich region and are flanked by short intact direct repeats that are derived from the site of insertion. The 3' terminus of the Alu element usually consists of a run of As that is only occasionally interspersed with other bases and it does not contain an RNA polymerase III terminator.

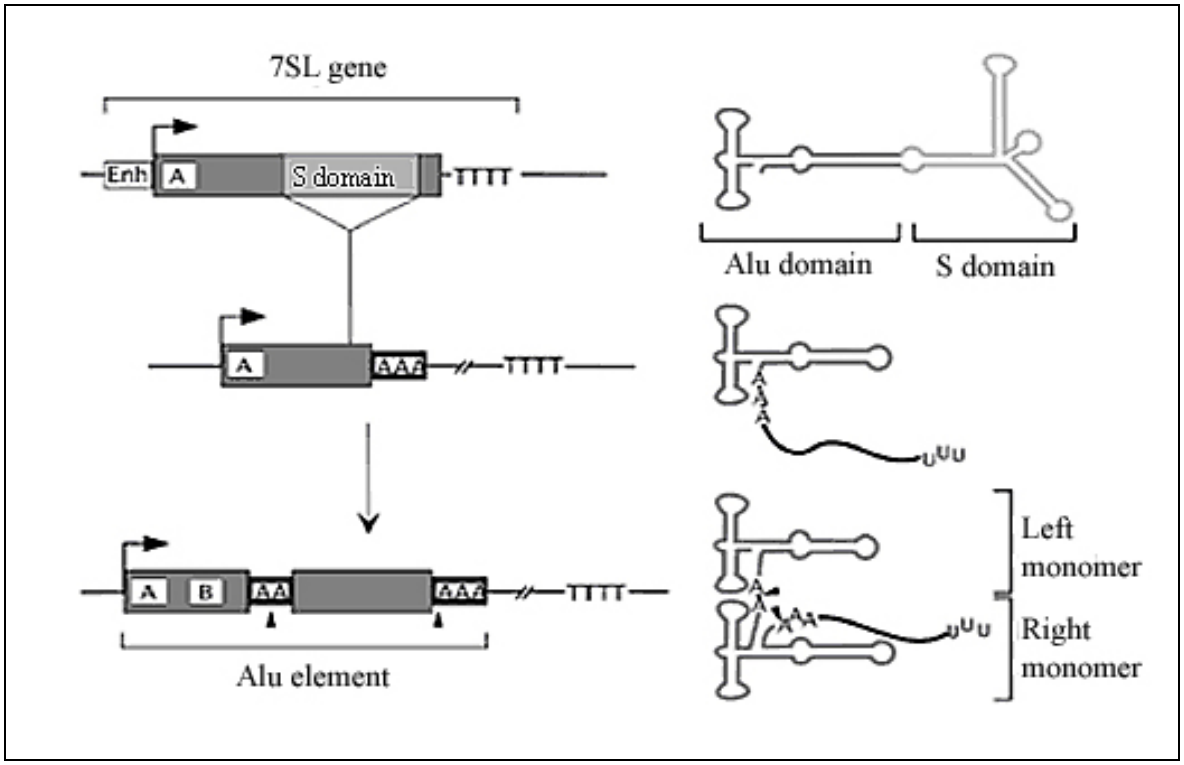


Figure 1-1 Evolutionary pathway for generation of the Alu elements (left) and corresponding RNA secondary structures (right).
The S domain of the 7SL was deleted and Alu acquired a poly-A tail. Two such monomers then fused to generate 'modern' dimeric Alu. (adapted from Dewannieux et al., 2003)

The amplification of B1, B2 and Alus is thought to occur by the reverse transcription of their RNA polymerase III-derived transcript in a process called retrotransposition, using enzymatic machinery of L1 LINEs (Dewannieux et al., 2003; Dewannieux and Heidmann, 2005). Although SINEs contain an internal promoter, they do not encode a protein.

The rate of amplification of human Alu elements has not been uniform; most of them duplicated more than 40 million years ago (Shen et al., 1991). The

amplification rate varied throughout primate evolution, giving rise to variable copy numbers of each Alu subfamily. The PS subfamily of Alus comprises of four old subfamilies, including Alu J, Sx, Sq and Sp subfamily (Batzer et al., 1996). Alu J and Alu S represent about 83% of all Alu elements. The Alu Y subfamily is the youngest and the only one shown to still be capable of retrotransposition (Shen et al., 1991). Early in primate evolution, there was approximately one new Alu element insertion in every primate birth. By contrast, today the rate is estimated to be one Alu insertion in every 200 births (Deininger and Batzer, 1999). The rate of amplification has therefore decreased by at least two orders of magnitude throughout the expansion of the family. Several factors have been suggested to influence the amplification capability and these include low transcriptional rates of each family and the ability of the specific transcript to associate with the L1 retrotransposition machinery (Dewannieux et al., 2003; Dewannieux and Heidmann, 2005) and activity of L1 elements themselves (Han and Boeke, 2004).

SINEs are located throughout the genome, but they tend to be enriched in gene-rich regions (Korenberg and Rykowski, 1988). They can also be found in almost any location within a gene, except those in which they would totally disrupt the function of that gene (Deininger and Batzer, 1999). Therefore, very few SINEs are found within 5' noncoding or coding regions of exons. In contrast, insertions into the 3' untranslated regions (3'UTRs) of genes are found commonly and appear to have few negative effects. Nevertheless, the human Genetic Mutation Database suggests that Alu elements contribute to approximately 0.1% - 0.3% of human genetic diseases. This is due to Alu element insertion into different parts of the gene, resulting in altered gene expression, disrupted reading frames or disrupted splicing, but especially due to unequal recombination events caused by dispersion of Alu elements throughout the genome (Deininger and Batzer, 1999; Deininger and Batzer, 2002).

Because of their dispersed character, all SINEs are common in hnRNA as part of RNA polymerase II-derived mRNAs (Jelinek et al., 1978; Ryskov et al., 1983). As a result, an Alu sequence was identified in 5% of 1616 human full-length cDNAs, with 82% and 14% of these located in 3'-UTR and 5'-UTR, respectively (Yulug et al., 1995). On the other hand, RNA polymerase III-derived transcripts are very rare. Despite their abundance, very few Alu, B2 and even less B1 transcripts

(Carey et al., 1986; Maraia, 1991; Paulson and Schmid, 1986) can be detected under physiological conditions in cultured cells or mouse tissues, indicating that expression of these repetitive elements is tightly down-regulated. Being regarded as parasitic sequences, it is not surprising, as their transcriptional activity would be the first step towards multiplication, undesired for the host genome, but also for SINEs themselves. So far, there is no evidence that increased transcription leads to increased retrotransposition; as the colonisation of the genome by these elements can only occur in the germ-line lineage, somatic transposition would leave no heritable trace (Bird, 1997).

SINE transcription, however, increases in a number of situations, which suggests that they may have a function. The observation that Alu elements and similar elements in other animals behave like classic cell-stress genes suggests a role in the stress response (Li et al., 1999; Liu et al., 1995). Cell stresses other than heat shock, such as viral infection and transformation and translational inhibition, increase the abundance of human Alu RNA, suggesting that the level of these transcripts is sensitive to the translational state of the cell (see later).

Both B1 and Alu transcripts get 3' processed and accumulate as small cytoplasmic RNAs called scB1 and scAlu (Maraia, 1991; Matera et al., 1990). Both were shown to associate with SRP9/14 subunits of SRP, although B1 with lower affinity than Alu (Hsu et al., 1995; Sarrowa et al., 1997). SRP is a particle composed of 7SL RNA (see above) and 6 protein subunits. It interacts with translating ribosomes and samples the nascent polypeptide chains for the presence of an ER-targeting signal sequence. It tightly binds to the ribosome-nascent chain complex and transiently blocks chain elongation until the complex reaches the ER membrane. There it releases the ribosome and protein synthesis is resumed across the ER membrane (Keenan et al., 2001). The signal recognition and targeting activities were assigned to the S domain of 7SL and SRP54. The arrest and delay in nascent chain elongation requires SRP9/14 subunits and the Alu-like part of 7SL (Siegel and Walter, 1988). It was proposed that Alu in complex with specific proteins might have the 7SL's Alu-like part inhibitory function. It was shown *in vitro* that, indeed, AluRNA/ SRP9/14 had an inhibitory effect on protein translation. However, unlike the Alu-like part of 7SL, it was acting at the level of initiation, resulting in lower polysome levels (Hasler and Strub, 2006).

Alu RNA can also regulate protein levels by interaction with the double-stranded RNA (dsRNA)-regulated protein kinase (PKR), an inhibitor of protein translational initiation. At low concentration, flAlu RNA can activate PKR (Williams, 1999), suggesting that under normal physiological conditions it can contribute to inhibition of protein synthesis. However, overexpressed full length Alu RNA (flAlu) was shown to be capable of increasing protein synthesis by binding to and inactivating PKR (Chu et al., 1998). Increased levels of flAlu RNA caused by cellular exposure to different stresses could therefore regulate protein synthesis by antagonizing PKR activation. Viruses themselves exploit PKR activity by inhibiting it with dsRNAs (Williams, 1999). FlAlus, together with scAlus, were shown to bind SRP9/14 during adenoviral infection (Chang et al., 1996), suggesting that Alus might be exploited by virus to inhibit PKR and allow protein synthesis necessary for viral replication. In addition, full length Alu (specifically the right monomer), B1 and B2 can also stimulate reporter gene expression in a PKR-independent manner (Rubin et al., 2002).

Both B1 and B2 RNA increase after heat shock in mouse cells (Fornace and Mitchell, 1986; Li et al., 1999; Liu et al., 1995). This increase is, however, unique to the SINEs; there is no general increase in RNA polymerase III transcripts such as 7SL, 7SK or U6 (Liu et al., 1995). While transcription of many heat shock protein and chaperone genes increases too, there is a general repression of RNA polymerase II-transcribed genes (Sonna et al., 2002). It was discovered *in vitro* and *in vivo* that this repression can be mediated via B2 RNA binding to RNA polymerase II; this inhibits the activity of pre-initiation complex and blocks all detectable RNA production (Allen et al., 2004; Espinoza et al., 2004). Regions in B2 structure were defined that are required for this effect (Espinoza et al., 2007). B1 has no such effect (Allen et al., 2004).

SINEs also had a global effect on evolution of mammalian genomes. They were, for example, shown to carry and spread RNA polymerase II promoters (Ferrigno et al., 2001), contain alternative splice sites (Sorek et al., 2002; Sorek et al., 2004), and generally shape mammalian transcriptomes (Sela et al., 2007). Recently they were suggested to regulate mRNA as targets for mi-RNAs if present in their 3' UTR (Smalheiser and Torvik, 2006). miRNA and siRNA are part of a mechanism collectively referred to as RNA interference (RNAi) (see later). miRNA can either direct mRNA to degradation or interfere with translational

initiation, resulting in reduced levels of mRNA or protein or both (see later; reviewed in (Valencia-Sanchez et al., 2006). However, it may also act at the level of transcriptional silencing (Kawasaki and Taira, 2004; Morris et al., 2004). Altogether, via RNAi, miRNA can regulate mRNA and protein levels.

Alus have also been shown to be human chromosome binding sites for SNF2h/NuRD-mediated binding of hRAD21, suggesting that Alus may play a role in sister chromatid cohesion (Hakimi et al., 2002). Alus are however not the only identified binding sites. Recent paper mapped other cohesin binding sites in human genome (Wendt et al., 2008). 8811 sites for hRAD21 (SCC1) were identified, mostly in intergenic regions, introns and within 5 kb upstream or downstream of genes. Although repetitive elements were specifically removed from the analysis (including Alu elements), it is known that these regions are enriched by them (Deininger and Batzer, 1999). 89% of the identified hRAD21 sites were identical to CTCF binding sites (Wendt et al., 2008). CTCF was found to be required for cohesin enrichment at those sites. However, CTCF and cohesin could associate with DNA independently. Chromatin-bound cohesin levels were not reduced after CTCF depletion, showing that cohesins still associate, but are distributed more broadly (Parelho et al., 2008; Wendt et al., 2008).

Despite the above, the general opinion is that Alu elements may represent "selfish DNA", which may have a negative impact on the host, but can be tolerated. Selfish DNA may also occasionally have positive benefits, but only by chance (Deininger and Batzer, 1999).

1.2 RNA polymerase III transcription and its regulation

1.2.1 Type 2 promoters

Class III genes have three distinct types of promoter, type 1, type 2 and type 3. The type 2 promoter, which is used by SINEs, is also the most common promoter type (White, 2001). It consists of two essential, highly conserved sequence elements of about 10bp each: an A-block and a B-block (Galli et al., 1981). In the type 2 promoters, the A-block is found further upstream, generally within 20bp of the transcription start site (White, 2001). The spacing between the A- and B-blocks is not too restricted: an optimal A- to B-block separation is 30-

60bp; however, a distance of up to 365bp can still support transcription (Baker et al., 1986; Fabrizio et al., 1987). This variation is remarkable, considering that a single transcription factor, TFIIIC, binds simultaneously to both the A- and B-blocks (Schultz et al., 1989).

The A- and B-blocks have consensus sequences TGGCNNAGTGG and GGTTCGANN-CC, respectively. Point mutations in the A- and B-blocks have been found to confer a substantial effect on transcription efficiency (Liu and Schmid, 1993; Newman et al., 1983; Nichols et al., 1989).

1.2.2 Transcription initiation complex assembly on class III genes

The route to RNA polymerase III recruitment varies depending on the promoter type of the gene to be transcribed. Type 2 promoter is discussed below.

The A- and B- block sequences are recognised by a multisubunit complex called TFIIIC. TFIIIC is one of the largest and most complex transcription factors known, having six subunits in yeast, with an aggregate mass of more than 500 kDa (Geiduschek and Kassavetis, 2001; Paule and White, 2000; Schramm and Hernandez, 2002). Photocrosslinking experiments have revealed that this enormous and flexible transcription factor can span the entire length of a tRNA gene (Bartholomew et al., 1990). Although both A- and B-blocks are contacted by TFIIIC, the latter is the major determinant of its binding affinity (Baker et al., 1986).

Human TFIIIC is composed of 5 polypeptides, known as TFIIIC220, 110, 102, 90 and 63, according to their molecular masses (Kovelman and Roeder, 1992). Three of these TFIIIC subunits (220, 110 and 90) have been shown to possess histone acetyltransferase (HAT) activity, which may serve to remodel chromatin in the vicinity of class III genes to access the promoter (Hsieh et al., 1999a; Kundu et al., 1999).

The primary function of TFIIIC is to recruit TFIIIB and to position it just upstream of the transcription start site. TFIIIB consists of three proteins: TATA box-binding protein (TBP) and two TBP-associated factors, known as TFIIB-related factor 1 (Brf1) and B double prime (Bdp1) (Schramm and Hernandez, 2002). Human TBP, Brf1 and Bdp1 have apparent molecular masses of approximately 34, 90 and

160kDa, respectively. While Brf1 and Bdp1 are specifically involved in the transcription of class III genes, TBP is also used by the transcription machineries of RNA polymerase I and RNA polymerase II (Cormack and Struhl, 1992). Brf1 forms a tight association with TBP in solution; however, Bdp1 is only weakly associated with this complex, if at all, in the absence of a DNA template (Geiduschek and Kassavetis, 2001; Huet et al., 1994; Kassavetis et al., 1991; Schramm and Hernandez, 2002; Schramm et al., 2000).

The recruitment of TFIIIB to class III gene promoters by TFIIIC has been best studied in *S. cerevisiae*. During transcription initiation complex formation, DNA-bound TFIIIC initially contacts the Brf1 subunit of TFIIIB, and this is thought to occur via the *S. cerevisiae* equivalent of human TFIIIC102 (Kassavetis et al., 1992b; Schramm and Hernandez, 2002). Several subsequent interactions that occur between each of the TFIIIB subunits and various TFIIIC components are also likely to participate in the formation of a stable pre-initiation complex (Schramm and Hernandez, 2002). The human TFIIIC102, TFIIIC63 and TFIIIC90 subunits bind to Brf1 (Hsieh et al., 1999a; Hsieh et al., 1999b) and the TFIIIC102 and TFIIIC63 subunits bind to TBP (Hsieh et al., 1999a). These interactions between the TFIIIC102 and TFIIIC63 subunits, taken together with the fact that TFIIIC63 interacts with the A-block (Hsieh et al., 1999a), provides a link between the TFIIIB-interacting and DNA-interacting subunits of TFIIIC. Since TFIIIB contains TBP, it can bind independently of TFIIIC to a TATA box (Joazeiro et al., 1994). However, most of the type 2 promoters lack a TATA sequence and cannot be recognised this way.

TFIIIC and TFIIIB are essential for the recruitment of RNA polymerase III to the transcription start site of type 2 promoter-class III genes (Figure 1.2). RNA polymerase III is the largest of the eukaryotic nuclear RNA polymerases. It is composed of 17 subunits in yeast and humans, adding up to a 600-700kDa complex (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). Several factors, however, contribute to the selection of the transcription start site. Additional internal or flanking sequences commonly confer modulatory effects. Indeed, although the site at which initiation can occur is dictated primarily in relation to the A-block (Baker et al., 1987; Ciliberto et al., 1983), the precise start site within that region is determined by local sequence. Thus, RNA polymerase III favours initiation at a purine preceded by a pyrimidine

(Ciliberto et al., 1983; Fabrizio et al., 1987) and the upstream flanking region can also be influential. In most cases, the 5' flanking sequences have an overall stimulatory influence upon transcription, although repressive effects can also occur (DeFranco et al., 1981; Dingermann et al., 1982; Hipskind and Clarkson, 1983). Despite their modulatory effects, flanking regions are generally poorly conserved. Indeed, the 5' flanking regions of tRNA genes display little or no homology, even between different genes encoding the same tRNA isoacceptor (Kubli, 1981). This variation may provide a mechanism for differential regulation of tRNA genes in response to differing codon and amino acid demands in various cell types.

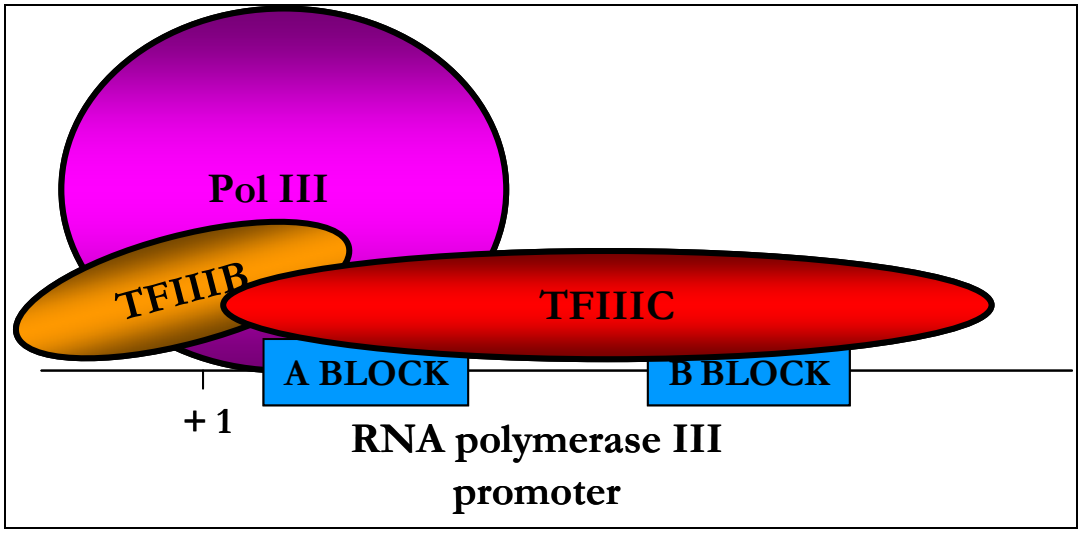


Figure 1-2 RNA polymerase III transcription complex assembled on type II promoter.

The 5' flanking sequence has also been shown to be important for 7SL RNA gene function (Ullu and Weiner, 1985). Alu elements ancestrally derived from this gene acquire a new 5'-flanking sequence after being transposed and this could lead to markedly decreased efficiency in transcription. Studies of Alu elements transcription showed that indeed the 5'-flanking region is important and alterations can result in a significant decrease in transcription *in vitro* (Chesnokov and Schmid, 1996; Roy et al., 2000; Shaikh et al., 1997). This could be due to a reasonable TATA box (Shaikh et al., 1997) or an Ap1 (activator protein 1 transcription factor) binding site, which was shown to be responsible for stimulating transcription (Chesnokov and Schmid, 1996). These assays were further done *in vivo* in HeLa cells (a cervical cancer cell line). Chimeric constructs of 7SL upstream sequence and Alu gene significantly increased Alu

transcription, while other Alu flanked just by vector sequence was hardly detectable (Roy et al., 2000). In other work, expression of Alu elements was tested with or without the added 7SL enhancer sequence. Levels of transcripts produced from Alu elements without the 7SL enhancer sequence were 3-5 times lower, indicating that the marked Alu sequence should be transcription/transposition competent regardless of its flanking DNA (Dewannieux et al., 2003).

Still, the principal determinant that dictates the general location where TFIIIB and RNA polymerase III is positioned is TFIIIC. The interface between these factors is quite flexible and allows TBP to scan the region within 30 bp for an optimal site for TFIIIB. Also, RNA polymerase III has certain sequence preferences and will look for optimal initiation sites (Joazeiro et al., 1996). For RNA polymerase III recruitment, all the three TFIIIB subunits are required; however, only Brf1 and TBP have been shown to make direct contacts with RNA polymerase III (Schramm and Hernandez, 2002). The majority of direct interactions occur between Brf1 and RNA polymerase III subunits RPC32, RPC39 and RPC62 (Wang and Roeder, 1997), although TBP can also associate with RPC39 (Wang and Roeder, 1997). In addition to these interactions, TFIIIC has been shown to interact with the RNA polymerase III subunit RPC62 via TFIIIC63 (Hsieh et al., 1999a).

1.2.3 Transcription initiation, elongation and termination by RNA polymerase III

Following RNA polymerase III recruitment, the two strands of DNA around the transcription start site are separated to form a transcription bubble (Geiduschek and Kassavetis, 2001). This melting of the DNA helix allows the polymerase to access the template strand, and is required before transcription can proceed. DNA melting is performed by the polymerase, although the Brf1 and Bdp1 components of TFIIIB also play an active role (Geiduschek and Kassavetis, 2001; Kassavetis et al., 1998; Kassavetis et al., 2001; Schramm and Hernandez, 2002). Thus, TFIIIB serves not only to recruit RNA polymerase III, but also participates in the formation of an open promoter complex.

Once the DNA strands have been separated, RNA synthesis can be initiated, and the polymerase progresses into the gene and dissociates from promoter-bound TFIIIB without significant pausing or arrest (Bhargava and Kassavetis, 1999). As RNA polymerase III progresses into the gene, the bubble of melted DNA moves with it (Kassavetis et al., 1992a). Although TFIIIC assembles within the transcribed regions of the majority of class III genes, this large factor is not dissociated from promoters during elongation (Paule and White, 2000). It is surprising that the DNA-bound transcription complex of TFIIIC and TFIIIB effects very little the progression of the polymerase during transcription. During transcription in the normal direction, the presence of TFIIIC delays RNA polymerase III for just 0.2 s (Matsuzaki et al., 1994). However, unlike RNA polymerase I and RNA polymerase II, RNA polymerase III does not require any accessory factors for efficient chain elongation (Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002). It is believed that the main reason is that RNA polymerase III-transcribed genes are extremely short. SINEs, some of them being over 300 nt long, constitute the longest RNA polymerase III-derived transcripts. Also, elongation of RNA polymerase III does not proceed at a uniform rate (Matsuzaki et al., 1994) and in yeast, RNA polymerase III subunit C11 was found to be involved in the pausing (Chedin et al., 1998). It shares significant homology with RNA polymerase II-specific elongation factor TFIIS. It may be that C11 subunit performs similar function to TFIIS.

Termination by RNA polymerase III occurs independently of other factors too: four or more T residues within the template strand of a class III gene are sufficient to signal the accurate and efficient termination of transcription (Bogenhagen and Brown, 1981; Cozzarelli et al., 1983). It has been proposed that the La antigen is involved in termination (Gottlieb and Steitz, 1989a; Gottlieb and Steitz, 1989b; Maraia et al., 1994). Immunodepletion of La from cell extracts was found to reduce RNA polymerase III output *in vitro*, which led to the suggestion that La could act as a transcriptional termination factor that mediates nascent transcript release (Gottlieb and Steitz, 1989a; Gottlieb and Steitz, 1989b). The presence of La on RNA polymerase III templates was also shown *in vivo*, although its function remains to be confirmed (Fairley et al., 2005).

Following the synthesis of the first transcript, RNA polymerase III is known to be recycled on the same DNA template for several further rounds of transcription (Dieci and Sentenac, 1996). The process is likely to involve a direct coupling between termination and re-initiation as demonstrated by findings that run-off termination on truncated class III genes does not allow efficient recycling and re-initiation (Dieci and Sentenac, 1996). This enables a stable complex on a yeast tRNA gene to direct subsequent cycles 5- to 10-fold more rapidly than the first. During multiple round transcription, synthesis of each tRNA molecule takes ~35 s, whereas initiation of the first transcription takes ~5 min. As a consequence, the slow initial step of polymerase recruitment is avoided, making the production of subsequent RNAs by RNA polymerase III more efficient (Dieci and Sentenac, 1996). Human RNA polymerase III can also be recycled, as was shown for VA and tRNA genes (Jahn et al., 1987). Addition of human recombinant La to isolated RNA polymerase III transcription complexes assembled on the VA1 promoter from mammalian cell extracts led to increases in transcription, apparently due to enhanced RNA polymerase III recycling and reinitiation (Maraia, 1996; Maraia et al., 1994). Newer findings indicate that yeast TFIIIB participates in this RNA polymerase III recycling on shorter transcripts and for longer transcripts, TFIIIC is also required (Ferrari et al., 2004).

1.2.4 Regulators of RNA polymerase III transcription

Transcription of RNA polymerase III genes is essential for sustained protein synthesis and is therefore a fundamental determinant of the capacity of a cell to grow. It gets upregulated in many situations involving cell growth. This process is often regulated through overexpression of transcription factors or release from repressors that control RNA polymerase III output in healthy cells. These factors have been well studied (see below). However, in the case of SINEs, often more global factors are involved in the regulation, such as DNA methylation and chromatin. In the following paragraphs, aspects and situations which were shown to affect the expression of SINEs are mentioned, excluding chromatin-mediated regulation which is going to be described in section 1.3.

The first way in which RNA polymerase III output is increased is by increased level of one or more of the limiting transcription factors on which it depends. A

variety of viruses have been shown to stimulate RNA polymerase III transcription this way, to meet an increase in biosynthetic demand. Adenoviral infection or SV40 transformation studies revealed that TFIIIC activity is increased (Hoeffler et al., 1988). In adenoviral infection, deregulation is largely due to the adenoviral oncoprotein E1A. Mutant virus strains lacking E1A show little or no activation of VA (Berger and Folk, 1985; Sollerbrant et al., 1993). Furthermore, purified recombinant E1A can stimulate VA1 transcription by up to 50-fold in HeLa extracts (Datta et al., 1991). However, E1A does not bind to the VA1 gene to exert a direct effect (Datta et al., 1991) but influences transcription through the general RNA polymerase III factors. HeLa cells infected with wild-type adenovirus display a significant elevation in TFIIIC2 activity (Hoeffler et al., 1988). This is a manifestation of a selective increase in the level of the TFIIIC110 subunit, seemingly through an induction of TFIIIC110 mRNA by E1A (Sinn et al., 1995) that raises the proportion of the transcriptionally active TFIIIC form (Hoeffler et al., 1988). All the five subunits of TFIIIC are overexpressed at both the mRNA level and protein level in fibroblasts transformed by Simian virus SV40 or polyomavirus (Felton-Edkins and White, 2002; Larminie et al., 1999).

TFIIIB is also activated during adenoviral infection, SV 40 and EBV transformation and infection by human papillomaviruses (HPVs). Several of them achieve that through binding to RB protein. RB is a retinoblastoma tumour suppressor protein which has strong capacity to repress RNA polymerase III transcription (White et al., 1996). When bound by RB, TFIIIB is unable to interact with either TFIIIC or RNA polymerase III (Sutcliffe et al., 2000). Viruses encode oncoproteins that disrupt RB-mediated repression of TFIIIB by binding to the RB pocket. These include the E1A product of adenovirus, the E7 product of HPV and the large T antigen of SV40, all of which have been shown to release TFIIIB from repression and therefore stimulate RNA polymerase III transcription (DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988). During EBV infection the stimulation of TFIIIB is achieved via stimulation of expression of Bdp1 subunit, which is enough to increase EBER expression *in vivo* (Felton-Edkins et al., 2006).

TFIIIB is bound and repressed not only by RB but also by p53 (Cairns and White, 1998; Chesnokov et al., 1996). When bound by p53, TFIIIB is unable to interact with TFIIIC and be recruited to RNA polymerase III templates (Crighton et al., 2003). Full induction of Alu genes requires another adenovirus oncoprotein, E1B

(Panning and Smiley, 1995). E1B is able to bind and inactivate p53, suggesting that adenovirus infection may also overcome the regulatory effects of p53 on RNA polymerase III transcription (Ko and Prives, 1996). The HPV oncoprotein E6 was also shown to target wildtype p53 for degradation (zur Hausen, 2000).

p53 appears to function as a general repressor of class III gene expression; however, these genes display differential sensitivity to the repressive effects of p53, with genes such as Alu and U6, possessing weak promoters, appearing most susceptible (Cairns and White, 1998; Chesnokov et al., 1996).

TFIIIB serves as a direct target for repression by p53. Co-fractionation and co-immunoprecipitation experiments demonstrated that p53 associates with endogenous TFIIIB in a relatively stable complex at physiological ratios (Cairns and White, 1998). In wild-type fibroblasts TFIIIB is limiting, but disruption of the p53 gene conferred a specific increase in TFIIIB activity and RNA polymerase III transcription. Furthermore, the inhibition by p53 of *in vitro* RNA polymerase III transcription can be specifically relieved by the addition of excess TFIIIB (Cairns and White, 1998). It has also been shown that the N-terminal region of p53, which possesses a TBP-binding site, is sufficient to bind TFIIIB (Chesnokov et al., 1996). Point mutations that abolish the binding of free TBP similarly abolish TFIIIB binding and, moreover, also abrogate the ability of p53 to repress RNA polymerase III transcription (Chesnokov et al., 1996). These data suggest that p53-repression of RNA polymerase III transcription involves a direct interaction with TBP within the TFIIIB complex. Once TFIIIB has been assembled into a transcription complex, however, it becomes significantly less susceptible to p53-repression (Cairns and White, 1998). Conversely, when bound by p53, TFIIIB cannot be recruited to promoters, as shown by chromatin immunoprecipitation of tRNA genes in living cells (Crighton et al., 2003). P53 was also shown to recruit chromatin remodelling and corepressor complexes such as SIN3 (Murphy et al., 1999).

TFIIIB further interacts with the proto-oncogene product c-myc (Gomez-Roman et al., 2003). c-myc is deregulated in a wide range of malignancies, including Burkitt's lymphoma, neuroblastomas and colon cancers (Dang, 1999; Nesbit et al., 1999). Depletion of c-myc by RNAi showed that it contributes to elevated levels of RNA polymerase III transcription in cancer cells (Felton-Edkins et al.,

2003). Moreover, c-myc significantly contributes to the levels of RNA polymerase III transcripts in normal mammalian cells (Gomez-Roman et al., 2003). Gene occupancy by TFIIIB increases rapidly in response to c-myc that can be found at RNA polymerase III promoters, including B2 SINEs (Gomez-Roman et al., 2003). It is then followed by recruitment of RNA polymerase III and induction of transcription (Kenneth et al., 2007). c-myc has a strong stimulatory effect on RNA polymerase III transcription both in human and mouse cells. When c-myc knockout and matched wild type fibroblasts are compared, the knockout fibroblasts show approximately sevenfold lower expression of B2 RNA. Human SINEs were not tested (Gomez-Roman et al., 2003).

RNA polymerase III transcript levels (including SINEs) are substantially reduced when F9 embryonal carcinoma (EC) cells differentiate into parietal endoderm upon induction with retinoic acid and cAMP (Murphy et al., 1983; White et al., 1989). This reduction in RNA polymerase III transcription rate was shown to be mediated again via this key transcription factor TFIIIB through its specific down-regulation (White et al., 1989). There is a significant decrease in the Brf1 subunit of TFIIIB (Alzuherri and White, 1998) and some decrease in the level of TBP (Alzuherri and White, 1998; Alzuherri and White, 1999; Perletti et al., 2001). Despite the fact that TBP is utilised to transcribe all three classes of genes, there is no overall change to RNA polymerase II transcriptional activity (White et al., 1989).

There are many more regulators of RNA polymerase III transcription, but these have not yet been connected to regulation of SINEs transcription and they are therefore not going to be mentioned here.

1.3 Chromatin and its effect on transcription

The human genome would extend to about 2m if unravelled. In order to fit into the nucleus, the DNA assembles with histone and non-histone proteins into chromatin and is further compacted into chromosomes. The chromatin proteins that serve to compact DNA in vivo are not merely a packing material, but provide a dynamic structure that is utilized by the cell to regulate gene expression.

1.3.1 Chromatin structure

Chromatin is generally comprised of 147 bp of DNA wrapped 1.65 turns around an octamer of histone molecules, the linker DNA between adjacent histone octamers, and members of a class of linker histones that bind the linker DNA and nucleosome core (Kornberg and Lorch, 1999; Zlatanova et al., 1999). The four histone subnits, H2A, H2B, H3 and H4 are amongst the best-conserved proteins in eukaryotes. Adjacent nucleosomes are connected by linker H1 DNA, which binds at the point where DNA enters and exits the subunits (Crane-Robinson, 1997). Progressive coiling of nucleosomes leads to compact higher-order chromatin structures. Arrays of nucleosomes compact to form 30-nm chromatin fibre and two competing classes of models have been suggested in which nucleosomes are either arranged linearly in a one-start higher order helix or zigzag back and forth in a two-start helix (Dorigo et al., 2004). A variety of evidence suggests that electrostatic interactions between nucleosomes are the driving force in chromatin fibre compaction. These interactions are favoured by increasing salt concentrations, which reduce the repulsive forces between linker DNA (Sun et al., 2005) and are likely to be modulated by post-transcriptional modifications that alter the charge of the very long histone tails (see below). The structure can also be modulated by other proteins, including the linker histone H1 and the HMGN non-histones. H1 and HMGN seem to have opposite effects on the structure of chromatin. Linker histone H1 influences the degree of chromatin compaction and its removal leads to decondensation (Fan et al., 2005). In contrast, HMGN (high mobility group N) proteins are nucleosome binding proteins that reduce the compaction, probably via their negatively charged C-terminal domains (Bustin, 2001). The chromatin fibres are assembled further into large domains, usually of 40-100 kb, containing non-histone proteins performing both structural and regulatory functions. These domains undergo further folding within the chromosome.

1.3.2 Chromatin regulates gene expression

Historically, chromatin was classified into two forms, heterochromatin and euchromatin (Sarma and Reinberg, 2005). Euchromatin is the region of

chromatin that is decondensed and is thought to represent loci that are transcriptionally active. Heterochromatin is highly compacted chromatin with regions of silenced DNA. Heterochromatin is further classified into constitutive heterochromatin and facultative heterochromatin. Constitutive heterochromatin is the region that is juxtaposed to centromeres of human chromosomes and is irreversibly silenced, while facultative heterochromatin referrers to regions which are silenced but can become transcriptionally active. Active and silent regions of chromatin are often considered to have ‘open’ and ‘closed’ chromatin structures, respectively (Felsenfeld and Groudine, 2003).

Chromatin fibre structures can be studied through nuclease accessibility. DNase I and micrococcal nuclease (MNase) are the two most commonly used nucleases for these studies (Rando, 2007). Transcriptionally active regions are considered to be more sensitive to DNaseI nuclease digestion than inactive regions (Gazit et al., 1982), while this is not seen with MNase that cuts linker DNA (Gilbert et al., 2004). Only recently was the structure of human 30nm chromatin fibre studied in detail. It was shown that human heterochromatin is surprisingly heterogeneous in structure and that there is no structural division between heterochromatin and euchromatin (Gilbert et al., 2004). There does not seem to be a simple correlation between gene expression and open chromatin fibres. Conversely, in regions of low gene density, active genes could be found with large domains of compact fibres. These findings challenge the historical view of the structural division of the two forms of chromatin.

Significant advances have been made in recent years to understand how chromatin and its modification and remodelling contribute to gene regulation. Both non-histone and histone proteins play a role, together with modifications of DNA itself. Chromatin remodelling complexes have been identified, the SWI/SNF family being the best characterised in humans (Langst and Becker, 2001; Tsukiyama, 2002). Chromatin-remodelling complexes mobilize nucleosomes, involving the breaking and reforming of histone-DNA contacts which cause the histone octamers to move short distances along the DNA. The precise mechanism is still unknown (Becker and Horz, 2002). Interplay exists between chromatin remodelling and histone modifications (see below) which results in gene-specific transcriptional activation or repression that is generally affected by the binding of transcriptional activators or repressors and their interplay.

1.3.3 Histone posttranslational modifications and gene regulation

During the past decade strong evidence has built up demonstrating that posttranslational modifications of the core histones H2A, H2B, H3 and H4 are associated with transcriptionally active and inactive DNA sequences. Histones are small basic proteins consisting of a globular domain and a more flexible and charged NH2-terminus, called the histone tail that protrudes from the nucleosome. Multiple modifications decorate each histone tail within the nucleosome, including some amino acids that can be modified in several different ways. Covalent modifications of histone tails known so far include acetylation, phosporylation, methylation, ubiquitylation and SUMOylation on various residues.

Histones contain a high proportion of amino acids with basic side chains, which are positively charged in physiological conditions and they are attracted by electrostatic interactions to the negatively charged DNA. Histone modifications result in a change in the net charge of nucleosomes (with the exception of methylation), which could loosen inter- or intranucleosomal DNA-histone interactions, thus control access of DNA-binding proteins such as transcription factors. This idea is supported by the observation that acetylated histones are easier to displace from DNA (Zhao et al., 2005). Attached chemical moieties also alter nucleosome surface and promote the association of chromatin-binding proteins (Berger, 2007).

1.3.3.1 Lysine acetylation

There are at least two different mechanisms by which acetylation and deacetylation of histone lysines regulates chromatin-based processes (Shahbazian and Grunstein, 2007). In one case, acetylation (or deacetylation) of many residues is coordinated, and the combined effect dictates function. In the other case, acetylation (or deacetylation) of specific residues has precise effects. It is becoming evident that a combination of these two mechanisms dictates the functional outcome of histone acetylation and deacetylation.

There is abundant evidence that acetylation of histone H3 and histone H4 (H3 and H4) are associated with active transcription (Kurdistani et al., 2004; Pokholok et al., 2005; Strahl and Allis, 2000). In human cells, lysine modified by

acetylation include K9, K14, K18 and K56 at histone H3 and lysines K5, K8, K12,K16 at histone H4 (Berger, 2007). The combination of a general effect in addition to a position-dependent effect of acetylation is apparent in transcription. For example, a study showed that the acetylation of many different lysine residues correlates with transcription, but individual mutations in H4K5, H4K8 and H4K12 have minor effects on transcription (Dion et al., 2005). Combined mutations, however, lead to cumulative changes in the expression of a group of genes. In contrast, H4K16 has a specialized role in transcription. If mutated it leads to changes in gene expression different than those caused by individual and combined mutations in the other H4 tail lysines (Dion et al., 2005).It was shown that the hypoacetylated state of H4K16 correlates with gene activity and with the binding of Bdf1 (Kurdistani et al., 2004).

In general, acetylated lysines may decrease the histone-DNA interaction and promote accessibility of the DNA for transcription. Moreover, an acetylated lysine no longer has a basic side chain, allowing it to be recognized by bromodomains found in many chromatin-associated proteins including transcription complexes (Agalioti et al., 2002; Lee et al., 1993; Vettese-Dadey et al., 1996).

Acetylation of lysines is established via histone acetyltransferases (Brown et al., 2000) and removed by histone deacetylases (Cress and Seto, 2000).

Histone acetyltransferases interact with transcriptional coactivators such as p300, CBP and PCAF, that physically connect many DNA-binding factors to the basal transcription machinery (Ogryzko et al., 1996; Yang et al., 1996).

Histone deacetylases (HDACs) have been identified as common components of diverse transcription regulators, mainly corepressors, indicating that deacetylases play a general role in repression of gene expression. In humans, eight deacetylases have been identified, HDAC1-HDAC8 (Cress and Seto, 2000). Like acetyltransferases, deacetylases are found as part of multiprotein complexes. Two corepressor complexes, Sin3 and NuRD, have been well characterised (Knoepfler and Eisenman, 1999). In mammalian cells, both complexes contain HDAC1 and HDAC2 (Knoepfler and Eisenman, 1999; Zhang et al., 1999). The deacetylase complex can be recruited mainly via DNA binding

proteins such as MBDs (Zhang et al., 1999) and p53 (Murphy et al., 1999), or via interaction with other corepressors such as coREST (You et al., 2001).

1.3.3.2 Lysine methylation

A large body of evidence demonstrated that histone lysine methylation is involved at many levels in the regulation of gene expression. However, the precise mechanisms by which it contributes to transcription stay mostly unresolved (Shilatifard, 2006). The most-studied sites of lysine methylation lie in the tails of H3 and H4 histones. Historically, it was thought that methylation of lysines 4, 36, and 79 of histone H3 (H3K4, H3K36 and H3K79) occurs primarily at transcriptionally active genes (Bannister et al., 2005; Bernstein et al., 2005; Pokholok et al., 2005), whereas methylation of H3K9, H3K27 and H4K20 have been connected to transcriptionally repressed genes and heterochromatic regions (Cao et al., 2002; Peters et al., 2001; Rice et al., 2003; Schotta et al., 2004).

It is clear today that lysine methylation displays the highest degree of complexity among known covalent histone modifications and this complexity is further multiplied by the fact that lysine methylation can occur several times (mono-, di- or trimethylation). Recent detailed studies showed that the level of modification is very important. Active genes were characterized by high levels of H3K4me1, H3K4me2, H3K4me3, H3K9me1 around transcription start sites and elevated levels of H3K36me3, H3K27me1, and H4K20me1 downstream of transcription start sites and throughout the entire transcribed regions (Barski et al., 2007; Vakoc et al., 2006). H3K9me3 could also be found at actively transcribed promoters (Squazzo et al., 2006; Vakoc et al., 2005).

In contrast, inactive genes were characterized by high levels of H3K9me2, H3K27me3, H3K79me3 and H4K20me3 in promoter and gene-body regions and low or negligible levels of H3K4 methylation at promoter regions and low or negligible levels of H3K36me3, H3K27me1, H3K9me1, and H4K20me1 in gene-body regions (Barski et al., 2007; Schotta et al., 2004). H3K9me3 was found to be associated with inactive genes in both of the studies and has long been associated with heterochromatin formation (Bannister et al., 2001).

Lysine methylation is established via histone methyltransferases (Sims et al., 2003) and can be removed by histone demethylases (see below).

Histone methyltransferases (HMTs) transfer methyl groups to histone tails. The best studied are HMTs containing a SET domain, 130- to 140-amino acid motif responsible for transfer of the methyl group to histone lysines (Jenuwein et al., 1998). SUV39H1 was described as an HMT whose activity is specific for H3K9 methylation (Rea et al., 2000). Methylation of H3K9 creates a motif that is specifically recognized and bound by the chromodomain of heterochromation protein 1 (HP1) (Lachner et al., 2001). In fission yeast (there is no H3K9 HMT in budding yeast (Sims et al., 2003)), the disruption of the Clr4 gene (which encodes another HMT) resulted in the loss of localization of Swi6 (homologue of HP1), illustrating that H3 methylation is required in the recruitment of HP1 and heterochromatin assembly *in vivo* (Nakayama et al., 2001). The activity of Suv39h1 and Clr4 and both H3K9 methylation and HP1 binding have critical roles in heterochromatin formation (Nakayama et al., 2001) (44,58,59 in big review) str 251. G9a and SETDB1 are two other well characterised HMTs. G9a is able to methylate other residues than just H3K9 (Tachibana et al., 2001) and both G9a and SETDB1 contribute to H3K9 methylation mainly in euchromatic rather than heterochromatic regions (Schultz et al., 2002; Tachibana et al., 2005). The connection with HP1 provides a molecular explanation for the general correlation of K9 methylation with transcription silencing or repression. H3K9 can also inhibit transcription through its interaction with HDACs (Stewart et al., 2005).

Post-translational histone modifications, such as acetylation, are reversible. By contrast, lysine methylation of histones was long thought to be irreversible. Recently, lysine-specific demethylase enzymes such as the lysine-specific demethylase 1 (LSD1) enzyme (Shi et al., 2004), the jumonji C (JmjC)-domain-containing histone demethylase 1 (JHDM1) (Tsukada et al., 2006) and JMJD2 family (Cloos et al., 2006; Whetstine et al., 2006) were identified. LSD1 and JHDM1 were shown to mediate demethylation of di- and monomethylated histones. JmjC-domain-containing members of the JMJD2 family efficiently reverse trimethylation (Cloos et al., 2006; Whetstine et al., 2006).

1.3.3.3 Lysine ubiquitination and sumoylation

Through proteosome-dependent degradation, the covalent modification of specific histone lysines by ubiquitin or a small ubiquitin-related modifier sumo plays a role in regulating transcription. Sumoylation can play a role in transcriptional repression (Shiio and Eisenman, 2003), while ubiquitination of lysine residue at histones H2A and H2B is regarded as a positive mediator of transcription (Zhang, 2003). Ubiquitination was also shown to be linked to lysine methylation (Daniel et al., 2004; Henry et al., 2003). The ubiquitin attachment involves E1 activation, E2 conjugation and E3 ligase enzymes. Bre1 was identified as E3 ligase for H2B ubiquitination (Wood et al., 2003). Ubiquitination is a reversible process and H2B was shown to be deubiquitinated by Ubp8 (Henry et al., 2003).

1.3.3.4 Arginine methylation

The catalytic module that methylates specific arginines is known as the PRMT (protein R methyltransferase) domain. It transfers the methyl group from SAM to the guanidine group of arginines to produce monomethylarginine and dimethylarginine (Zhang and Cheng, 2003). Methylation of specific arginines in histone H3 (R17 and R26) and H4R3 correlates with the active state of transcription. For example, methylated H4R3 facilitates H4 acetylation and enhances activation of transcription by a nuclear hormone receptor (Wang et al., 2001).

1.3.3.5 Serine phosphorylation

Phosphorylation of the histone serines can be established by several kinases such as Aurora B or PKA and reversed by the phosphatase 1 (PP1) family (Hsu et al., 2000). Phosphorylation of the histone H3 serine10 is the best characterized with regards to transcription (Clements et al., 2003). It correlates with mitosis and chromosome condensation but also with active transcription. It enhances H3K14 acetylation by additional interactions with HAT which then results in promotion of transcription. Phosphorylation may thus play a role in regulation of other modifications that occur adjacent to it.

1.3.4 Linker histone H1 and gene regulation

Another important component of chromatin structure is the linker histone H1. H1 plays an important structural and functional role in chromatin. The presence of bound H1 also has a strong inhibitory effect *in vitro* on nucleosome mobility (Pennings et al., 1994). It interferes with chromatin remodelling complex activities when present on the chromatin fibre (Horn et al., 2002). It is often depleted on active chromatin and can cause inhibition of transcription *in vitro* (Bresnick et al., 1992; Shimamura et al., 1989; Smith and Hager, 1997). It was therefore long considered as a global repressor of gene activity through its compaction of chromatin. The concept of H1 as a general repressor of chromatin activity was then challenged *in vivo*. In higher organisms, studies on H1 are complicated by the presence of several subtypes, encoded by separate genes. In mice, there are six somatic subtypes (H1a-e and $H1^0$), which differ in primary sequence and in relative abundance from tissue to tissue (Fan et al., 2003; Fan et al., 2005). In the mouse, deletion of one or two H1 subtypes results in a compensatory upregulation of other subtypes, resulting in the normal level of H1/nucleosome and no apparent phenotype (Fan et al., 2003; Fan et al., 2001; Sirotkin et al., 1995). Cells and tissues can tolerate very low levels of H1 and only a small percentage of genes are affected in their activity (Fan et al., 2003; Fan et al., 2005).

1.3.5 HMGN and gene regulation

Although removal of histone H1 leads to small changes in global transcription levels (Fan et al., 2005), there is evidence on the other hand that non-histone architectural proteins such as HMGN can modulate transcription (Bustin, 2001; West et al., 2004). HMGN (high mobility group N) proteins alter chromatin structure by unfolding chromatin and they are considered to be associated with actively transcribed genes. There are currently four members of the family, but details about their role in transcription remain mostly unknown mainly due to the fact that these proteins are only present in higher eukaryotes (West, 2004). $Hmgn1^{-/-}$ mice appear normal but the expression of some of their genes is altered (Birger et al., 2003). Recently, overexpression of two splice variants of another member of the HMGN family, HMGN3 indicated that HMGN may play a role in Glyt1 (glycine transporter 1) expression (West et al., 2004).

1.3.6 DNA methylation and its mediators

DNA methylation is a covalent modification of cytosine by the addition of a methyl group to the 5 position of the nucleotide ring. It appears that DNA and histone methylation have a cyclical and mutually reinforcing relationship, and both are required for stable and long-term epigenetic silencing (Cheung and Lau, 2005). Direct functional links between DNA and histone methylation have been uncovered. For example, in *Neurospora* and *Arabidopsis*, genetic evidence indicates that H3K9 methylation is a prerequisite for DNA methylation to occur (Tamaru and Selker, 2001). Loss of Suv39H1/2 in knockout mouse cells also altered the DNA methylation pattern of their pericentric heterochromatin (Lehnertz et al., 2003). On the other hand, examples of ablation of DNA methylation affecting H3 methylation and other histone modifications have also been found in *Arabidopsis* and human cells (Espada et al., 2004; Tariq et al., 2003). It is also connected to deacetylation by HDACs and binding of chromatin remodelling complexes and corepressors.

It is known that the presence of methylated CpG can interfere with binding of some transcription factors to their cognate sites as shown in Figure 1.3a (Tate and Bird, 1993). Exclusion of transcription factors and other proteins by DNA methylation of their cognate DNA binding sites can directly affect the transcription of the associated genes (Hark et al., 2000; Kim et al., 2003). Also, DNA methylation can directly influence the translational positioning of a nucleosome at specific DNA sequences *in vitro* and could lead to masking of essential regulatory elements by nucleosomes (Davey et al., 1997). The methyl group is thought to make the double helix more rigid, thereby restricting DNA affinity for the histone octamer, which results in an overall effect on the positioning of the nucleosome in the chromatin structure (Davey et al., 2004). DNA methylation also affects binding of linker histone H1. That has an effect on nucleosome structure, but not on chromatin compaction (Gilbert et al., 2007). In addition to these direct mechanisms of repression, there is now evidence for indirect repression mechanisms that are mediated by proteins that bind to methylated DNA (Figure 1.3b).

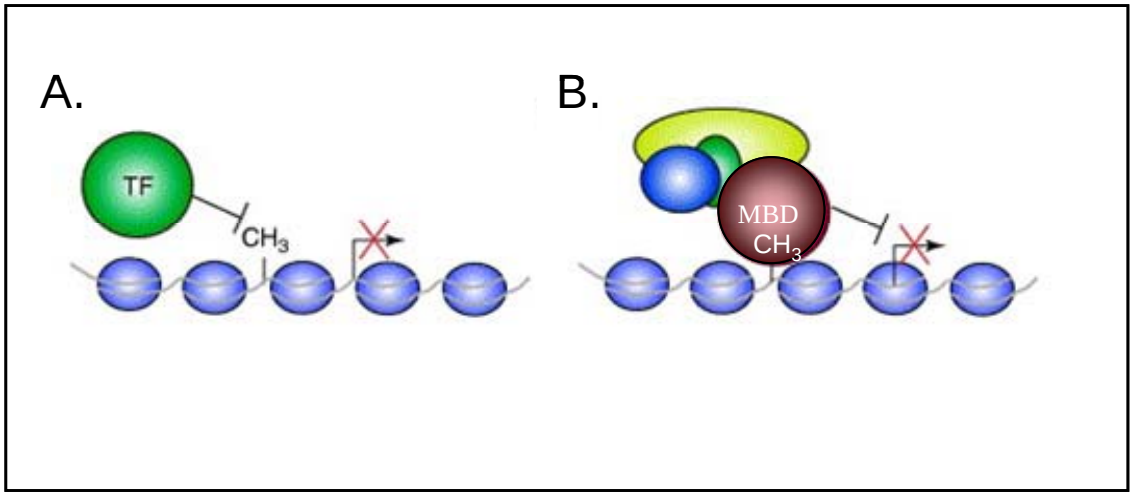


Figure 1-3 DNA methylation-mediated repression.
A. DNA methylation directly inhibits binding of some transcription factors. B. MBD proteins directly recognize methylated DNA and recruit corepressors and/or chromatin remodeling complexes to modify surrounding chromatin. (adapted from Klose and Bird, 2006).

1.3.6.1 MBD proteins

Following the initial demonstration that extracts of human cells contain proteins that bind to methylated DNA (Huang et al., 1984), and the discovery of the MeCP1 complex (Meehan et al., 1989), that has been shown to bind methylated promoters and repress transcription both *in vitro* and *in vivo* (Boyes and Bird, 1991), a number of proteins have been identified. MeCP2 was discovered in 1992 (Lewis et al., 1992; Meehan et al., 1992); MBD1 (formerly PCM1) was identified in 1997 by a search of DNA sequence databases. It shared similarity with MeCP2 in its MBD domain. It was shown to bind to methylated DNA and to repress transcription from methylated templates (Cross et al., 1997). In addition to MeCP2 and MBD1, three other proteins that contain a methyl-CpG binding domain were identified – MBD2, MBD3 and MBD4 (Figure 1.4) – and together with MBD1 further characterised (Hendrich and Bird, 1998). With the exceptions of MBD2 and MBD3, sequence similarity between the proteins is limited to the MBDs themselves. MBD2 and MBD3 show high conservation between human and murine genes (97.6 and 93.8% amino acid identity, respectively), whereas the human and murine homologues of MBD1 and MBD4 are less conserved (70.9 and 65.5%, respectively). All these genes produce alternatively spliced variants. MBD2 and MBD4 bind specifically to methylated DNA only and it appears to be independent of sequence context. Localisation studies of GFP-fused proteins showed that MBD1, MBD2, and MBD4 colocalised with major satellite DNA in mouse cells, but

localisation of MBD2 and MBD4 was disrupted in cells lacking a functional DNA methyltransferase gene. This suggested that MBD2 and MBD4 are capable of binding methylated DNA *in vivo* as well as *in vitro*. MBD1 was also capable of binding methylated DNA *in vivo,* but bound to the same heterochromatic sites in DNA methyltransferase-deficient ES cells (Hendrich and Bird, 1998)

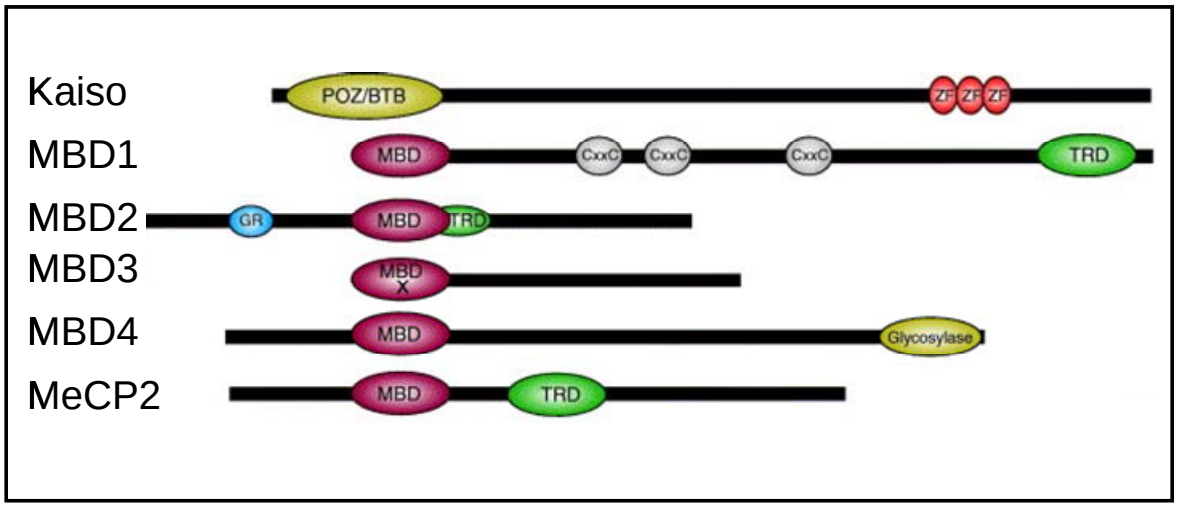


Figure 1-4 A family of MBD proteins. Six mammalian MBDs have been characterised so far. Kaiso is an atypical MBD, because it depends on a zinc-finger domain (ZF) to recognize methylated DNA and a POZ/BTB domain to repress transcription. MBD1 uses its methyl-binding domain (MBD) to bind methylated DNA sequences. In addition, MBD1 contains three zinc-binding domains (CxxC), one of which binds specifically to non-methylated CpG dinucleotides, and a C-terminal transcriptional repression domain (TRD). MBD2 possesses an MBD that overlaps with its TRD domain, and a GR repeat at its N terminus. MBD3 contains a well-conserved MBD domain that does not recognize methylated DNA owing to crucial amino acid changes. MBD4 binds methylated DNA through an MBD domain and has a C-terminal glycosylase domain that is important for its function in DNA repair. MeCP2 is the founding member of the MBD protein family and contains a conserved MBD domain and an adjacent TRD domain. (adapted from Klose and Bird, 2006).

1.3.6.2 MeCP2

MeCP2 binds preferentially to single symmetrically methylated CpG and was found to bind to chromosomes at sites known to contain methylated DNA through its methyl-CpG binding domain (MBD) (Nan et al., 1993; Nan et al., 1996). On mouse chromosomes, it binds prominently to the highly methylated major satellite located near centromeres (Lewis et al., 1992), whereas on human or rat chromosomes which do not contain highly methylated satellite DNAs, MeCP2 was broadly distributed throughout chromosome arms (Nan et al., 1997). DNA methylation is necessary for MeCP2 localisation as mutant cells with low levels of genomic DNA methylation show inefficient localisation of MeCP2 protein (Nan

et al., 1996). MeCP2 further contains a transcriptional repression domain (TRD) (Nan et al., 1997). Because of its broad distribution in the genome, MeCP2 was found to be a global transcriptional repressor of methylated DNA and this repression was dependent on the function of both MBD and TRD domains (Nan et al., 1997). MeCP2 has no difficulties accessing its target sequences in chromatin. When preassembled chromatin containing H1 linker histone was challenged with MeCP2, a specific loss of H1 detection was observed. This suggested that MeCP2 could displace histone H1 from chromatin in order to access its binding sites. The underlying mechanism of MeCP2 repression was found to be through the Sin3A/HDAC corepressor complex (Jones et al., 1998; Nan et al., 1998). The region of MeCP2 that localises with the TRD associates with mSin3A, HDAC1 and HDAC2, but mSin3A is the preferred binding partner and HDACs have much weaker affinity for MeCP2 . Transciptional repression *in vivo* is relieved by the deacetylase inhibitor trichostatin A (TSA), indicating that deacetylation of histones is an essential component of this repression mechanism. However, repression is not completely alleviated by TSA, indicating that a component of repression by the TRD may be deacetylase-independent, consistent with the observation that mSin3A retains some ability to repress transcription even in the absence of associated HDACs (Laherty et al., 1997).

However, only a small amount of mammalian MeCP2 interacts with Sin3A and this interaction is not stable (Klose and Bird, 2006). This suggests that MeCP2 is not an obligate component of the Sin3a corepressor complex and may therefore engage a more diverse range of cofactors for repressive function. On one of the most well-characterised neuronal-specific genes, NaCh II, MeCP2 forms a complex with co-REST (an associated corepressor of repressor element RE-1 silencing transcription factor) and is also associated with the HMT SUV39H1 (histone lysine methyltransferase, suppressor of variegation 39H1), further recruiting HP1 (Lunyak et al., 2002). HMT SUV39H1 methylates specifically H3K9 (Rea et al., 2000) and that recruits HP1 (heterochromatin protein 1), which mediates gene silencing (Bannister et al., 2001). MeCP2 was shown to direct H3K9 methylation *in vivo* through interaction with an unidentified H3K9-specific HMT (Fuks et al., 2003). MeCP2 also associates with Dnmt1, suggesting a mechanism for co-ordinated methylation and gene repression during DNA replication (Kimura and Shiota, 2003). As mentioned above, MeCP2 may also repress in a histone deacetylase-independent manner. Such repression activity

was originally identified in *in vitro* experiments in which naked DNA was incubated with MeCP2 and HeLa nuclear extracts (Nan et al., 1997). Since it may be anticipated that nucleosomes were not assembled under the assay conditions, it was speculated that transcriptional inhibition was achieved through the histone deacetylase-independent pathway. This pathway was later shown to be active on the SV40 enhancer/promoter and was not relieved by TSA (Yu et al., 2000). MeCP2 also interacts with the Brahma component of the SWI/SNF complex *in vivo* and is functionally linked to repression. MeCP2 and Brahma assembly occurs on genes methylated in cancer and the FMR1 gene in fragile X syndrome. This is the first time MeCP2 was shown to recruit a chromatin remodelling complex and use it for repression (Harikrishnan et al., 2005). MeCP2 was also shown to mediate chromatin compaction by forming complexes with nucleosomal arrays. By mutating MeCP2 sequence, it was shown that it is independent of its MBD, which led to the conclusion that MeCP2 compacts chromatin independently of DNA methylation (Georgel et al., 2003). This would be in agreement with data showing that chromatin secondary structure is not altered in the absence of DNA methylation, but it leads to altered binding of linker histone H1, independent of MeCP2 (Gilbert et al., 2007). Involvement of MeCP2 in maintaining compact chromatin secondary structure (Georgel et al., 2003) seems unlikely, given that MeCP2 binding was shown to be DNA methylation-dependent (Nan et al., 1996).

MeCP2 is required in neurons for normal brain function and mutation in the sequence causes Rett syndrome (Amir et al., 1999), a progressive neurological disorder that affects almost exclusively girls. Rett syndrome results from mosaic expression of mutant and wild-type MeCP2 alleles in the brain caused by the random inactivation of one X-linked MeCP2 allele during early female development. How MeCP2 inactivation causes Rett syndrome is not clearly understood. Most missence mutations in MeCP2 are tightly clustered at the methyl-CpG binding domain causing decreased binding to methylated DNA (Ballestar et al., 2000; Yusufzai and Wolffe, 2000). It implies that methyl-CpG binding by MeCP2 is essential for proper brain function. MeCP2 can also act as a transcriptional repressor and mutations in TRD are also very common in Rett syndrome (Ballestar et al., 2000; Yusufzai and Wolffe, 2000). Whether this function has relevance to Rett syndrome depends on identification of target genes in the brain. To better understand MeCP2 involvement in Rett syndrome,

several MeCP2 mouse models have been produced. MeCP2-null mice were prepared using *cre/lox* recombination (Chen et al., 2001; Guy et al., 2001). These mice had no apparent phenotype until 6 weeks old, when rapid regression started, leading to eventual death at ~8 weeks. Good parallels in character and the time of onset of symptoms have been found between female mouse heterozygous for the MeCP2-null allele and Rett syndrome patients (Guy et al., 2001). Mice with conditional expression of MeCP2 expressing *cre* under nestin (neuronal progenitors-specific) promoter were then produced (Chen et al., 2001; Guy et al., 2001). Mice with nestin-cre MeCP2 conditional mutation showed the same phenotype. It implied that the MeCP2 mutation in the brain is sufficient to produce the same phenotype as the MeCP2-null mice. Recently, $MeCP2^{lox\text{-}Stop,cre}$ animals were produced (Guy et al., 2007). They have MeCP2 inactivated by insertion of *lox-Stop* cassette, but it can be conditionally activated under the control of its own promoter and regulatory elements by cassette deletion using the cre-ER/TM system (Guy et al., 2007). Female MeCP2 $^{Stop/+,cre}$ mice behaved similarly to $MeCP2^{+/-}$ female mice. Upon treatment with TM (tomofixen) resulting in MeCP2 activation, *Stop/+,cre* females with clear neurological phenotype progressively reverted to a phenotype close to a wild type. The fact that viable but defective neurons can be repaired is a very important discovery as Rett syndrome patients show abnormal neuronal morphology, but not neuronal death (Armstrong et al., 1995).

1.3.6.3 MBD1

Similar to MeCP2, MBD1 is an abundant chromosomal protein (Ng et al., 2000). MBD1 binds to methylated DNA and, possessing a TRD domain, it can actively repress gene transcription (Ng et al., 2000). Transcriptional repression is dependent on both MBD and TRD domains and on deacetylation, as TSA treatment restores transcription to over 75% of control levels. However, it is not depleted by antibodies to the histone deacetylase HDAC1 like MeCP2. Thus, the deacetylase-dependant pathway by which MBD1 actively silences methylated genes is likely to be different from MeCP1 and MeCP2 (Ng et al., 2000). Uniquely among MBD proteins, it was shown that a major MBD1 isoform also contains the CXXC-3 domain (Figure 1.4) that binds specifically to nonmethylated CpG dinucleotides (Jorgensen et al., 2004). The MBD1 protein therefore makes use of two distinct DNA binding domains to target CpGs, the MBD requiring methylated

CpG and the CXXC-3 domain requiring nonmethylated CpG. Although only some MBD1 isoforms carry the CXXC-3 DNA binding domain, this new finding affects the previous view of MBD1 as a protein that exclusively interprets the DNA methylation signal. The biological significance of the dual DNA binding capacity is currently unknown (Jorgensen et al., 2004). MBD1 interacts with two HMTs, SUV39h1 and SETDB1 (histone H3 K9 specific methyltransferases) and with p150, a component of the chromatin assembly factor-1 (CAF-1) (Fujita et al., 2003a; Reese et al., 2003; Sarraf and Stancheva, 2004). MBD1 association with CAF-1 and SETDB1 at replication foci appears to facilitate H3K9 tri-methylation before histones are loaded onto DNA, indicating that the H3K9 methylation is maintained simultaneously with DNA methylation via the replication-coupled CAF-1/MBD1/SETDB1 complex (Sarraf and Stancheva, 2004). MBD1-containing chromatin associated factor (MCAF) and methylpurine-DNA glycolase (MBP) interact with the TRD of MBD1 and repress transcription of reporter genes *in vivo* (Fujita et al., 2003a; Watanabe et al., 2003).

1.3.6.4 MBD2 and MBD3

Mammalian MBD2 is a methyl-CpG binding protein, but mammalian MBD3 is not (Hendrich and Bird, 1998). MBD3 behaves differently than the other MBD proteins, failing to specifically bind methylated DNA *in vitro* or colocalize with major satellite *in vivo* (Hendrich and Bird, 1998).

MBD3 was identified as one of the seven subunits of the second most prominent histone deacetylase multisubunit complex, the Mi-2/NuRD (the nucleosome-stimulated ATPase Mi-2/nucleosome remodelling histone deacetylase) complex. NuRD complex is composed of the SWI2/SNF2 helicase/ATPase domain-containing Mi2 protein, the two histone deacetylases HDAC1 and HDAC2, the two histone-binding proteins RbAp46 and RbAp48 (Zhang et al., 1998), and polypeptides of 70 and 32 kD later identified as metastasis-associated proteins MTA2 and MBD3, respectively (Zhang et al., 1999). MBD3b is the major splice form of MBD3 in the complex and it only contains a portion of the methyl-CpG binding domain. It is embedded within the NuRD complex and it does not bind methylated DNA. It however mediates the association of MTA2 with the core histone deacetylase complex. Thus, the function of the NuRD complex might not depend on an intrinsic DNA-binding subunit, but upon recruitment by other DNA-

binding proteins. Mammalian MBD2 shows a strong preference for binding to methylated DNA *in vitro* and *in vivo* (Hendrich and Bird, 1998). It has been shown that in HeLa cells MBD2 associates with HDAC and can repress transcription in a TSA-dependant manner (Ng et al., 1999). MBD2 is not part of the NuRD complex but it was found to interact with it (Zhang et al., 1999). The addition of NuRD to an *in vitro* methylated DNA-binding assay containing MBD2 resulted in the production of a DNA-protein complex migrating slower than the MBD2-DNA protein complex confirming that MBD2 tethers NuRD complex to methylated DNA, raising the possibility that MBD2 might recruit the NuRD complex to methylated DNA *in vivo* (Zhang et al., 1999). More recent data, however, suggested that MBD2/NuRD and MBD3/NuRD are distinct protein complexes with different biochemical and functional properties (Le Guezennec et al., 2006).

When the MeCP1 complex discovered in 1989 (Meehan et al., 1989) was characterised in more detail, MBD2 was found to be part of it (Ng et al., 1999), rather then MBD1 as previously reported (Cross et al., 1997). It was probably due to cross-reacting of anti-MBD1 antibody with MBD proteins unknown at the time. MeCP1 methylation-mediated repressing activity was found to be due to a complex containing 10 major polypeptides including MBD2 and all of the known NuRD components, including MBD3. MBD2 associates here with the NuRD in MeCP1 *in vivo* and therefore it probably targets the nucleosome remodeling and histone deacetylase NuRD complex to methylated DNA (Feng and Zhang, 2001). MBD2 was also shown to interact with the Sin3A complex (Boeke et al., 2000).

MBD2 and MBD3 were also shown to interact with two members of the p66 protein family, which are novel proteins involved in transcriptional repression (Brackertz et al., 2002). Dnmt1 has also been identified in a complex that contains both MBD2 and MBD3, binds to hemimethylated DNA and is located at the replication focus during late S phase, suggesting that MBD2 and MBD3 may act to repress transcription of newly synthesized DNA (Tatematsu et al., 2000). The genes for both MBD2 and MBD3 have been deleted in mice (Hendrich et al., 2001) and Mbd3 knockout mice die during early embryogenesis, whilst Mbd2 knockout mice appear to be largely normal except for defective maternal behaviour.

1.3.6.5 MBD4

MBD4 is the only member of the MBD family of proteins that does not appear to be involved in transcriptional repression (Hendrich and Bird, 1998). Apart from the MBD domain, it has a glycosylase domain and it was shown to have a G/T mismatch glycosylase activity, as well as 5-methylcytosine DNA glycosylase activity (Hendrich et al., 1999), so it is possibly involved in DNA demethylation (see later).

1.3.6.6 Kaiso

Kaiso protein is a more recent addition to the family of methyl-CpG binding domain proteins. It is unrelated in structure to other MBD proteins (Figure 1.4). It belongs to the BTB/POZ family of zinc finger proteins (Prokhorchuk et al., 2001). *In vitro*, Kaiso recognizes DNA sequences that contain at least two methyl-CpGs, and represses transcription from reporter templates in a methyl-CpG-dependent manner (Prokhortchouk et al., 2001). *In vivo*, it is required to maintain DNA-methylation dependent transcription silencing during early *Xenopus laevis* development. Developmental arrest and apoptosis can be rescued by injection of human Kaiso mRNA into the embryos (Ruzov et al., 2004). Kaiso was found to reduce the enhancer-blocking activity of CTCF (Defossez et al., 2005). CTC-binding factor (CTCF) is a DNA-binding protein of vertebrates that plays essential roles in regulating genome activity through its capacity to act as an enhancer blocker (Ohlsson et al., 2001). Insulators are DNA elements that maintain partition between transcriptionally active and inactive chromatin, and they can be subdivided into two functional classes: barrier elements, which stop the spread of heterochromatin, and enhancer blockers, which prevent an enhancer from activating transcription in a neighbouring repressed region (West et al., 2002). These data suggest that the Kaiso-CTCF interaction negatively regulates CTCF insulator activity (Defossez et al., 2005).

1.3.6.7 DNA methyltransferases

In mammalian genomes, DNA methylation is found predominantly in the context of CpG dinucleotides. In fact, 70% of all CpG dinucleotides are methylated in mammals (Fraga et al., 2003), with the exception of CpG islands, which are CG-rich regions mostly coincident with the promoter of protein-coding genes. The human genome is predicted to contain around 29,000 CpG islands (Lander et al., 2001). The mechanism(s) by which CpG islands escape methylation when the vast majority of CpGs are targeted for methylation are not yet clear, but one possibility is that local chromatin structure may exclude the methylation machinery.

DNA methylation is essential for normal development and to faithfully maintain genome function in adult cells. Patterns of DNA methylation are highly dynamic during mammalian development, during which the epigenome is reprogrammed with the erasure of genome-wide methylation so that cell- or tissue-specific methylation patterns can be established *de novo* (Dean et al., 2003; Okano et al., 1999; Santos et al., 2002).

As a consequence of the dynamic state of DNA methylation, two different methylation processes occur: *De novo* methylation establishes the methylation state; maintenance methylation copies it onto daughter DNA strands after DNA replication. To date, five mammalian DNMTs have been identified (Hermann et al., 2004).

Dnmt1 is the most abundant methyltransferase in mammalian cells and is essential for genomic stability. It is believed to be the primary maintenance DNA methyltransferase as it normally restores DNA methylation to symmetrical CpG nucleotides in a semi-conservative manner during or shortly after DNA replication (Yoder et al., 1997). During mammalian development, DNA demethylation of the maternal genome during preimplantation results as a consequence of the exclusion of Dnmt1 from the nucleus (Carlson et al., 1992).

The role of Dnmt2 in establishing or maintaining the epigenome is not yet clear; it has no detectable methyltransferase activity *in vitro* (Okano et al., 1998).

Dnmt3a and Dnmt3b function as *de novo* methyltransferases and play distinct roles in establishing methylation patterns during embryonic development. Deletion of Dnmt3b results in multiple developmental defects in the mouse, including substantial hypomethylation of centromeric minor repeats, and Dnmt3a null mice are viable but die at about four weeks of age (Okano et al., 1999). Deletion of both Dnmt3a and Dnmt3b in the mouse yields a more severe phenotype than either single mutant with a reduction in global methylation (Okano et al., 1999). Studies of deletions of DNA methyltransferases showed that only Dnmt1 mutants manifest marked loss of genomic cytosine methylation (Li et al., 1992; Okano et al., 1999; Okano et al., 1998). Although Dnmt3^{-} ES cells showed only partial loss of DNA methylation at the time (Okano et al., 1999), during prolonged culturing virtually no DNA methylation remained (0.6%), possibly due to Dnmt1 failure to maintain the DNA methylation levels (Gilbert et al., 2007).

Dnmt3L is a DNA methyltransferase 3-like protein, which lacks the catalytic motifs that characterize the DNA cytosine-5-methyltransferases but is related to the active methyltransferases Dnmt3a and Dnmt3b in framework regions (Aapola et al., 2000). Deletion of Dnmt3L does not prevent oogenesis, but the heterozygous offspring of homozygous mutant females die before mid-gestation as a result of biallelic expression of imprinted genes normally methylated and silenced on the allele of maternal origin. Dnmt3L was shown to collaborate with Dnmt3a to generate genomic methylation patterns on maternal imprinted genes in oocytes. However, male mice that lack Dnmt3L are viable but sterile, with a complete absence of germ cells in adult males (Bourc'his et al., 2001). It was shown using Dnmt3L-deficient male germ cells that Dnmt3L is involved in de novo methylation of dispersed and tandem repeated sequences, and not imprinted genes. It is specifically required for de novo methylation and heritable silencing of interspersed repeated sequences in a brief perinatal period in the non-dividing precursors of spermatogonial stem cells. In Dnmt3L-deficient spermatocytes they remain unmethylated and transcribed at high levels. Loss of Dnmt3L from early germ cells also causes meiotic failure in spermatocytes, which do not express Dnmt3L. This result indicates that the Dnmt3L protein might have a function in the de novo methylation of dispersed repeated sequences in a premeiotic genome scanning process that occurs in male germ cells (Bourc'his and Bestor, 2004). In addition, Dnmt3L appears to play a

supporting role in stimulating methylation activities of Dnmt3a and Dnmt3b in vitro and these directly interact with the N-terminus of Dnmt1 and all three latter enzymes co-operate to establish and maintain methylation patterns throughout the genome (Kim et al., 2002; Liang et al., 2002).

1.3.6.8 DNA demethylation

DNA demethylation is associated with the erasure and subsequent resetting of imprinted marks in the development of gametes, the paternal pronucleus during fertilisation and the preimplantation period. Asynchronous demethylation of the paternal genomes occurs rapidly after fertilisation and it is believed to be an active process as it occurs in the absence of DNA replication.

Several attempts have been made to identify and characterise the mechanisms of demethylation and two distinct ways of demethylation have been described. First is a passive demethylation as a result of the absence of DNA methyltransferases maintaining DNA methylation during DNA replication (Matsuo et al., 1998); the second is an active demethylation (Reik et al., 2001).

MBD2b was identified as an active demethylase by some (Bhattacharya et al., 1999; Detich et al., 2002), but this was not seen by other researchers (Boeke et al., 2000; Ng et al., 1999). Against MBD2b activity as a demethylase is also the fact that mutant mice that have MBD2 with an inactive MBD domain showed the same demethylation profile as wild type mice (Santos et al., 2002). In *Arabidopsis*, ROS1 protein has been shown to have glycosylase activity *in vivo* and mutations in its sequence resulted in hypermethylation and transcriptional silencing of specific genes (Kapoor et al., 2005). The recombinant protein had glycosylase activity only on methylated templates, bringing evidence that a base excision mechanism is involved in active demethylation (Kapoor et al., 2005). DNA break and repair involvement in demethylation has since been reported in mammalian cells (Kress et al., 2006). *In vitro,* thymine excision activities of two glycosylases, TDG (thymine DNA glycosylase) and MBD4, have been proposed to be compatible with the cleavage products detected (Zhu et al., 2000).

Recently, DNA methyltransferases Dnmt3a and Dnmt3b were shown to be able to demethylate as well as methylate CpGs via deamination (Metivier et al., 2008). Both Dnmt3a and DNMT3b are able to deaminate cytosine and methylated

cytosine *in vitro*. It was proposed that T:G mismatches generated by deamination of methylated cytosine could then be removed through base excision repair of MBD4 and TDG. General abasic sites could then be repaired by activities of endonucleases, polymerases and DNA ligases (Waters et al., 1999). $MBD4^{-/-}$ mice, however, do not show any developmental defects suggestive of perturbed DNA demethylation, but rather show an increase in frequency of C:T transitions, consistent with a role of MBD4 in DNA repair mainly (Millar et al., 2002). Dnmt3a and TDG were shown to associate and influence each other *in vitro* (Li et al., 2007) and the involvement of TDG in DNA repair after deamination is currently favoured (Metivier et al., 2008). As DNA strand breaks would bear the risk of damaging genome integrity, these mechanisms of active demethylation must be tightly regulated and understanding of these processes is necessary for understanding DNA methylation not only as a stable epigenetic mark but also as an integral component of transcription.

1.3.7 RNAi

RNA interference (RNAi) or RNA silencing is a regulatory mechanism mediated by short RNAs called miRNA and siRNA. miRNAs and siRNAs are ~21-26-nucleotide (nt) RNA molecules. Although both types of molecules can be functionally equivalent and interchangeable, they are distinguished by their mode of biogenesis (Carmell and Hannon, 2004). miRNAs are produced from transcripts that form stem-loop structures. These are processed in the nucleus by a complex comprised of at least two components: the RNase III enzyme Drosha, and a protein called Pasha in *Drosophila* or DGCR8 in mammals (Landthaler et al., 2004). Initial cleavage is followed by transport to the cytoplasm of about 70-nt pre-miRNA, which is further processed by the cytoplasmic RNase III endonuclease Dicer complex (Provost et al., 2002). Final processing by Dicer appears coupled to assembly of the miRNA into the RNA-induced silencing complex (RISC), which is the effector of RNAi (Gregory et al., 2005). In contrast, siRNAs are produced from long double-stranded RNA (dsRNA) precursors, which can be either endogenously produced or exogenously provided. Processing of siRNAs is also Dicer-dependent (Provost et al., 2002). Because both miRNA and siRNA are found in the same complex (RISC), they are thought to be interchangeable and this complex can mediate both cleavage and translational inhibition of target mRNA (Mourelatos et al., 2002; Zeng et al., 2003). The key component of the RISC

complex is an Argonaute protein. Argonaute proteins directly interact with the miRNA/siRNA through a PAZ domain (Ma et al., 2004; Song et al., 2003). The Argonaute protein family of mammalian argonaute proteins is diverse, with only Argonaute 2 (Ago2) being capable of mRNA cleavage (Liu et al., 2004).

1.3.7.1 RNA induced posttranscriptional gene silencing

The first manner in which miRNAs and siRNAs control gene expression is post-transcriptional, by directing endonuclease cleavage of the target mRNA by RISC complex. Studies indicate that the minimal composition of the RISC complex is the miRNA/siRNA and Ago2 (Liu et al., 2004). The products of the cleavage appear to be degraded by the same mechanisms as bulk mRNA involving decapping and deadenylation (Yamashita et al., 2005). miRNA can also target mRNAs for decapping directly without involvement of the RISC complex. Several activators of decapping are concentrated in cytoplasmic processing bodies (P-bodies) and mammalian argonaute proteins were shown to concentrate there too. Reporter mRNAs that are targeted for translational repression by endogenous or exogenous miRNAs become concentrated in P-bodies in a miRNA-dependent manner (Liu et al., 2005). A third way that miRNAs silences mRNAs posttranscriptionally is by interfering with their translation. It was revealed by multiple examples, where silencing by miRNAs resulted in no change or change smaller than was observed at the protein level (Pillai et al., 2005; Zeng et al., 2003). Argonaute proteins were again shown to be involved (Pillai et al., 2005). However, other Argonaute proteins than argonaute2 must be involved, as translational repression in response to miRNAs remains intact in Ago2-null cells (Liu et al., 2004).

Post-transcriptional gene silencing achieves specificity through RNA-RNA sequence recognition and base pairing. However, RNA can also form base pairs with DNA and through RNA interference affect gene expression at the level of genomic DNA (see below).

1.3.7.2 RNA-derived DNA and/or histone methylation and transcriptional gene silencing

RNA-directed DNA methylation was originally discovered with a viroid system in plants (Wassenegger et al., 1994). A link to RNAi was reinforced when it was

shown that the process requires a dsRNA processed into small RNAs, that, when homologous to promoter regions, trigger promoter methylation and transcriptional gene silencing (Mette et al., 2000). RNA directed DNA methylation in *Arabidopsis* starts by dsRNA trigger and site specific *de novo* methylation of cytosine that can occur in all cytosines, not just in CG dinucleotides (Aufsatz et al., 2002a). Unlike RNAi-based heterochromatin which can spread over several kilobases from the RNA-targeted DNA sequence (Hall et al., 2002), RNA-directed *de novo* DNA methylation is largely confined to the target region with only the homologous DNA sequence becoming methylated (Aufsatz et al., 2002a). The maintenance of DNA methylation and transcriptional gene silencing requires MET1 DNA methyltransferase (Aufsatz et al., 2004) which cooperates with SNF2-like chromatin remodelling protein, DDM1 (Lippman et al., 2004) and histone deacetylase HDA6 (Aufsatz et al., 2002b). Other DNA methyltransferases such as DRM2 have been implicated in maintaining DNA methylation (Cao et al., 2003). RNA-directed histone H3K9 methylation was shown to require SUVH4 HMT (Jackson et al., 2002). By experimental mutations, other components were found necessary for RNA-directed histone and DNA methylation in plants, such as Argonaute 4 (Zilberman et al., 2003), Dicer-like 3 (DCL3), RNA-dependent RNA polymerase 2 (RDR2) and silencing-defective 4 (sde4) (Chan et al., 2004), linking it further to RNAi. Whereas Argonaute 4 seems to have a role specifically in transcriptional gene silencing where its mutation correlates with the loss of H3K9me2 and transcriptional gene silencing (Xie et al., 2004; Zilberman et al., 2003; Zilberman et al., 2004), Argonaute1 can contribute to both posttranscriptional and transcriptional silencing (Kim et al., 2006; Vaucheret et al., 2004).

However, RNAi-mediated transcriptional gene silencing does not necessarily involve DNA methylation. RNAi was also described to be connected with assembly of heterochromatin in fission yeast (Volpe et al., 2002), where there is no endogenous DNA methylation (Antequera et al., 1984). In contrast to *Arabidopsis*, it shows that transcriptional silencing can be achieved without DNA methylation. Here, transcription of the two strands of the centromeric repeats generates dsRNAs. Dicer protein then cleaves dsRNAs to small RNAs that guide the HMT Clr4 to the respective site on the chromosome resulting in H3K9 methylation and recruitment of HP1 homologue Swi6 resulting in silencing (Bannister et al., 2001; Volpe et al., 2002). Deletion of components of the RNAi

pathway such as Argonaute1, Dicer or RNA-dependent RNA polymerase disrupts heterochromatin-mediated silencing which correlates with loss of H3K9 methylation and Swi6 association (Hall et al., 2002; Volpe et al., 2002). An RNAi effector complex called RITS complex (RNA-induced initiation of transcriptional gene silencing) is then tether to silenced loci by H3K9 methylation. This tethering results in generating more siRNAs from the locus and promotes the RNAi-derived heterochromatin maintenance in a self-enforcing loop mechanism (Noma et al., 2004).

1.3.7.3 RNA-directed DNA and histone methylation in mammals

Whether transcriptional gene silencing in mammals involves RNA-directed DNA methylation like in plants, or is established without it remains to be determined. Recent observations revealed that target genes might be silenced by RNA-associated silencing in mammalian cells at the level of the chromatin (Kim et al., 2006; Morris et al., 2004; Ting et al., 2008). siRNA were shown to mediated transcriptional repression that includes DNA methylation of the silenced gene (Kawasaki and Taira, 2004; Morris et al., 2004). The silencing is abolished by the addition of 5-azacytidine and TSA (Morris et al., 2004) or by suppression of Dnmt1 and Dnmt3b expression (Kawasaki and Taira, 2004). There is also an induction of H3K9 methylation (Kawasaki and Taira, 2004) The precise mechanism is not known (Kawasaki and Taira, 2004; Morris et al., 2004).

However, high controversy surrounds RNA-directed DNA methylation. Kawasaki's paper (Kawasaki and Taira, 2004) has been retracted together with other papers from the lab, and there has not been much work published in following years. Moreover, other papers ruled out involvement of DNA methylation in RNAi-directed silencing (Park et al., 2004; Ting et al., 2005); silencing of dsRNA-targeted promoter was achieved even in HCT DKO cells, which have less than 5% genomic methylation and lack the capacity to methylate DNA (Ting et al., 2005). This was consistent with data from *S. pombe*, in which silencing effects and heterochromatin formation induced by dsRNAs were achieved in the absence of DNA methylation (Volpe et al., 2003; Volpe et al., 2002).

So is there at least a potential for RNA-directed DNA and histone methylation transcriptional gene silencing in mammals? There are two issues. Firstly, do mammals have the necessary components for this pathway? Some components have mammalian homologues or closely related counterparts. DNA methyltransferases Dnmt1 and Dnmt3a/3b are mammalian homologues of Met1 and DRM2, respectively (Goll and Bestor, 2005). DDM1 which cooperates with Met1 (Lippman et al., 2004) has a mammalian homologue, the lymphoid specific helicase (LSH)(Dennis et al., 2001). Another SNF2 like protein DRD1 is related to mammalian ATRX SNF2 subfamily (Gibbons et al., 2000). DICER and Argonaute1 are also present in mammalian cells (Kim et al., 2006; Ting et al., 2008). So, it seems like mammals have much of the machinery necessary for RNA directed DNA methylation. Secondly, do trigger RNAs exist in the nucleus? RNA-directed DNA methylation in plants clearly takes place in nucleus. dsRNAs corresponding to repetitive elements have been detected in the hnRNA fraction in the nucleus of mammalian cells (Kumar and Carmichael, 1998). However, while plant's DCL3 (Dicer-like 3) localises to nucleus, human DICER is cytoplasmic (Billy et al., 2001). Whether there is a mechanism to produce small RNAs from dsRNAs in the nucleus or whether small RNAs could translocate to nucleus is currently not known. Certainly mature miRNAs were shown to be present both in nuclear and cytoplasmic cellular fractions (Meister et al., 2004).

RNAi-dependent DNA methylation therefore remains a vital and controversial issue. Further work from Morris' group brought more evidence that histone methylation is connected with RNAi-derived transcriptional silencing. siRNA-targeted promoters were shown to establish H3K9me2 and H3K27me3 (Weinberg et al., 2006), and follow-up study showed that both histone methylation modifications are mediated via Argonaute1 and its recruitment of HMT activity (Kim et al., 2006). It was also shown there that only negligible amounts of DNA methylation were detected at the siRNA-targeted promoters (Kim et al., 2006). Recently, however, DICER was identified to be important for maintaining aberrant hypermethylation of selected genes in HCT116 cells (Ting et al., 2008). Because DICER null cells are not viable (Bernstein et al., 2003), Dicer helicase domain knock out HCT116 cells were used for the study. These Dicer^{ex5} cells were previously shown to have defects in miRNA processing (Cummins et al., 2006), but observed selective demethylation of some genes was surprising. Control experiments showed that for the identified genes, promoter

hypermethylation and transcriptional silencing require DICER to maintain the hypermethylated status in these cells. The only well-defined role for DICER is to process dsRNAs therefore it suggests that RNA molecules may be involved in this hypermethylating and silencing pathway.

The evidence available so far suggests following putative mechanisms for RNA-mediated transcriptional gene silencing suggested from the initial work (Figure 1.5). Firstly, the nuclear delivered siRNAs might bind to an argonaute such as Argonaute1 or 2, possibly involving HDACs as siRNA-mediated transcriptional silencing was shown to be TSA sensitive (Morris et al., 2004). The siRNA/Ago complex then searches until a match is made between the antisense strand of siRNA and the target promoter region. Then it recruits HDACs. The HDACs activity then leads to nucleosomal compaction which may or may not be marked with DNA methylation and/or histone methylation (Figure 1.5a). Alternatively, the siRNAs may complex with HP1 and SUV39H1. HP1 contains a hinge region that has been found to exhibit RNA binding activity (Muchardt et al., 2002). Also Dnmt3a was shown to bind siRNA (Jeffery and Nakielny, 2004). The siRNAs may then function to direct the chromatin remodelling complexes which are known to interact with HDACs, such as NuRD, to the target gene resulting in histone deacetylation, DNA methylation, and silencing (Figure 1.5b). A third scenario could be that siRNAs somehow affiliate with a chromatin remodelling complex like Sin3, subsequently facilitating DNA methylation of the target gene and recruitment of the Sin3, possibly via MBD proteins. Both MeCP2 and MBD2 were shown to interact with RNA through their RG repeats region with high affinity (Jeffery and Nakielny, 2004)(Figure1.5c). Alternatively, chromatin remodelling complexes could be recruited via argonaute/siRNA complex rather than siRNA on its own (Figure 1.5d). Or, a putative transcriptional silencing complex can be guided to the targeted promoter by promoter associated RNA. That may be recognised by the antisense strand of the siRNA or possibly endogenous antisense RNAs as it was recently described by Morris et al. (Han et al., 2007).

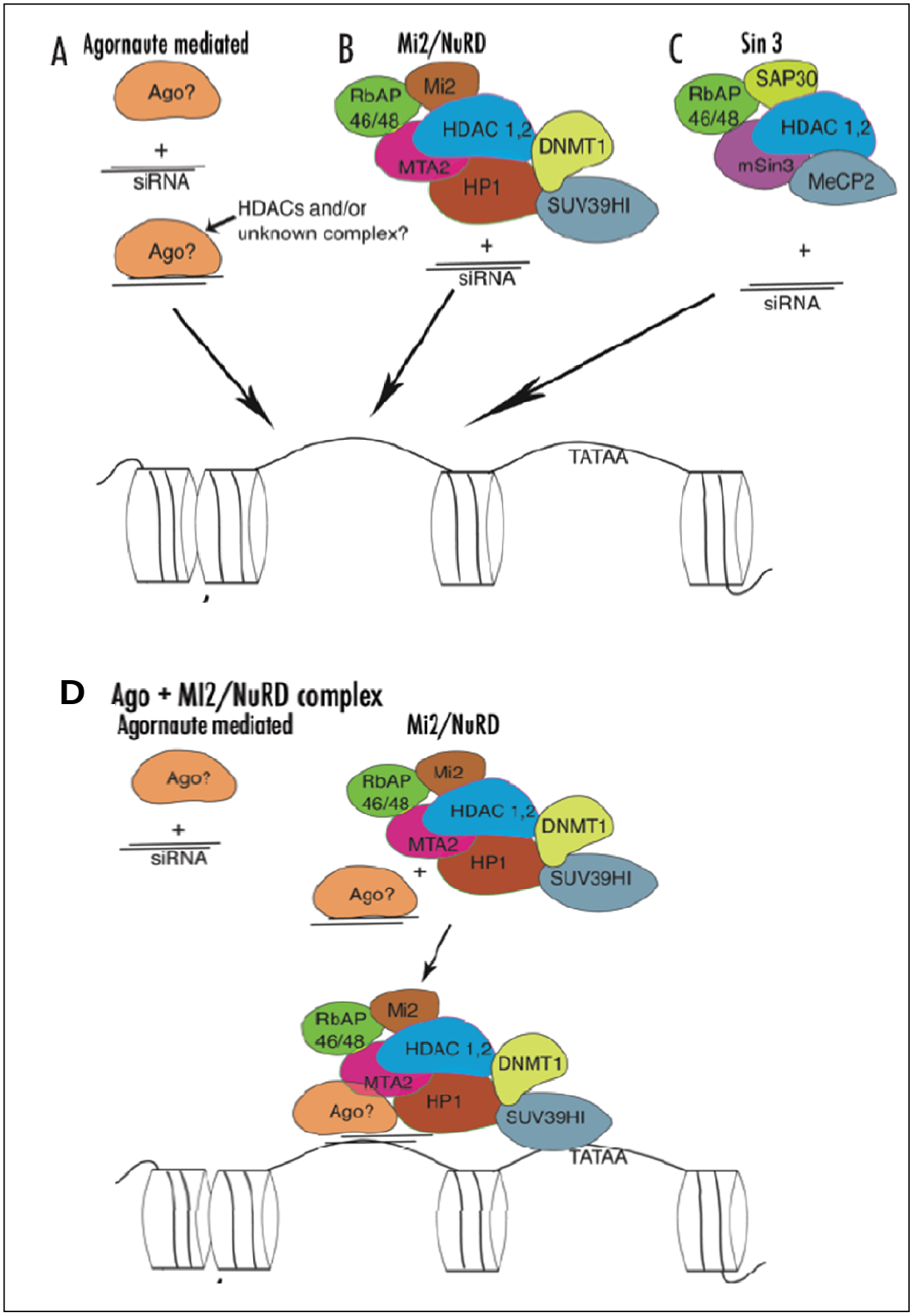


Figure 1-5 Putative mechanisms for siRNA mediated transcriptional silencing. Four scenarios are presented here. (A) Silencing via siRNA/argonaute complex. (B) siRNA might bind through HP1 and direct the HP1 and its cognate complex to a target gene. (C) siRNA somehow affiliates with chromatin remodeling complexes interacting with MBD proteins. (D) Involvement of multiple complexes including siRNA/argonaute and chromatin remodeling and corepressor complexes such as NuRD. (adapted from (Kawasaki et al., 2005)

1.3.8 The effect of chromatin and DNA methylation on RNA polymerase III transcription

DNA methylation appears to affect only specific categories of class III genes. Apart from Alus, template methylation can repress RNA polymerase III transcription of tRNA and VA *in vitro* and in the case of VA1 also in transfected cells (Juttermann et al., 1991; Kochanek et al., 1993; Liu and Schmid, 1993). It can also inhibit tRNA genes if injected into *Xenopus* oocytes, but a 5S rRNA gene are unaffected (Besser et al., 1990).

DNA methylation-mediated silencing has long been investigated as a possible cause of SINE's transcriptional repression. Alu sequences are CpG rich and these are highly methylated in the mammalian genome (Fraga et al., 2003). In fact, more than 90% of methylated CpG dinucleotides in the human genome occur in retrotransposons (Bird, 2002). Association of H3K9 methylation, which is a marker of heterochromatin and is specifically associated with inactivation of gene expression (Lachner and Jenuwein, 2002; Lachner et al., 2001), was demonstrated with Alu elements (Kondo and Issa, 2003), suggesting that H3K9 methylation may be related to the suppression of Alu elements through DNA methylation.

DNA methylation was shown to be connected with Alu transcriptional silencing both *in vitro* and *in vivo* (Li et al., 2000; Liu et al., 1994; Liu and Schmid, 1993). HeLa cells were treated with 5-azacytidine to demethylate DNA and the DNA demethylation verified using enzymatic methyl-sensitive restriction digests. Alu transcripts were then detected using primer extension and northern blot. Treatment with 5-azacytidine resulted in a 5- to 8-fold increase in full length Alu transcript expression, while the effect on scAlu RNA (left monomer only Alu) was much less pronounced. Also, methylated and unmethylated Alu constructs were transfected into human cells (Li et al., 2000). Depending on the amount of transfected methylated template, methylated constructs were 9- to 20-fold less active.

However, some silenced Alus when demethylated with 5-azacytidine remained inactive (Liu et al., 1995), suggesting that the regulation is more complex. Alu

elements were shown to be the main target sequences for MeCP2 binding *in vivo* (Koch and Stratling, 2004). However, MeCP2-mediated repression of Alu elements was not so far demonstrated. In transient transfection assays, MeCP2 had no inhibiting effect on the AluSx reporter construct, although it was shown to repress an L1 reporter construct in the same assay (Yu et al., 2001). The mechanisms of DNA methylation-mediated SINE repression remain unknown.

The effect of DNA methylation and chromatin on SINE expression was mainly studied in cell stress conditions where SINE expression was shown to be upregulated (see below).

There is an increase in SINE RNA levels in viral infections such as adenovirus infection (Li et al., 2000; Panning and Smiley, 1995; Russanova et al., 1995). Viral infections and transformation can increase RNA polymerase III transcription through deregulation of transcription factors and their regulators as it was described in section 1.2.4. However, studies suggested that this upregulation can be global, not specific to RNA polymerase III activity (Li et al., 2000; Russanova et al., 1995). Treatments with other agents which lead to cell stress response were shown to increase SINE expression. Heat shock, ethanol and cycloheximide treatment greatly induces levels of B1,B2 and Alu RNA (Fornace et al., 1989; Fornace and Mitchell, 1986; Liu et al., 1995). While the basal and induced levels of B2 elements are higher than B1, the kinetics of their response is essentially coincident (Liu et al., 1995). SINE function in these situations was described in section 1.1.2. Because many SINE members are transcribed, it was believed to be achieved via a global repressive mechanism; DNA methylation was considered.

DNA methylation-mediated repression of SINEs was studied during cell stress conditions in three cell lines, K562, HeLa and 293. K562 cells have naturally low levels of endogenous DNA methylation (Li et al., 2000). There is a 5-fold increase in Alu RNA levels in K562 cells compared to HeLa or 293 cells. However, when K562 and HeLa cells were infected with adenovirus 2, treated with cycloheximide or heat-shocked, there was very little effect on Alu RNA levels in K562 cells compared to HeLa cells. That these differences were not caused by different levels of methylation was shown by studying activities of methylated and unmethylated constructs transfected into K562 cells. Exposure of K562 cells

containing the constructs to heat shock or cycloheximide increase expression from both templates only ~ 2- to 3-fold. Taken together, this demonstrated that cell stress does not relieve the methylation-mediated repression in K562 cells. Alus activity therefore can be increased via cell stress leaving DNA methylation levels constant.

In vitro transcription assays performed using nuclei from infected and uninfected HeLa cells incubated with uninfected extracts revealed that increase Alu activity in infected cells is due to increased accessibility of Alu templates to RNA polymerase III complexes (Russanova et al., 1995). K562 cells were shown to have more open chromatin than HeLa cells, as measured by the nucleosomal repeat unit length (Li et al., 2000). After adenoviral infection, there is an increase in nucleosomal spacing in HeLas but very little effect on nucleosomal spacing in K562 cells. Similar results were obtained after treatment of HeLa and 293 cells with heat shock or cycloheximide. Accessibility of α-satellite DNA was measured and it was shown that both in control K562 cells and in adenovirus-infected HeLa cells the chromatin accessibility, including α-satellite, is greater than in control HeLa cells. This suggested that the opening is not Alu-specific or RNA polymerase III specific.

A variety of studies have established that RNA polymerase III transcription can be inhibited by the presence of histones (Almouzni et al., 1990; Gottesfeld and Bloomer, 1982; Morse, 1989). In yeast, some data suggest that TFIIIC competes for DNA access with histones (Marsolier et al., 1995). Another report showed that yeast RNA polymerase III can transcribe through nucleosomal DNA by mobilizing histones along the templates (Studitsky et al., 1997). The susceptibility of class III genes to nucleosomal repression is template-dependent. tRNA genes are highly resistant to repression by histones as, removal of H1 from murine fibroblasts makes little or no difference in accessibility of tRNA genes to transcription factors. On the other hand, B2 SINEs seem highly susceptible (Carey and Singh, 1988; Russanova et al., 1995). In HeLa chromatin preparations, Alu genes seem to be silenced by chromatin, while tRNA and 5S rRNA genes seem accessible to transcription factors in the same extracts (Russanova et al., 1995). However, H1 was shown to have little effect on Alus in human cells (Russanova et al., 1995). Acetylation can facilitate the access of transcription factors to chromatinised promoter sequences (Lee et al., 1993). Thus, evidence that

human TFIIIC possesses histone acetyltransferase (HAT) activity (Hsieh et al., 1999a; Kundu et al., 1999) suggests that TFIIIC performs a role, in addition to its function as an assembly factor, to weaken the interaction of nucleosomes with the transcribed region of at least some class III genes.

Nucleosome positioning was shown before to have an effect on Alu activity (Englander and Howard, 1995; Englander et al., 1993). It was shown that Alu elements possess the capacity to fix the rotational and translational positions of tetramer or octamer particles reconstituted in vitro (Englander et al., 1993). The reconstitution of an Alu element with octamers of core histones was shown to result in the complete abrogation of in vitro RNA polymerase III-dependent template activity. It was further shown that transcription could be fully abolished when a CpG methylated Alu template was reconstituted with (H3/H4)2 tetramers. The nucleosome positioning capacity of Alu elements was further examined within native chromatin (Englander and Howard, 1995) and it was shown that a significant fraction of human Alu elements is associated with rotationally positioned nucleosomes.

1.4 Project aims and general strategy

The general aim of this PhD project is to further examine if and how DNA methylation and chromatin are involved in transcriptional regulation of SINEs.

The first aim was to assess the involvement of methyl-CpG-binding domain proteins in silencing SINEs. The presence of MeCP2 will be re-examined to include B1 and B2 families and association of other MBD proteins will be tested. The presence of methyl-CpG-binding proteins at chromosomal SINEs would provide more evidence about the mechanism of DNA methylation regulation. If ChIP confirms the presence of methyl-CpG-binding proteins at Alu, B1 and B2 genes, the presence of chromatin-associated complexes which would contribute to SINE regulation will be investigated.

The second aim was to seek further support for above mechanisms by testing if a DNA methylation-free *in vivo* environment leads to dissociation of methyl-CpG-binding domain proteins from SINE promoters and whether this will have an effect on SINEs activity. Human and rodent cells with reduced levels of DNA methylation will be used. Also, cDNA extracted from wild-type and MeCP2-knockout mice tissue will be used to look for changes in expression of B1 and B2.

This will provide insight into the effect on SINEs of DNA methylation and chromatin-mediated regulation.

2 Chapter 2- Materials and methods

2.1 Cell culture

2.1.1 Bacterial culture

All strains of *Escherichia coli* were grown in Luria Broth (LB; 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per litre pH 7.5). LB plates were made by adding 7.5 g bacto agar to 500 ml LB. All media were autoclaved before use and supplemented with ampicillin (50µg/ml). The plasmids used in this study contained the ampicillin resistance gene encoding β-lactamase and so were selected for by the presence of ampicillin.

2.1.2 Budding yeast culture

Saccharomyces cerevisiae (*S. cerevisiae)* strain GG BY 62 (his4-912Δ lys2-128Δ ura3-52) was streaked to plates containing YPD complete media (50 g/l) and 2% (w/v) yeast agar and incubated at 30°C. Single colony was picked and streaked into new YPD/agar plate or 5 ml YPD pre-culture was inoculated and shaken by orbital shaker at 30°C overnight. This was then added into fresh YPD media and grown to exponential phase (1 x 10^7 cells/ml) in volume needed for further application. Following transformation, cells were also grown on Formedium minimal media lacking uracil (Formedium Ltd).

2.1.3 Mammalian cells culture

Mammalian cell culture was performed in a class II hood, using aseptic technique and sterile equipment and reagents. All cell types were grown in humidified atmosphere containing 5% CO_2 at 37°C. HeLa cells, CCL 39 cells (Chinese hamster lung fibroblasts) and BALB/c 3T3 (A31) cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin and 1% streptomycin (all Sigma). HCT116 and HCT116 Dnmt1 $^{-/-}$ Dnmt3b $^{-/-}$ (HCT DKO) cells were grown in RPMI 1640 (+ L glutamine) supplemented with 10% FBS, 1% penicillin and 1% streptomycin, 1% sodium pyruvate (GIBCO Invitrogen), 25% glucose and 1 M HEPES. *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ mouse fibroblasts were grown in DMEM supplemented with 15% FBS, 1% penicillin and 1% streptomycin, 1% non-

essential AMK (GIBCO Invitrogen), 1% sodium pyruvate and 0.1% β mercaptoethanol.

Cells were passaged when subconfluent (approximately every 2 to 3 days) using buffered trypsin (0.05% trypsin, 0.02% EDTA; Sigma). Cells were also stored by cryo-freezing. For cryo-freezing, trypsinised cells were resuspended in 70% DMEM (plus penicillin and streptomycin), 20% FBS and 10% DMSO or in 90% FBS and 10% DMSO. 1 ml aliquots were transferred to cryo-tubes, and frozen overnight at -80˚C, before transferring to liquid nitrogen for permanent storage.

2.2 Plasmid DNA

2.2.1 Plasmids

The following table lists all the plasmids used in this study.

Gene	Description of plasmid
7SL	pUC19 vector containing the *HindIII-EcoRI* fragment of human 7SL gene
Alu Jo	pGEM®-5Zf(+) (Promega) vector containing the *SpeI-SacI* fragment of Alu Jo chromosome 19
pYES2int	pYES2int is integrating version of pYES2 Invitrogen plasmid with removed 2 µl ORI (origin of replication). It contains ura3 gene
7SL	pYES2int containing the *HindIII-EcoRI* fragment of human 7SL gene
Alu Jo	pYES2int containing the *XbaI-SpeI* fragment of human Alu Jo

Table 2-1 Plasmids

Alu Jo and 7SL were digested out of pGEM®-5Zf(+) and pUC19 vector, respectively, and subcloned into pYES2int vector (Invitrogen, modified) using restriction digest, gel purification, ligation and control restriction digest (below) or PCR (section 2.13).

2.2.2 Restriction digest

Plasmids were digested as follows: 2-5 µl DNA was digested using 1 µl restriction enzyme for a single digest or 1 µl of each enzyme for a double digest, together with 2 µl of appropriate enzyme buffer in a 20 µl final volume. The reaction was incubated for 1 h at the optimal temperature for the enzymes' activity.

2.2.3 Ligations

A standard ligation reaction was carried out as follows: 1 µl plasmid was mixed with 5 µl insert, 2 µl of 10 × T4 DNA ligase buffer (1 × final concentration) and 1 µl T4 DNA ligase. The reaction mix was incubated at 10°C overnight and the following day transformed into DH5α competent cells using the standard protocol (Section 2.2.6, 2.2.7). The resulting colonies were screened by restriction analysis (Section 2.2.2) to confirm the presence of inserted DNA.

2.2.4 Agarose gel electrophoresis

For the required percentage gel, routinely 1%, the appropriate amount (1 g/100 ml) of agarose was dissolved in 1 × TBE (45 mM tris-borate, 1 mM EDTA) then 2-3 µl ethidium bromide was added to give a slab of agarose gel. Samples for analysis were diluted by the addition of 6 × loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 0.15% (w/v) Ficoll) before being loaded on the agarose gel. Gels were run at 100 V in 1 × TBE buffer for about an hour. Gels were viewed using a UV transluminator and photographed using E.A.S.Y. imaging software.

2.2.5 DNA extraction from agarose gel

The QIAquick Gel Extraction Kit (QIAGEN) was used to purify DNA from agarose gels according to manufacturer's instructions. Briefly, DNA was excised from the gel using a sterile scalpel. 3 × gel volumes of Buffer QG (supplied) was added to the gel and dissolved at 50°C. The melted agarose-DNA mix was applied to a QIAquick spin column and centrifuged for 1 min. The supernatant was discarded and the DNA was now bound to the column. The column was then washed with 750 µl of buffer PE (supplied) containing EtOH. The column was centrifuged for 1

min to remove buffer and then spun for a further minute to remove excess ethanol. Finally DNA was eluted by adding 30 or 50 µl of nuclease-free water and stored at -20°C.

2.2.6 Transformation of competent cells using heat shock

For plasmid storage and propagation, *E.coli* XL-1 blue supercompetent cells (Stratagene) were transformed. These cells were stored at –80°C and thawed on ice prior to use, to prevent loss of transformation efficiency. 10-20 ng of plasmid DNA was added to 50 µl of thawed cells and mixed gently. The mixture was incubated on ice for 30 min, with occasional gentle agitation. Following this time, cells were heat-shocked for exactly 45 sec at 42°C, then transferred to ice for a further 2 minutes. 450 µl of SOC medium (LB, 0.04% glucose, 10 mM MgSO4, 10 mM MgCl2), which had been pre-heated to 42°C, was then added and cells were incubated at 37°C for 1 h on an orbital shaker (225-250 rpm). Subsequently, 150 µl of the transformation mixture was plated on LB-agar (2% LB, 2% agar) containing 50 µg/ml of the selective antibiotic ampicillin, and then incubated at 37°C overnight to allow colony formation. An isolated bacterial colony was selected from a streaked LB-agar plate, and used to inoculate 5 -10 ml of LB medium containing 50 µg/ml ampicillin. This mini-culture was incubated at 37°C overnight on an orbital shaker (300rpm). 1 ml was frozen in 20% sterile glycerol for stock, the rest used for Miniprep or the QIAGEN Plasmid Maxi Kit, according to the manufacturer's instructions.

2.2.7 Transformation of competent bacterial cells using electroporation

The BIORAD *E.coli* pulser was used in electro-transformation using the method adapted from the BIORAD manual. To 50µl of competent DH5α *E.coli* cells, 1-2 µl of the required plasmid DNA was added and left on ice for 1 min. The DNA/cell mix was transferred to an ice cold cuvette, and the cuvette inserted into the cuvette holder of an electroporator, which was set at 2.4 V. Both pads were kept pressed until the electroporator beeped. 1 ml of SOC media was added immediately and this mix then transferred to a 1.5 ml screw top microfuge tube and incubated in a 37°C shaker for 45-60 min. Transformed cells were plated

onto LB plates containing appropriate antibiotics and incubated at 37°C overnight.

2.2.8 Plasmid preparation using QIAprep® Spin Miniprep Kit (QIAGEN)

Plasmid DNA was purified from overnight *E.coli* culture using the QIAprep® Spin Miniprep Kit (QIAGEN) using the protocol in the manufacturer's manual. After 16 h incubation at 37°C, cells were collected at 13,000 rpm for 1 min and then resuspended in 250 µl buffer P1 (50mM Tris/HCl pH 8.0, 10 mM EDTA, RNase A 100 µg/ml). Cells were then lysed by addition of 250 µl buffer P2 (0.2 M NaOH, 1% SDS) and incubated for 5 min. 350 µl of buffer N3 (2.55 M KOAc pH 4.8) was added and the mixture was then centrifuged at 13,000 rpm for 10 min to pellet cell debris. Supernatant was put through a QIAprep column to allow DNA to bind to column. The column was washed with 750µl buffer PE and spun twice to remove all trace of buffer. Finally to elute the DNA, 50µl of nuclease-free water was added. DNA was stored at -20°C.

2.2.9 Plasmid preparation using the QIAGEN Plasmid Maxi Kit

For large scale plasmid DNA preparation, the QIAGEN Plasmid Maxi Kit was used. 250 ml of bacterial cell culture was harvested by centrifugation (Sigma Laboratory Centrifuge 4K15) at 6000g for 15 min at 4°C, then resuspended in 10 ml of Buffer P1 (500 mM Tris pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Cell lysis was performed by adding 10 ml of Buffer P2 (200 mM NaOH, 0.1% SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed at room temperature for 5 min before lysates were neutralised by adding 10 ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5). Addition of Buffer P3 caused the precipitation of potassium dodecyl sulphate, SDS-denatured proteins, chromosomal DNA and cell debris. Precipitation was enhanced by 20 min incubation on ice. The SDS-denatured proteins and chromosomal DNA were co-precipitated with the detergent, whilst the plasmid DNA remained in solution due to a lack of close protein associations. Centrifugation at 20000g for 30 min was performed (at 4°C) to separate precipitated debris from soluble material. Following this centrifugation, the supernatant containing plasmid DNA was

promptly removed and applied to a QIAGEN-tip 500, pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to bind tightly. The resin was then washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol), before eluting the purified plasmid DNA into a Falcon tube with 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris pH 8.5, 15% isopropanol). DNA was precipitated with 10.5 ml of isopropanol. The sample was then centrifuged at 15000g for 30 min at 4˚C. Following this, the supernatant was carefully decanted out leaving the pelleted plasmid DNA, which was then washed with 70% ethanol and recentrifuged. The pellet was air-dried for approximately 10 min, and then resuspended in an appropriate volume of sterile distilled H_2O. All plasmid DNA stocks were stored at -20˚C.

2.3 Competent yeast stable transformation

To make competent *S. cerevisiae* cells GGBY62 strain was used. It is a his4-912Δ lys2-128Δ ura3-52 strain (collection of Dr McInerny). It was streaked to YPD complete media (50 g/l) 20 g/l yeast agar and a few days later, 5 ml YPD preculture was inoculated and shaken by orbital shaker at 30˚C. This was then added into fresh 100 ml of YD media and grown to exponential phase (1 x 10^7 cells/ml). Cells were spun at 2000 rpm and washed with sterile H_2O, spun and resuspended in 1 ml sterile 1 x TE/LiAc. Cells were spun again at 2000 rpm and resuspended in 0.25 ml 1 x TE/LiAc. 50 µl yeast cell suspension was mixed with 5 µl transforming linearised DNA (Alu Jo or 7SL in pYES2int linearised with *StuI*) and 5 µl of salmon sperm DNA (10 mg/ml, Sigma). 300 µl sterile PEG (40% PEG4000, 1 x TE, 1 x LiAc made fresh from sterile 50% PEG4000, 1 x TE, 1 x LiAc) and mixed thoroughly. This was incubated for 30 min in roller drum for 30 min. 40 µl dimethyl sulphoxide (DMSO) was added and cells were heat shocked at 42˚C for 15 sec. Cells were then spun briefly (6 sec) at 4000 rpm, pellet resuspended in 1 ml 1 x TE and spread onto plates containing minimal media without uracil (1.9 g/l, Formedium). They were left to grow at 30˚C for several days.

2.4 Transient transfection of Alu Jo and VAI

CCL39 cells were transfected using the Lipofectamine™ reagent (Invitrogen™ Life Technologies Inc.) The transient transfection with Lipofectamine™ required cells to be at a confluency of ~75% at the time of transfection. 7 µg of plasmid DNA extracted using the QIAGEN Plasmid Maxi Kit was used per 10 cm dish. Alu Jo in pGEM-5zf(+) plasmid was transfected either alone (7 µg) or co-transfected with VAI (in pUC19, 3.5 µg of each) or VAI alone was transfected as a negative control. Per each dish, plasmid DNA and 0.5 ml of OptiMEM were mixed, which was then mixed with 0.5 ml OptiMEM and 30 µl Lipofectamine™. The Lipofectamine™-DNA-OptiMEM solution was incubated for 45 min in the dark at room temperature. During this time, 10 cm plates were washed with 1 ml of OptiMEM and 4 ml of OptiMEM was then added and left until the end of the 45 min incubation time. At this point, solution of the Lipofectamine™-DNA-OptiMEM mix was added to each plate and incubated at 37°C for 3 h. Media on plates containing transfected cells was replaced with DMEM after 3 hours, and cells grown for 24 hours or until confluent. RNA was extracted using TRI reagent (Section 2.6.1) and used for cDNA synthesis or primer extension.

2.5 Genomic DNA isolation

2.5.1 Isolation of yeast genomic DNA

10 ml of *S.cerevisiae* cells were grown for 2-3 days at the permissive temperature until saturation point (absorbance of 0.5 – 0.6 at 600 nm). Cells were harvested by centrifugation at 2000 rpm for 5 min. The cell pellets were then resuspended in 0.5 ml dH_2O and transferred to a 1.5 ml screw cap microfuge tube where they were pelleted by a short 5 sec spin. Supernatant was discarded and the cells were resuspended in the residual liquid before adding 0.2 ml of solution A (2% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA), 0.2ml phenol:chloroform (1:1) and 0.3 g acid washed glass beads. Cells were lysed using the HYBAID Ribolyser at 3×40 sec bursts at setting 4. 400µl of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) was added followed by centrifugation at 14000 rpm for 5 min. The aqueous layer only, containing both DNA and RNA, was transferred to a fresh 1.5 ml microfuge tube. 1 ml of 100% ethanol was added to the aqueous phase, gently mixed by inverting to avoid breakage of large DNA

fragments and put at -80°C for 10 min. This was then pelleted by centrifugation at 13000 rpm for 2 min and supernatant discarded. The pellet was resuspended in 400 µl of TE and 3 µl Ribonuclease A (10 mg/ml) and incubated at 37°C for 5 min to digest unwanted RNA. 1 ml of 100% ethanol and 8 µl of 5 M ammonium acetate was then added, gently mixed by inversion and left at -70°C for 25-30 min. The DNA was pelleted by centrifugation at 13000 rpm for 2 min and the supernatant discarded. The pellet was air-dried and resuspended in 100 µl TE and the DNA stored at -20°C.

2.5.2 Isolation of mammalian genomic DNA

Mammalian cells were grown on tissue culture dishes until subconfluent. Cells were detached from dishes using buffered trypsin (0.05% trypsin, 0.02% EDTA; Sigma), collected to 15 ml falcon tubes, spun at 500g and supernatant was discarded. Cells were resuspended in 10 ml ice cold PBS, spun at 500g and supernatant discarded. This step was repeated. Cell pellet was resuspended in 0.5 ml of digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8, 25 mM EDTA pH 8, 0.5% SDS) per dish (1 x 10^7 cells). 5 µl of proteinase K (10 mg/ml) was added and cells were incubated with shaking at 55°C for 12-18 h. Nucleic acids were extracted with equal volume of phenol/chloroform (1:1). Samples were spun for 10 min at 1700g in a swinging bucket rotor. The aqueous layer was transferred into a new tube and 1/10 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol were added. The DNA immediately formed a stringy precipitate. It was picked with a glass pipette and transferred to fresh 1.5 ml microfuge tubes containing 70% ethanol. Samples were spun at 13000g for 2 min, ethanol removed and samples air-dried for 10 min. DNA was resuspended in 1 ml sterile H_2O and incubated for 1 h at 65°C to facilitate solubilisation.

2.5.3 Quantification of DNA

DNA concentration was determined by measuring absorbance at 260 nm, and using the following calculation: DNA concentration (µg/ml) = absorbance at 260 nm x 50 x dilution factor.

2.6 Isolation of total RNA

2.6.1 RNA extraction from mammalian cells

Total cellular RNA was extracted from all mammalian cells grown on 10 cm tissue culture dishes using TRI reagent (Sigma), according to the manufacturer's instructions. Media was aspirated from the cells, then cells were scraped into TRI reagent and transferred to sterile 1.5 ml microfuge tubes. 1 ml of TRI reagent was used per plate. Samples were incubated for 5 min at room temperature, to allow the complete dissociation of nucleoprotein complexes, then 0.2 ml of chloroform was added to each. Thorough mixing of chloroform and TRI reagent was ensured by vortexing each sample for 15 sec. Samples were incubated at room temperature for a further 5 min, then centrifuged at 12000g for 15 min at 4˚C. This centrifugation separated the samples into 3 phases: a lower organic phase containing proteins, a middle interphase containing DNA, and an upper aqueous phase containing RNA. The RNA-containing phase was carefully removed, and transferred to a fresh microfuge tube. To precipitate RNA, 0.5 ml of isopropanol was added, samples were mixed by vortexing for 10 seconds, then incubated for 10 min at room temperature. Subsequently, samples were centrifuged at 12000g for 10 min at 4˚C. Following centrifugation, the supernatant was discarded, and the remaining RNA pellet was washed using 1 ml of 75% ethanol, made using diethylpyrocarbonate (DEPC)-treated H_2O (0.1% DEPC). Samples were centrifuged for a further 5 min at 12000 g (4˚C), then the supernatant was aspirated off, and RNA pellets left to air dry for approximately 10 min. Once dry, RNA was resuspended in 30-50 µl of DEPC-treated H_2O. To aid resuspension, samples were incubated at 50˚C for 15 min. All RNA samples were stored at -80˚C.

2.6.2 RNA harvested from mouse kidneys

MeCP2 wild type, female heterozygous and male knock-out mouse kidneys were obtained from Dr Mark Bailey (Guy et al., 2001). Table 2 below lists all animals used.

Mouse	Date of birth	MeCP2 genotype	Strain
Ko671	20/08/02	+/+	C57
Ko672	20/08/02	+/-	C57
Kob13	28/02/03	-/y	C57/Balb/6
Kob14	28/02/03	+/y	C57/Balb/6
Ko771	28/03/03	+/y	C57

Table 2-2 MeCP2 mice

Following removal, kidneys were immediately snap-frozen in liquid nitrogen and stored in a -80°C freezer. Prior to handling, tissue was put onto dry ice. Tissue was crushed using a pestle and mortar in liquid nitrogen, then transferred to sterile microfuge tubes. 1 ml of TRI reagent was added per kidney. Subsequently, the homogenate was centrifuged at 12000g for 5 min to remove any insoluble material. The supernatant containing RNA was transferred to a fresh tube and RNA extracted as described above.

2.6.3 RNA extraction from yeast

RNA extraction from budding yeast required the growth of a 200 ml culture of cells to exponential phase and harvesting at 3000 rpm for 5 min. Supernatant was discarded and the cell pellet was resuspended in 1 ml STE (0.32 M sucrose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0) and transferred to a screw cap microfuge tube and centrifuged at 13000 rpm for 5 sec. Cells could be stored at -80°C or used further. The cell pellet was resuspended in 200 µl STE and 600 µl NTES (100 mM NaCl, 5mM EDTA, 50 mM Tris-HCl pH 7.5, 1% (w/v) SDS) was added together with 500 µl of water saturated hot phenol at 65°C and 0.3 g acid-washed glass beads. Cells were lysed using a Hybaid Ribolyser for 3 × 40 sec bursts at setting 4. After centrifugation at 13000 rpm for 5 min, the upper aqueous phase and protein interface was transferred into a fresh microfuge tube containing 500 µl hot phenol. The mixture was again ribolysed for a 1 × 40 sec burst and again spun for 5 min. This step was repeated with room temperature phenol. The aqueous phase was then transferred to 400 µl of phenol/chloroform (1:1) at room temperature, ribolysed and spun. The aqueous phase was then transferred to a second 400 µl aliquot of phenol/chloroform, ribolysed and spun. The aqueous phase was then transferred to a 300 µl aliquot of chloroform and ribolysed. Finally after spinning for 5 min the aqueous phase was transferred to a fresh microfuge tube where 3 volumes of 100% ethanol and one tenth sample

volume of 3M sodium acetate pH 5.2 were added. The RNA was precipitated overnight at -20°C or for a couple of hours at -70°C. RNA was then pelleted by centrifugation at 13000 rpm for 10 min and supernatant was discarded. The pellet was washed in 70% ethanol in RNAse-free dH_2O and centrifuged for 1 min. Ethanol was removed and the pellet was left to air dry. Pellet was then resuspended in 50 µl of RNAse-free dH_2O and dissolved by incubating at 65°C with frequent pipetting.

2.6.4 Quantification of RNA

Spectrophotometer was employed to measure the absorbance of each sample at 260 nm, and the following formula was used to calculate the RNA concentration: RNA concentration (µg/ml) = absorbance at 260 nm x 40 x dilution factor.

2.7 Protein extraction

2.7.1 Preparation of extracts

Extracts for polyacrylamide gel electrophoresis were prepared from all mammalian cells grown on 10 cm dishes. Dishes with cells were placed on ice and washed twice in ice cold PBS (phosphate-buffered saline). Cells were then scraped directly into cell lysis buffer (20 mM HEPES pH 7.8, 150 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 10 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT, 0.5 µg/ml leupeptin, 1 µg/ml trypsin inhibitor, 0.5 µg/ml aprotinin and 40 µg/ml bestatin) and transferred to sterile microfuge tubes. 250 µl of buffer was used per plate. The cell lysates were incubated on ice for 15 min, and then passed through a 26-guage needle three times. Cell debris was collected by centrifugation at 12000g for 10 min and supernatant collected, snap-frozen on dry ice and stored at -80°C.

2.7.2 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. The colour change produced upon mixing this reagent with protein can be quantified by measuring

absorbance at 595 nm, and is directly proportional to the concentration of protein in the sample. For each experiment, a standard curve was constructed by measuring the absorbance (using a spectrophotometer) of 0, 2, 4, 6, 8, 10 and 12 μg of BSA in 1ml of Bradford's reagent. 1 or 2 μl (depending on the colour change) of the whole cell extracts were added to 1 ml of reagent. Absorbance readings at 595 nm were performed in duplicates, and the protein concentration of each sample determined from the standard curve.

2.8 Western blot analysis

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

Whole cell lysates (prepared as described above) containing 30-50 μg of protein were resolved by denaturing SDS-PAGE on 10% or 12% (unless otherwise indicated) polyacrylamide minigels (resolving buffer contained 375 mM Tris pH 8.8, 0.1% SDS), 4% polyacrylamide stacking gels contained 125 mM Tris pH 6.8, 0.1% SDS. Prior to loading, samples were boiled for 4 min in 1 x protein sample buffer (62.5 mM Tris pH 6.8, 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue). Electrophoresis was performed in 1 X SDS running buffer (0.1% SDS, 76.8 mM glycine, 10 mM Tris pH 8.3) at 200 V for 1 h.

2.8.2 Western blot analysis

Following resolution by SDS-PAGE, proteins were transferred to a Hybond™-P membrane (Amersham) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system. Transfer was carried out in 1 x transfer buffer (76.8 mM glycine, 10 mM Tris pH 8.3, 16.5% methanol) at 70 V for 1 h at room temperature or 50 V at 4°C overnight. Membrane was then stained using 1 x Ponceau S solution to ensure efficient transfer of the protein to the membrane, and subsequently washed with 1 x TBS (2.5 mM Tris pH 7.6, 15 mM NaCl). Membrane was then cut into pieces of membrane containing proteins of interest and these were separated into dishes and blocked in milk buffer ((1 x TBS, 0.5% Tween-20, 5% skimmed milk powder (Marvel)) for 1 hour at room temperature. After 1 h, membranes were transferred into fresh milk buffer and primary antibody was added in appropriate dilution and incubated for 2 h. The primary antibodies

utilised are listed in Table 4 (Section 2.16). After incubation, membranes were washed 3 times for 5 minutes in 1 x Western buffer (32.5 mM Tris, 150 mM NaCl, 0.5% Tween-20) to remove excess primary antibody. Subsequently, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako), diluted 1 in 1000 in milk buffer, for 1 h at room temperature. Excess secondary antibody was then removed by three 5 min washes and two 15 min washes in 1 x Western buffer. Following a final 5 min wash in 1 x TBS (2.5 mM Tris pH 7.6, 15 mM NaCl), the HRP-conjugated (horseradish peroxidase) secondary antibodies were detected using the enhanced chemiluminescence (ECL) method (Amersham). Chemiluminescence detection, using the ECL™ Western Blotting analysis system (Amersham Pharmacia), was performed by adding equal volumes of reagent 1 and reagent 2 to the membrane, incubating at room temperature for 1 min, and then exposing the membrane, covered in Saran Wrap, to ECL film (Amersham Pharmacia) for different lengths of time before developing using the X-OMAT film processor.

2.9 Northern blot

2.9.1 Total RNA separation by electrophoresis and membrane transfer

1 g of agarose was dissolved by boiling in 63 ml of dH_2O and 20 ml of 5 x MNE (120 mM MOPS, 25 mM NaOAc, 5 mM EDTA pH 7) and then cooled to approximately 60°C. 17 ml of formaldehyde was added and gently mixed. The mixture was poured into a horizontal gel mould and left to set for 30 min. 10-20 µg RNA was mixed with 20 µl of RNA buffer (600 µl formaldehyde, 200 µl formamide, 240 µl 5 x MNE, 160 µl dH_2O) and 1 µl of 0.5 mg/ml ethidium bromide. Samples were incubated at 60°C for 5 min to denature RNA secondary structure and then loaded onto the formaldehyde gel. RNA was then separated by electrophoresis at 60 V for 2.5 h in 1 x MNE. The gel was visualised under a UV transilluminator to ensure effective RNA separation and equal loading.

The gel was then washed in 0.1 M sodium phosphate buffer pH 6.5 (61 ml 1 M Na_2HPO_4, 39 ml 1 M NaH_2PO_4 made to 1 litre with dH_2O) with gentle shaking for 10 to 15 min. For capillary transfer of the RNA, the gel was inverted and placed on a wick of Whatmann 3MM chromatography paper, which had been pre-soaked

in and was fed from a reservoir of 0.1 M sodium phosphate buffer pH 6.5. A gel-sized piece of pre-soaked Hybond N nylon membrane (Amersham Pharmacia), followed by 2 pieces of pre-soaked Whatmann paper, were placed on the gel ensuring no air bubbles between layers. A stack of folded paper towels was then added followed by a 0.5 kg weight, to ensure efficient transfer of RNA to the membrane by capillary action. Pieces of autoradiography film were placed at the edges of the gel to prevent the paper towels from contacting the wick. The transfer was allowed to proceed for 16 to 18 hours, and then RNA was fixed to the membrane by UV exposure and baking in a 70˚C oven for 1 h.

2.9.2 Probe preparation, radiolabelling and purification

To specifically detect the RNA of interest, radiolabelled complementary DNA probes were used: the Alu Jo probe was prepared from a 349bp *SacI-SpeI* fragment of Alu Jo in pGEM-5zf(+) plasmid (Promega), and the probe for Gal1 transcript was prepared from the 523bp *SpeI-SacI* fragment of pYES2int plasmid (Invitrogen, modified). A Megaprime DNA Labelling Kit (Amersham Pharmacia) was used to label the probes by random oligonucleotide priming, according to the manufacturer's instructions. 5 µl of random hexamer oligonucleotides were mixed with 2 µl (25 ng) of the DNA fragment to be probed, and 26 µl with DEPC-treated H_2O. This was heated at 95˚C for 5 min to denature the DNA. Slow cooling of the mixture to room temperature allowed the random hexamer oligonucleotides to anneal to the DNA. 10 µl of reaction buffer (dATP, dGTP, dTTP in Tris pH 7.5, β-mercaptoethanol and $MgCl_2$), 2 µl (2U) of DNA polymerase I Klenow fragment, and 50 µCi of [α-^{32}P] dCTP (Amersham Pharmacia) were added, and labelling was allowed to proceed at 37˚C for 1 h. The labelled DNA was then denatured by heating at 100˚C for 5 min, and then chilled and stored on ice until used.

To achieve higher specifity of the probe and decrease background radioactivity, the probe was purified from unincorporated [α-P^{32}] dCTP using a size-exclusion column. Sephadex G-50 was prepared by adding two volumes of TE and autoclaving. The plunger of a 1 ml syringe (Plastipak) was removed and a small amount of glass wool (silane treated, Supelco) was used to plug the end of the syringe before a microfuge tube was placed on the end of the syringe. Both syringe and microfuge tube were placed into a 50 ml centrifuge tube and

Sephadex G-50 was added to the syringe and centrifuged for 5 min at 3000 rpm. TE was removed and the process repeated until 0.7 ml of Sephadex G-50 remained in the syringe. A fresh microfuge tube was then placed on the bottom of the syringe and the radiolabelled probe (made up to 100 µl with dH_2O to increase volume added to column) was added to the Sephadex G-50 column and centrifuged for 5 min at 3000 rpm. The probe was collected and the labelled DNA was denatured by heating at 100˚C for 5 min, and then chilled and stored on ice until used.

2.9.3 Hybridisation and analysis

Prior to hybridising the membrane with an appropriate radiolabelled probe, it was pre-hybridised in a Techne hybridisation oven for 2-4 h at 42˚C in 18 ml of hybridisation buffer (10 ml formamide, 4 ml P buffer (1% BSA, 1% polyvinyl-pyrrolidone, 1% ficoll, 250 mM Tris-HCl pH 7.5, 0.5% sodium pyrophosphate, 5% SDS), 4 ml 50% dextran sulphate (Pharmacia), 1.16 g NaCl). The hybridisation buffer was heated to dissolve salt at 42˚C and then 200 µl salmon sperm DNA (Sigma, denatured at 95˚C for 5 min and chilled on ice) was added. Denatured radiolabelled probe was added to the hybridisation buffer and hybridisation carried out at 42˚C overnight. The following day the hybridisation buffer was poured away and the membrane was first washed twice for 5 min each time in 2 x SSPE (20 x SSPE stock is 3 M NaCl, 20 mM NaH_2PO_4, 200 mM EDTA pH 7.4) at 42˚C. The membrane was then washed in 2 x SSPE + 0.5% SDS at 65˚C for 15 min to remove unspecific binding. The signal to background ratio was monitored using a Geiger counter and if background was still high, the 65˚C wash was repeated. Finally, the membrane was rinsed in 0.1 x SSPE. The membrane was then exposed to autoradiography film for an appropriate length of time. To reprobe the membrane, it was boiled in DEPC-treated H_2O for 5 minutes and exposed to autoradiography film overnight to check there is no detectable radioactivity. Then it was pre-hybridised and probed as before.

2.10 *In vitro* transcription assay

In vitro transcription of class III genes was reconstituted using 20 µg of HeLa nuclear extracts (Computer Cell Culture Center, Mons, Belgium) to provide the basal RNA polymerase III transcription components.

2 µl (250 ng/ml) of plasmid DNA containing the templates Alu Jo in pGEM-5zf(+) plasmid (Promega) and 7SL in pUC19 was mixed with 2µl of dH_2O. The following 25 µl mix was added to the template: 2 µl of HeLa nuclear extract, 2 µl of dH_2O, 13 µl LDB (12 mM HEPES pH 7.9, 60 mM KCl, 7.2 mM MgCl2, 0.28 mM EDTA, 1.2 mM DTT, 10% glycerol), 2 µl TC(NE) (0.5 mM of each rATP, rCTP and rGTP in DEPC EDTA), 1 mM CP/ED (creatine phosphate) and 10 µCi of [α-32P]UTP (Amersham Pharmacia). *In vitro* transcription without [α-32P]UTP was sometimes performed if the RNA product was subsequently used for primer extension. If unlabelled IVT was performed, 0.5 mM rUTP were added instead of of [α-32P]UTP. Transcription was carried out at 30°C for 1 hour. Transcription was stopped by the addition of 250 µl of 1 M ammonium acetate/0.1% SDS containing 20 µg of yeast tRNA (which stabilises the newly synthesised RNA in the samples). Phenol/chloroform extraction was then performed, to remove protein and DNA, by adding 250 µl of phenol/chloroform (1:1) to each sample. Samples were mixed thoroughly by vortexing, and then centrifuged at 13000g for 5 min. 200 µl of the resulting upper aqueous layer was then transferred to a fresh microfuge tube containing 750 µl of 100% ethanol. The samples were mixed by repeated inversion, and left at -20°C overnight to precipitate RNA. The following day, samples were centrifuged at 13000g for 30 min to pellet the precipitated RNA. The supernatant was carefully removed and discarded, then pellets were washed using 750 µl of 70% ethanol (prepared using DEPC-treated H_2O) and re-centrifuged at 13000g for 5 min. Again, the supernatant was discarded. RNA pellets were dried at 50°C for 5 min. Once dry, cold RNA was mixed with 4 µl of DEPC-treated H_2O and kept at -80°C. 4 µl of formamide loading buffer was added to radioactive RNA. Samples were then vortexed for 30 min to ensure complete resuspension of the RNA, and heated at 95°C for 2 min. Electrophoresis and autoradiography of radiolabelled transcripts was performed, as described in section 2.17 for PCR products.

2.11 Primer Extension

RNA from CCL cells transfected with Alu Jo (0.25 µg) and RNA polymerase III template VA1 (0.25 µg), was analysed by primer extension. RNA from *in vitro* transcribed Alu Jo was used as a positive control. VA1 (5'-CACGCGGGCGGTAACCGCATG-3') or Alu Jo (JoINR primer 5'- GAC AGT GTC TCA

CTC TGC TAC C -3' or PxuniqR primer 5'- GTA ATT CTT TTG TAG AGA CAG ACT CAC -3') primers were [γ-^{32}P]ATP end-labelled using T4 polynucleotide kinase (Promega). 50 ng of each primer was mixed with 2 µl T4 kinase buffer, 40 µCi of [γ-^{32}P]ATP, 2 µl of T4 kinase and 11 µl DEPC-treated H_2O. The mix was incubated at 37°C for 1 h and then stopped by addition of 1 µl 0.5 M EDTA and heated to 65°C for 10 min. This was stored overnight at -20°C if necessary.

For each primer extension reaction, 10 µg of RNA (made up to 10 µl with DEPC-dH_2O) were mixed with 10 µl of the relevant labelled probe diluted 1:10 in First Strand Buffer (Invitrogen) and incubated at 80ºC for 10 min. Samples were then immediately transferred to a heat block at 50°C, then briefly spun and incubated further for 2 h. This allowed the labelled probe (primer) to hybridise to the target sequence. Tubes were then pulse-microfuged and moved to a heat block at 42 °C. 30 µl of an elongation mix (23 µl DEPC-dH_2O, 0.5 µl 0.1 M DTT, 5 µl 5 mM dNTP mix (5 mM in DEPC- dH_2O), 0.5 µl 5 mg/ml actinomycin D, 0.5 µl RNASin (Promega) and 0.5 µl (100 U) of Superscript II Reverse Transcriptase (Invitrogen) was then added to the samples to initiate reverse transcription and the reaction was allowed to proceed for 1 h at 42ºC. Reaction products were ethanol precipitated overnight as described above. Pellets were resuspended in 4 µl of formamide loading buffer (98% (v/v) formamide, 10 mM EDTA pH 8.0, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF) was added to each sample, which was then vortexed for 1 h to ensure the RNA was fully dissolved. 1.5 µl of each sample was loaded on a pre-run 7% (v/v) polyacrylamide sequencing gel and analysis continued as described in section 2.17.

2.12 Nuclear run

2.12.1 Extraction of nuclei

HeLa cells were grown in 10 cm tissue culture dishes as described above until 80% confluent. Cells were trypsinised with buffered trypsin (0.05% trypsin, 0.02% EDTA; Sigma) and collected into 50 ml falcon tubes. Cells were spun at 500g for 5 min and washed with cold PBS and then spun and kept on ice. Cells were then counted using a haemocytometer. Typically, 5 dishes of cells were used, which gave about 3 x 10^7 cells. Cells were spun at 500g for 5 min, washed in cold PBS

again, spun and resuspended in 3 ml of extraction solution A (0.5% NP-40, 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) with added 20 µl/ml of solution A of protease inhibitor mix ((PIM, consisting of 1.6 ml of bestatin (2.5 mg/ml), 50 µl of leupeptin (1 mg/ml), 100 µl of trypsin inhibitor (1 mg/ml), 24 µl of aprotinin (2.2 mg/ml), 70 µl of pepstatin (1 mg/ml) and 150 µl of E-64 (2 mg/ml)), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM DTT on the day of use. The 3 ml of suspension were transferred into an ice cold loose homogeniser and homogenised by 10 strokes up and down. The suspension was kept on ice. Two disposable Beckman tubes were filled with 4 ml of extraction solution B (1.8 M sucrose, 5 mM Mg acetate, 0.1 mM EDTA, 10 mM Tris pH 8 with freshly added PIM (10 µl/ml of solution B), 1 mM DTT and 1 mM PMSF). 6 ml of extraction solution was also added to the suspension in homogeniser and gently mixed with a pipette. This mix was then layered carefully on top of the extraction solution B in the disposable Beckman tubes. Tubes were carefully transferred to the chilled Floor Beckman L8-55R Ultracentrifuge, SW 40 Ti rotor and spun at 30000g for 45 min at 4°C. In the meantime, 1 ml of extraction solution C was prepared (25% glycerol, 5 mM Mg acetate, 0.1 mM EDTA, 50 mM Tris pH 8 with freshly added 20 µl of PIM, 5 mM DTT and 1 mM PMSF). After the spin, the supernatant was discarded and any remaining liquid carefully dried. The pellet of nuclei was resuspended in extraction solution C (100 µl/1 x 10^7 nuclei) and aliquoted to cryotubes, snap-frozen in liquid nitrogen and kept in liquid nitrogen until used.

2.12.2 Probe and membrane preparation

10 µg of linearised plasmid (Alu Jo and 7SL in pYES2int plasmid) or 2 µg of DNA fragment (Alu Jo and 7SL) was used as a probe. 2 µl of the probes were also loaded on an agarose gel to check equal loading of all probes onto the membrane. Probes were dissolved in 0.5 M NaOH and boiled at 85°C for 15 min to denature DNA strands, then chilled on ice. In the meantime, Hybond™-N membrane (Amersham) was soaked in 10 x SSC (1.5 M NaCl and 0.15 M sodium citrate) for 10 min and put into a slot blot manifold (Amersham Biosciences) attached to a vacuum pump. Every slot of membrane was washed with some 10 x SSC and liquid run through the membrane using vacuum. Probe was diluted to 200 µl with 10 x SSC and applied onto the membrane using vacuum. Each slot of the membrane containing probe was then washed twice with 1 ml of 10 x SSC.

Membrane was then air-dried and probes were UV and heat cross linked to the membrane. Membrane was kept until needed.

2.12.3 Nuclear run-on assay

50 µl of run-on solution A (2 mM of each ATP,GTP, CTP and UTP, 0.1 M S-adenosyl methionine in DEPC-treated H_2O) was mixed with 10 µl DEPC-treated H_2O and run-on solution B (0.6 M KCl and 12.5 mM Mg acetate). 1 x 10^7 nuclei per reaction was carefully defrosted in a 30°C waterbath and the above mix together with 5 µl of RNasin and 100 µCi of very fresh [α-^{32}P] UTP (Amersham Pharmacia) was added. Samples were incubated at 30°C for 30 min. After incubation, 3 µl of DNase (Invitrogen) was added and samples incubated for another 10 min. RNA was then diluted with 10 µl 10% SDS and 100 µl TES (TE and 0.5% SDS) and mixed with 340 µl of phenol/chloroform, vortexed and spun at 13000g for 10 min. The top layer containing the RNA was transferred into a fresh 1.5 ml microfuge tube and mixed with 125 µl 3M NaOAc and 1 ml 100% ethanol. Tubes were vortexed briefly and spun at 13000g for 5 min. Supernatant was removed and pellet resuspended in 375 µl of TES. 125 µl of 3 M NaOAc and 1 ml of 100% ethanol were added and samples left to precipitate overnight at -20°

Next day, samples were spun at 13000g for 20 min. In the meantime membrane with probes was prehybridised in pre-warmed rapid hybridisation buffer (Rapid-hyb buffer, Amersham Biosciences) at 70°C for 1 h. Supernatant was removed from the samples and pellet was resuspended in 1 ml of pre- warmed hybridisation buffer and the mix added to the membrane and hybridised at 70°C overnight in a hybridisation oven. The following day, membrane was washed in 2 x SSC/0.1% SDS for 20 min in room temperature, twice in 0.5 x SSC/0.1% SDS at 65°C for 15 min and then covered in Saran Wrap and exposed to ECL film (Amersham Pharmacia) for different lengths of time before developing using the X-OMAT film processor.

2.13 Polymerase chain reaction

Polymerase chain reactions (PCRs) were performed using a Techgene thermal controller (TECHNE). Each reaction had a total volume of 20 µl and contained 2 µl of DNA or cDNA, 0.5 mM of the appropriate forward and reverse primers, 0.5

U of Taq DNA polymerase (Promega), 1 x Taq DNA polymerase buffer (Promega), 1.5 mM MgCl2, 0.2 mM of each non-radioactive dNTP, and 2 μCi of [α-^{32}P] dCTP (Amersham). Table 2.3 lists the primers used for PCR, their sequences, Tm and cycle numbers. Those used for reverse transcription PCR are labeled R, those used for chromatin immunoprecipitation assays are labeled C, primer extension P. It is also stated whether they were used for human or mouse DNA or cDNA (H or M respectively). Star indicates primers designed using UCSC Genome Browser (www.genome.ucsc.edu) and Primer3 software. The cycling parameters employed typically were 95°C for 3 minutes, number of cycles of [95°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds], 72°C for 5 minutes. Annealing temperature was typically 54-59°C or for primers with Tm over 70°C (whichever was lower from the primer pair) annealing temperature was selected 5°C below the Tm. Number of cycles was lower for cDNA and higher for DNA. PCR reaction products were diluted 1:1 with formamide loading buffer and resolved on 7% polyacrylamide sequencing gels as described in section 2.17.

Product			Forward (F) and reverse (R) primers	Tm°	Cyc-les
ARPP P0 (265bp)	R	H/M	F: 5'-GCA CTG GAA GTC CAA CTA CTT C-3' R: 5'-TGA GGT CCT CCT TGG TGA ACA C-3'	61.6 67.6	
tRNALeu (88bp)	R/C	H/M	F: 5'-GTC AGG ATG GCC GAG TGG TCT AAG GCG CC-3' R: 5'-CCA CGC CTC CAT ACG GAG ACC AGA AGA CCC-3'	81.8 80.8	24-28
tRNATyr (84bp)	R/C	H/M	F: 5'-CCT TCG ATA GCT CAG CTG GTA GAG CGG AGG-3' R: 5'-CCG AAT TGA ACC AGC GAC CTA AGG ATG TCC-3'	77.3 77.5	24-28
7SL (150bp)	R/C	H/M	F: 5'-GTG TCC GCA CTA AGT TCG GCA TCA ATA TGG-3' R: 5'-TAT TCA CAG GCG CGA TCC CAC TA-3'	76.0 75.6	13-25
5S (107bp)	R	H/M	F: 5'-GGC ATA CCA CCC TGA ACG C-3' R: 5'-CAG CAC CCG GTA TTC CCA GG-3'	67.7 70.6	18
B1* (102bp)	R/C	M	F: 5'-TGG TGG TGC ATG CCT TTA AT-3' R: 5'-CCT GGT GTC CTG GAA CTC ACT-3'	65.1 65.4	14-18
B2 (90bp)	R/C	M	F: 5'-GGG GCT GGA GAG ATG GCT-3' R: 5'-CCA TGT GGT TGC TGG GAT-3'	66.8 64.6	14-18
GAPDH (452bp)	R	M	F: 5'-TCC ACC ACC CTG TTG CTG TA-3' R: 5'-ACC ACA GTC CAT GCC ATC AC-5'	66.6 66.0	30-32
GAPDH (228bp)	R	H	F: 5'-GAA GGT GAA GGT CGG AGT C-3' R: 5'-GAA GAT GGT GAT GGG ATT TC-3'	61.3 60.7	15
ApoE* (184bp)	R	M	F: 5'-GTT TCG GAA GGA GCT GAC TG-3' R: 5'-AGC GCA GGT AAT CCC AGA AG-3'	63.9 65.3	24-26
ApoE* (233bp)	C	M	F: 5'-TTC GGA AGG AGC TGG TAA GAC-3' R: 5'-CGA CAG TCC CGT ACT CCT TC-3'	64.4 63.6	25-27
Actin* (205bp)	R	S.C.	F: 5'-CGT TCC AAT TTA CGC TGG TT-3' R: 5'-AGC GGT TTG CAT TTC TTG TTG-3'	63.6 66.0	18
ApoE* (164bp)	C	H	F: 5'-CAG CGG AGG TGA AGG ACG TC-3' R: 5'-CTC CTC CTC TCC CCA AG-3'	69.4 59.8	24-26

p53BP2* (189bp)	R	M	F: 5'-GTT GGT TTC GGC GAG AAG G-3' R: 5'-GAA GCC AAG CGA GAA CGA G-3'	66.8 65.1	25
Alu19* 3001-3301 (249bp)	C	H	F: 5'-CTC ACG ATC ATG GCT AAC TGC-3' R: 5'-GCC TGT AAT TCC AGC TGC TC-3'	64.6 63.8	30
Set1* 64441 64921 (493bp)	C	M	F: 5'-GCA TGC ATA CCA CTC CAC AC-3' R: 5'-CAG AGA ATC TGC AGT CGT ATT TCC-3'	64.1 64.8	30
Set2* 64741-65281 (557bp)	C	M	F: 5'-CTG CCT TCA GAC ACA CCA GAA G-3' R: 5'-GAT GGA AGA GGT TTT GCC AAG-3'	66.1 64.6	30
tRNA Sec (74bp)	R/C	M/H	F: 5'-GGA TGA TCC TCA GTG GTC-3' R: 5'-GGT GGA ATT GAA CCA CTC-3'	57.7 57.7	24
ALU Jo genomic* (419bp)	C	H	F: 5'-CTA CTC AAA ATA TTA AAC ATA GGC-3' R: 5'-GCT GCA ACG CTG CTA TGA AC-3'	54.8 65.9	26-30
PxUNIQR*	R/P	H	R: 5'-GTA ATT CTT TTG TAG AGA CAG ACT CAC-3'	59.4	25
H19* (161bp)	R/C	M	F: 5'-AGA GCT GGA GGA GAG TCG TG-3' R: 5'-TCC TCT CCA ACC CTA GCT CAG-3'	63.8 64.6	25
Jo INF,R* (255bp)	R	H	F: 5'-CTT ACA CGT GTC ATC CCA GC-3' R: 5'-GAC AGT GTC TCA CTC TGC TAC C-3'	63.2 60.5	22
U1 (186bp)	C	H	F: 5'-CCC TGC CAG GTA AGT ATG-3' R: 5'-CAC GAA GGA GTT CCC GTG-3'	58.0 64.4	28
Pv51/ JoINR-Alu cons (174bp)	R	H	F: 5'-ACC ATC CCG GCT AAA ACG GTG A-3' R: 5'-GAC AGT GTC TCA CTC TGC TAC C-3'	72.0 60.5	27-30
scAlu (JoINF-PxuniqR) (113bp)	R	H	F: 5'- CTT ACA CGT GTC ATC CCA GC -3' R: 5'- GTA ATT CTT TTG TAG AGA CAG ACT CAC -3'	63.2 59.4	26-30
Alu chr 6 (396bp)	C	H	F: 5'-CCA GAA AAT TAC CAA TTA GTT C-3' R: 5'-GGG CCT ATT GAC TAT GCT TAC-3'	56.0 59.1	25
Alu chr 10 (442bp)	C	H	F: 5'-GAT TCT CAA CAG CAG AAT TCC ATG CC-3' R: 5'-CAT GTT TGA GAA TGT CTA CTT CTT AG-3'	71.1 58.0	25
Alu chr 22 (371bp)	C	H	F: 5'-GTT CTG ACA CAC TTG GAG AAA GTG-3' R: 5'-GTT GTT GTT ATT GCA CAA CTC AAC-3'	63.8 62.1	25

Table 2-3 Primer information
R=RT-PCR; M=mouse; H=human, S.C=S.cerevisiae,* primers designed using UCSC genome browser.

2.14 Reverse transcription PCR (RT-PCR)

RNA harvested from cells or mouse kidneys was DNase I-treated prior to cDNA synthesis using a DNA-free kit (Ambion), according to the manufacturer's instructions. 10 µg of RNA was diluted with DEPC-treated H_2O to give a final

volume of 25 µl. To this, 2.5 µl of 10 x DNase I Buffer (Ambion) and 1 µl (2 U) of DNase I (Ambion) were added. Samples were incubated at 37˚C for 30 min. To inactivate the DNase I, 5 µl of DNase Inactivation Reagent slurry (Ambion) was added, and samples incubated for 2 min at room temperature. Subsequently, samples were centrifuged (in an Eppendorf Centrifuge 5415R) at 10000g for 1 min to pellet the Inactivation Reagent. The supernatant containing DNA-free RNA was transferred to a fresh sterile microfuge tube, and stored at –80˚C. 1.2 µg of this RNA was used for cDNA synthesis.

1.2 µg (3 µl) of RNA and 200 ng (2 µl of 1:10 dilution) of random hexanucleotide primers (Roche) were diluted in DEPC-treated H_2O to give a final volume of 24 µl. Primer annealing was carried out for 10 min at 80˚C. Following this, samples were transferred to ice and 1 µl (200 U) of Superscript II reverse transcriptase (Invitrogen Life Technologies), 8 µl of 5 x First Stand Buffer (Invitrogen Life Technologies), 4 µl of 0.1 M dithiolthreitol (DTT) (Invitrogen Life Technologies) and 2 µl of 10 mM dNTPs (Promega) were added to each. One sample was also treated with the same mix but no reverse transcriptase was added. Reverse transcription was then allowed to proceed for 1 h at 42˚C. The reaction was stopped by heating to 70˚C for 15 min. cDNAs were stored at –20˚C. Polymerase chain reaction using cDNA as a template was then performed as described above. No superscript samples served in the PCR analysis as a control that no DNA contamination is present. PCR was also performed with 1:2 diluted cDNA to check whether it gave a weaker product and therefore confirm that the amount of cycles used was quantitative. Radiolabelled PCR products were loaded on 7% polyacrylamide sequencing gels and visualised by autoradiography as described in section 2.17.

2.15 Chromatin immunoprecipitation (ChIP) assay

Mammalian cells were grown on 10 cm tissue culture dishes. One dish (1×10^7 cells) was used per condition. To crosslink cellular DNA and proteins, cells were treated with 1% formaldehyde. Crosslinking was allowed to proceed for 10 min at 37˚C, then glycine was added at a final concentration of 0.125 M to stop the crosslinking, and plates were transferred to ice. 2 ml of ice cold PBS was added to each dish and cells were scraped and transferred to Falcon tubes. Tubes were centrifuged (in a Sorvall RT 6000 D) at 500g for 5 min at 4˚C, then cells were

washed twice by resuspension in ice-cold PBS, followed by centrifugation at 500g for 5 min. Following removal of supernatant after the final wash, cell pellet was snap-frozen on dry ice and tubes kept at -80˚C until used or used immediately for chromatin immunoprecipitation. Cell pellets were resuspended in 50 ml of PBS/0.5% NP-40, spun 5 min at 500g in a swing rotor and supernatant removed. Cells were resuspended in high salt buffer (0.5% NP-40/PBS, 1 M NaCl) and incubated on ice for 30 min. Cells were then centrifuged at 500g for 5 min, and washed once in 0.5% NP-40/PBS. Subsequently, hypotonic disruption was performed by resuspending cells in 50 ml of low salt buffer (0.1% NP-40, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl) and incubating on ice for 30 min. Following this, samples were centrifuged at 500g for 5 min at 4˚C, and the resulting pellets resuspended in 1 ml of low salt buffer. To obtain nuclei, samples were then passed through a 26-guage needle 3 times. 1.7 ml of low salt buffer was added and remaining cell membranes lysed with 300 µl of 20% sarkosyl (N-lauroyl sarcosine). Subsequently, lysed nuclei were transferred to a sucrose cushion (40 ml low salt buffer/100 mM sucrose) and centrifuged at 4000g for 10 min at 4˚C. The supernatant was discarded, and the pellet was resuspended in 3 ml of TE (10 mM Tris pH 8.0, 1 mM EDTA). This 3 ml was then applied to a second sucrose cushion and the centrifugation process repeated. The final pellet containing genomic DNA was resuspended in 2 ml of TE, and then the DNA was sheared into smaller fragments (500 bp-1 kb on average) by ChIP sonicator (Bioruptor, Diagenode) with 30sec on/off cycles for 10 min set according to the manufacturer's instructions. 0.2 ml of 11 x NET buffer (1.65 M NaCl, 5.5 mM EDTA, 5.5% NP-40, 550 mM Tris pH 7.4) was added to 2 ml of sonicated material, then this was transferred to microfuge tubes for centrifugation at 13000g for 5 min. The supernatants were then aliquoted evenly into microfuge tubes. Each aliquot was incubated in the presence of 5 µg (25 µl) of an appropriate antibody overnight at 4˚C on a rotating wheel. Antibodies used for ChIP analysis are listed in Table 4. As a negative control, one aliquot was incubated in the absence of antibody (beads only). Also, 10% of the aliquot volume was retained for use as an input control.

The following day, 100 µl containing a 1:1 mix of appropriate protein-A-Sepharose or protein-G-Sepharose beads (Sigma) and 1 x NET buffer were added for further 2 h incubation at 4˚C, and then recovered on disposable Polyprep columns (Pierce). Columns were washed 4 times in 5 ml of RIPA buffer (50 mM

Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), 4 times in 5 ml of LiCl buffer (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA pH 8.0) and 4 times in 5 ml of TE. Any unbound material was washed off. Columns were then closed with caps and beads were incubated in 200 µl of 1% SDS/TE for 10 min to detach immunoprecipitated material off the beads. This was then eluted to 1.5 ml microfuge tubes and incubation and eluting step repeated with another 200 µl of 1% SDS/TE. The pooled samples (and input DNA made up to a total volume of 400 µl with 1% SDS/TE) were incubated overnight at 42˚C with 0.125 mg/ml Proteinase K to degrade the antibodies and any other proteins present. Subsequently, genomic DNA was extracted using phenol/chloroform, as described above. Ethanol precipitation was performed overnight at -20˚C. The immunoprecipitated DNA was then resuspended in 40 µl TE. 2 µl of immunoprecipitated or input DNA was used per PCR reaction following the protocol outlined in PCR section. Primers and number of cycles of PCR are listed in Table 2.3 and they are indicated with letter C.

2.16 Sequential ChIP analysis

A31 and HeLa cells were grown on forty 10 cm tissue culture dishes until subconfluent and harvested as in section 2.15. Formaldehyde crosslinking, PBS washes and cell lysis were followed up to the sonication step, as described in the ChIP analysis protocol (section 2.15). 11 x NET buffer was added as before, samples spun briefly and the supernatants then aliquoted evenly into two microfuge tubes only. 5 µg (25 µl) of TFIIIC (Ab7) or RNA polymerase III (1900) antibodies and a negative control antibody were used and samples incubated overnight at 4˚C on a rotating wheel. 10% of the aliquot volume was retained for use as an input control. The following day, appropriate beads were added. Incubation, transfer to the disposable Polyprep columns (Pierce) and the washes were performed as described above up to the elution step. Columns were closed with caps and beads were incubated only once in 200 µl of 1% SDS/TE for 20 min. Samples were eluted to 1.5 ml microfuge tubes and diluted 1:10 in 1 x NET to 2 ml final volume. TFIIIC (Ab7) or RNA polymerase III (1900) precipitated samples were then aliquoted evenly into fresh microfuge tubes and incubated in the presence of 5 µg (25 µl) of appropriate antibody overnight at 4˚C on a rotating wheel. 10% of the aliquot volume was retained for use as an input control. Next,

appropriate beads were added and the rest of the protocol (columns, washes, elution and genomic DNA extraction) was performed as described for ChIP analysis above. The immunoprecipitated DNA was then resuspended in 40 µl TE. Inputs from the first chromatin immunoprecipitation step were diluted 1:10 (to match the dilution of the antibody-precipitated samples). 2 µl of immunoprecipitated or input DNA was used per PCR reaction following the protocol outlined in section 2.13.

Antibody	Protein	Source	Host	Type	Quantity µl	Beads
Ab7	TFIIIC 220	In house	Rabbit	serum	25	a
128	Brf1 (TFIIIB)	In house	Rabbit	serum	25	a
330	Brf1 (TFIIIB)	In house	Rabbit	serum	25	a
1900	RNA pol III (RPC 155)	In house	Rabbit	serum	25	a
06-866	Acetyl Histone H4	Upstate	Rabbit	serum	5	a
06-599	Acetyl Histone H3	Upstate	Rabbit	serum	5	a
07-523	Trimethyl Histone H3(K9)	Upstate	Rabbit	serum	5	a
FL 109	TFIIA	Santa Cruz	Rabbit	serum	25	a
C-18	TFIIB	Santa Cruz	Rabbit	serum	25	a
M 9317	MeCP2*	Sigma	Rabbit	monocl	6	a
	MBD2*	Stancheva	Rabbit	serum	25	a
IMG-306A	MBD1*	IMGENEX	Mouse	monocl	10	g
Sc-28710	Brm*	Santa Cruz	Rabbit	serum	25	a
Sc-7899	HDAC2*	Santa Cruz	Rabbit	serum	25	a
Sc-7872	HDAC1	Santa Cruz	Rabbit	serum	25	a
Sc-11378	Mi2	Santa Cruz	Rabbit	serum	25	a
Sc-994	mSin3A	Santa Cruz	Rabbit	serum	25	a
8WG16	RNA pol II	Covance	Mouse	monocl	3	g
554293	p53	BD Pharmingen™	Mouse	monocl	Western blot	-
Sc-1615	Actin (C-11)	Santa Cruz	Goat	serum	Western blot	-

Table 2-4 List of antibodies
*** indicates antibodies also used for Western blot analysis**

2.17 Polyacrylamide sequencing gels electrophoresis

Radiolabelled products of any analysis resulting in PCR, primer extension and *in vitro* transcription were diluted 1:1 with formamide loading buffer (98% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 5 mM EDTA), and resolved on 7% polyacrylamide sequencing gels containing 7 M urea and 0.5 X TBE (45 mM Tris, 45 mM boric acid, 0.625 mM EDTA pH 8.0), 200 µl 20% APS and 20 µl TEMED. Gels were pre-run at 40W for 30 min in 0.5 X TBE prior to loading samples (1.5 µl of each). Before loading, samples were heated at 95˚C for 2 min,

and then quenched on ice. Electrophoresis was carried out for 1 h at 40W in 0.5 x TBE, then gels were vacuum-dried for 1 h at 80˚C. Radiolabelled PCR products were visualised by autoradiography.

2.18 Quantification and statistical analysis of data

Data were quantified by densitometry using ImageJ software (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). ImageJ is a public domain Java image processing program. It quantifies gray values and the number of pixels found for each gray value for each band. It uses a simple graphical method that involves generating lane profile plots, drawing lines to enclose peaks of interest, and then measuring peak areas. Statistical analysis was performed using a Student's t-Test (www.physics.csbsju.edu; unpaired, unequal variance) or a t- test (Microsoft Excel; two-tailed distribution, unequal variance). A probability (p) value of less than 0.01 or 0.05 was taken as a statistically significant difference between two groups. Box and whisker plots were used as a graphic representation.

3 Chapter 3 – Transcriptional activity of human Alu elements

3.1 Introduction

Despite the abundance of Alu sequences in RNA polymerase II-generated heterogeneous nuclear RNA (Jelinek et al., 1978), it was reported that Alu elements are not generally transcribed by RNA polymerase III and these transcripts are rare *in vivo* (Liu et al., 1994; Paulson and Schmid, 1986). Some of them got copied into heterochromatic regions and are probably silenced as a consequence. However Alus are interspersed throughout the whole genome (Schmid and Deininger, 1975) and the majority of them is concentrated in euchromatic gene-rich regions therefore their incidence in heterochromatin is unusual (Gilbert and Allan, 2001; Korenberg and Rykowski, 1988). Some proportion of Alus accumulated mutations in their RNA polymerase III promoter resulting in its inactivation. Such a promoter can no longer drive transcription. Those with preserved promoters are not usually transcribed by RNA polymerase III *in vivo* (Liu et al., 1994; Paulson and Schmid, 1986) despite of being transcribed *in vitro* (Duncan et al., 1979; Fuhrman et al., 1981). Alu elements are highly methylated (Schmid, 1991). There is growing evidence that methylation may be the main silencing mechanism, but it will be addressed in the next chapters.

In this chapter, a member of the Alu family was selected that would have typical properties of the old Alu families. Members of the young families can still be transcriptionally active and therefore they are not the subject of this thesis (Batzer et al., 1996; Matera et al., 1990). All these aspects were considered when selecting the representative Alu. There are restrictions in this approach, as one selected Alu can be in its unique environment or have unique properties resulting in difficulties with transcriptional activity and therefore not be representative of the whole large family. Characterising one Alu element however allows one to examine specific aspects which can then be translated into understanding of other Alu elements. This approach was used in numerous studies before and helped elucidate properties of the Alu family. For example, studies of Alus in the α-globin locus led to the recognition that the family is RNA

polymerase III-transcribed (Duncan et al., 1979; Duncan et al., 1981). Alus from the ε-globin locus and their *in vitro* transcription were also studied (Di Segni et al., 1981) and led to characterisation of the Alu tRNA-like bipartite promoter (Paolella et al., 1983).

3.2 Results

3.2.1 Characterisation of an individual Alu and its activity in vitro

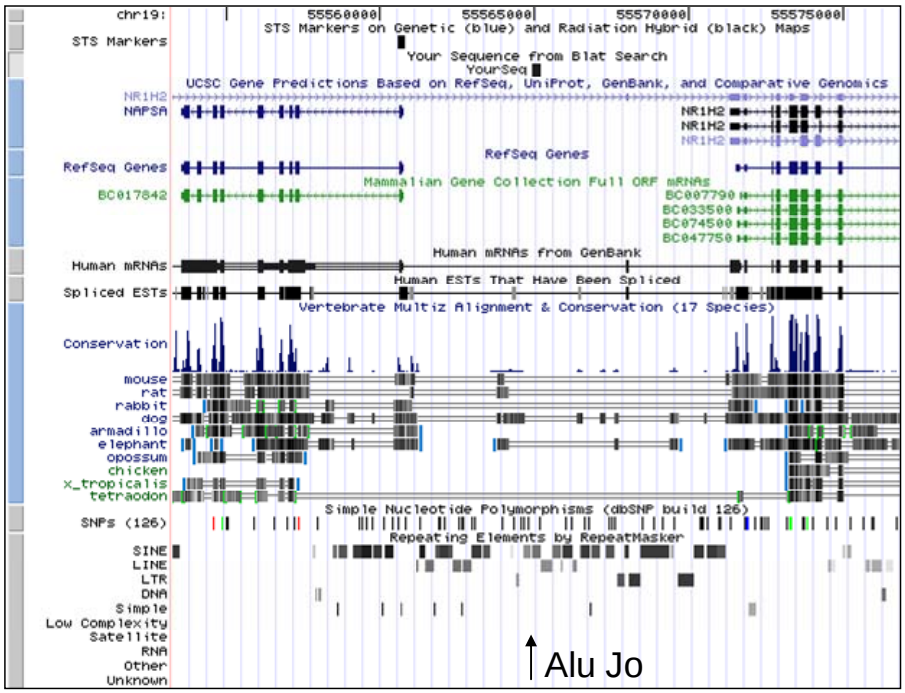
An Alu Jo element was obtained cloned into pGEM®-5Zf(+) (Promega) vector (Hever, kind gift). It was one amongst many human genomic fragments that bound strongly to a methyl-binding domain (MBD) column, suggesting that its DNA is methylated, as it is recognised by the MBD domain of MeCP2 protein. It is a full length 300 bp Alu element, consisting of left and right monomers. It belongs to the oldest family of Alus, family J (Batzer et al., 1996; Jurka and Smith, 1988), a large family which appeared very early in the ancestral human lineage and any silencing mechanisms would be therefore well established here. Its sequence with diagnostic mutations (Shen et al., 1991) is depicted in Figure 3.1a. It is located on human chromosome 19 in an intergenic location in a small cluster of other SINEs and LINEs (Figure 3.1b).

The first necessity for Alu elements' *in vitro* activity is a preserved RNA polymerase III promoter. One of the causes for low transcriptional activity of old Alus is that they often accumulated mutations in the promoter and the promoter is no longer active. The Alu promoter is a bipartite structure, as found in tRNA genes, with two highly conserved sequences, an A and B block, located approximately 60 nucleotides apart (Paolella et al., 1983). Previous *in vitro* transcription assays showed, that the fidelity of both A box sequence (Liu and Schmid, 1993) and B box sequence (Murphy and Baralle, 1983) of the RNA polymerase III promoter is a critical determinant of activity. Comparison of the Alu Jo's promoters with the consensus A and B block sequences revealed that the A block of Alu Jo exactly matches the consensus sequence, while there is a purine to pyrimidine mutation in the fifth position of the B block (Figure 3.1c). This position has not been reported as critical for promoter activity (Murphy and Baralle, 1983), but there is a possibility that Alu Jo's transcription might be impaired.

A.

5'GGCCGGGCATGGTGGCTTACACGTGTCATCCCAGCACGTTGGCAGGCTGAGGTGG
GAGGATTGC**TT**GAGCCCAGGAG**T**TCCAGACCA**G**CC**T**GGG**G**AA**C**A**T**AGTGAGTCTGTC
TCTACAAAAGAATTACCTGGGCTTGCTAGCGCATGCTTGTAGTCCCAGCTACTCTGGA
GACTGAGATGGCAGGATCACTTAGCCCAGGAGGTCGTGGCTGCAGTGAGCTGTGACT
ACGCCACCCCACTCCAGCCTAGGTAGCAGAGTGAGACACTGTCTCAAAACAAAACAAA
ACAAAAA3'

B.



C.

5'GGCCG**GGCATGGTGG**CTTACACGTGTCATCCCAGCACGTTGGCAGG

A box RGYNNRRYGG B box GTTCRANNC

CTGAGGTGGGAGGATTGCTTGAGCCCAGGA**GTTCCAGAC**CAG…….3'

Figure 3-1 Details of Alu Jo used in this study
(A) The Alu Jo's sequence. Diagnostic mutations determining its origin are highlighted in bold. (B) Alu Jo's location in the genome is shown using the UCSC Genome Browser (www.genome.ucsc.edu).(C) Alu Jo's promoter sequence. A and B box consensus sequences are aligned with Alu Jo's A and B box to determine mutations. Mutation in the B box is highlighted in red.

To determine whether, despite the mutation, the promoter can drive transcription *in vitro*, an *in vitro* transcription assay (IVT) was performed using the Alu Jo cloned into pGEM®-5Zf(+). The 7SL pUC19 construct was used here as a positive control. Briefly, plasmid DNA was mixed in a low salt buffer with HeLa nuclear extract containing basic RNA polymerase III transcription machinery components. rATP, rCTP and rGTP were added to the reaction together with [α-32P] UTP and transcription was allowed to proceed for 1 hour. Labelled transcript was then recovered and detected on a polyacrylamide sequencing gel.

In vitro, Alu Jo was transcribed, although at a lower level than 7SL (Figure 3.2a). It gave a product significantly longer than expected (around 550 bp). Alu Jo, like other Alus, lacks a RNA polymerase III terminator sequence, poly(T) (Schmid and Maraia, 1992), present in other RNA polymerase III templates (Bogenhagen and Brown, 1981). After the transcription of Alu sequence, RNA polymerase continues into the adjacent 3' sequence before it eventually dissociates (Fuhrman et al., 1981). This explains the length of the Alu Jo *in vitro* transcript. To confirm that it is indeed a transcript derived from the Alu Jo promoter, another *in vitro* transcription assay was performed without [α-32P]UTP. The unlabelled IVT product was then used as a template for a primer extension (Figure 3.2b). The primer extension method is used to determine the 5' end of the transcript and it was performed here using the Alu JoINR primer. A product of the expected size of approximately 270 bp was detected, along with other prematurely terminated cDNA. Alu transcripts have complex secondary structure (Labuda and Striker, 1989) which can be an obstacle for the reverse transcriptase (Sambrook and Russell, 2001b). This may explain the multiple truncated products.

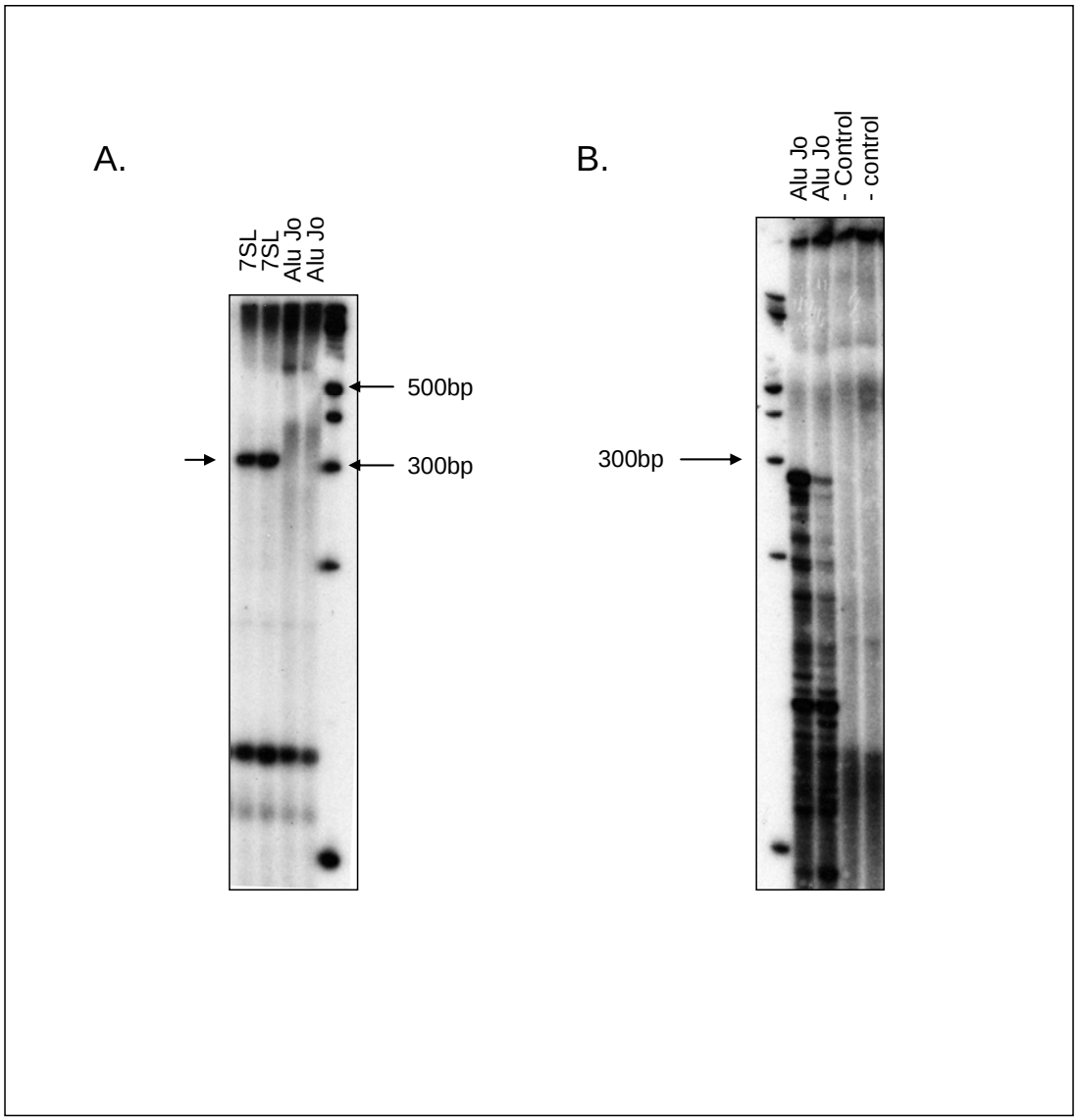


Figure 3-2 Alu Jo's promoter is active *in vitro.*
(A) Radiolabelled IVT using the Alu Jo cloned into the pGEM vector shows a transcript of around 550 bp long. It is presumably derived from the Alu Jo promoter as there is no other RNA polymerase III promoter in the pGEM vector. 7SL cloned into pUC19 served as a control. Reaction showed in duplicate. Marker sizes are indicated. Autoradiograph. (B) Primer extension using unlabelled Alu Jo IVT product as a template confirmed that the transcript obtained in the IVT reaction is indeed driven from the Alu Jo promoter. Product of expected 270 bp was obtained when radiolabelled JoinR primer was used for the extension. Yeast RNA was used as a negative control. Half of the unlabelled Alu Jo IVT product was used in the second lane. Marker sizes are indicated. Autoradiograph.

3.2.2 Transcriptional activity of Alu Jo in vivo

To test the activity of this promoter *in vivo*, Alu Jo was transiently transfected into CCL cells, a rodent line where there are no other similar sequences present. This tested whether it is capable of assembling the RNA polymerase III machinery on its promoter and being transcribed in a non-chromosomal context. Total RNA was extracted and assayed using the primer extension method (Figure 3.3a). Co-transfected VAI was used as a positive control for transfection efficiency (VAI panel). Briefly, primers were labelled with [γ-32P]ATP and allowed to hybridise with target RNA sequence extracted from cells. Reverse transcription was then used to extend the primers. Labelled products were recovered and detected on a polyacrylamide sequencing gel. cDNA complementary to the RNA transcript would match in length the distance between the primer and the 5' terminus of the RNA. Unlabelled RNA from *in vitro* transcribed Alu Jo was used as a positive control for the primer extension and RNA from CCL cells transfected with VAI only was used as a negative control. No transcript was detected for Alu Jo using JoINR primer design to detect full length transcript (data not shown). PxuniqR primer was then used to look at the left monomer only transcript as many dimeric Alus are processed to the left monomer only (Maraia et al., 1993; Matera et al., 1990). However, still no transcript was detected. Primer extension may not be sensitive enough for rare transcripts, because it does not contain a signal amplification process. cDNA was therefore made and RT-PCR performed (Figure 3.3b). Sample where no reverse transcriptase (Superscript) was added during cDNA synthesis was used as a control for genomic contamination. cDNA made from CCL cells transfected with VAI only was used as a negative control. RT-PCR using JoINF /JoINR primers detected full length transcript, presumably driven from Alu Jo's promoter because there is no RNA polymerase II promoter on the pGEM®-5Zf(+) plasmid. CCL cells are rodent Chinese hamster cells which do not have any human Alu sequences. They however have B1 sequences which are monomeric homologues of Alus. To make sure B1 sequences are not detected this primer pair is designed to detect dimeric Alu transcript. cDNA from CCL cells transfected with VAI template alone was used to check that, indeed, there was no endogenous transcript detected. RT-PCR also showed that the quantity of the Alu Jo transcript increased with the amount of available template. Alu Jo

transfected on its own (7 μg of DNA) gave stronger signal then when co-transfected with VAI (3.5 μg each). Endogenous 7SL shows equal levels of cDNA. This is evidence that Alu Jo can be transcribed *in vivo* from its own promoter by RNA polymerase III.

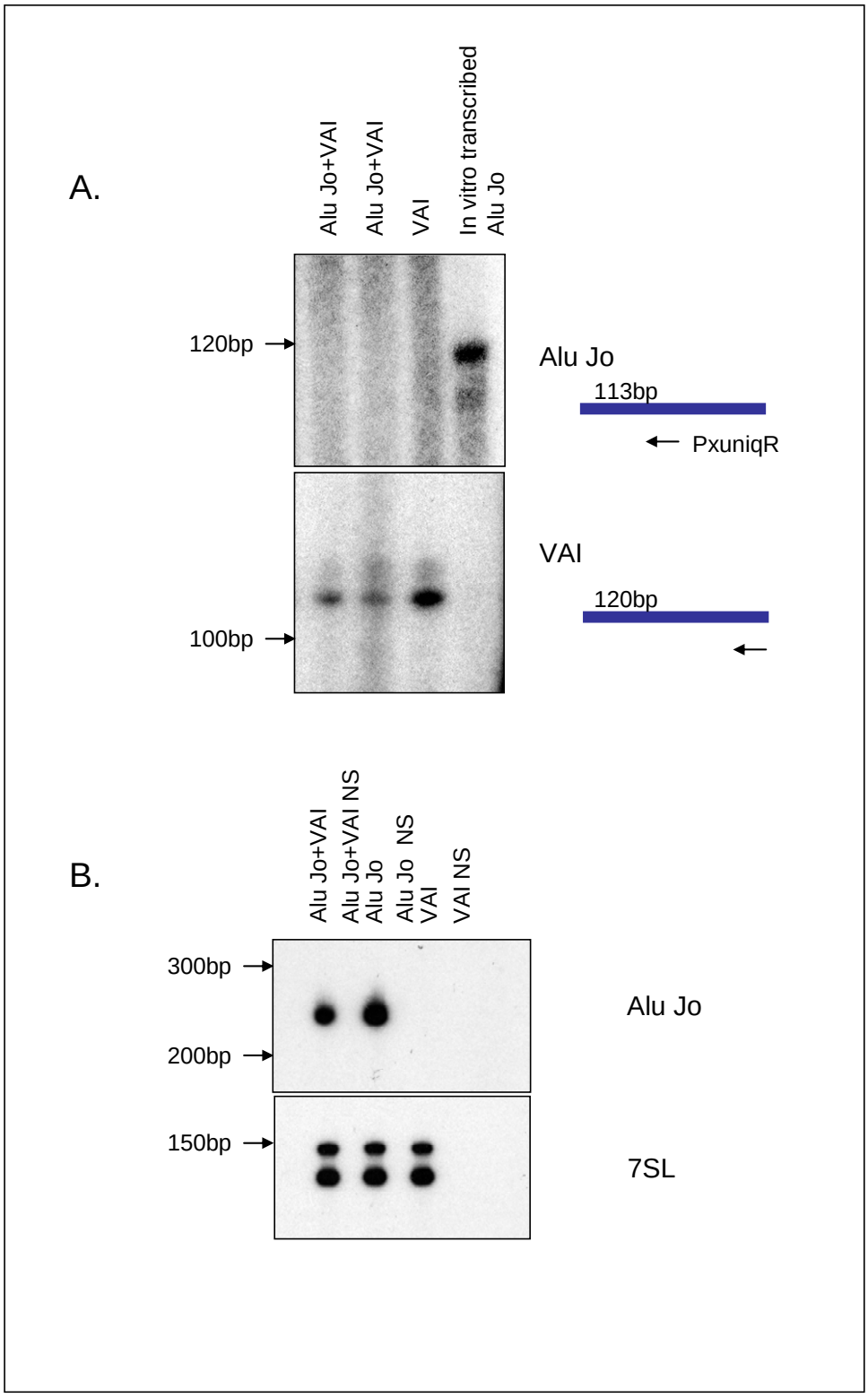


Figure 3-3 Alu Jo's activity in CCL cells
Alu Jo and VAI were transiently co-transfected into CCL cells and total RNA extracted from the cells was then analyzed for Alu Jo's transcriptional activity. (A) The primer extension using radiolabelled PxuniqR primer detected no transcript. Unlabelled Alu Jo IVT product and co-transfected VAI primer extensions were used as controls. The blue bars indicate the position of primers. Marker sizes are indicated. Autoradiograph. (B) Alu Jo transcript was detected by RT-PCR using the JoinF and JoinR primers. Endogenous 7SL served as control for cDNA loading. NS= sample with no Superscript added during cDNA synthesis served as a negative control for DNA contamination. Marker sizes are indicated. Autoradiograph.

To overcome the problem with secondary structure and to find out more about the rates of transcription, nuclear run-on analysis was employed to look at level of Alu Jo's transcription. It was performed using HeLa nuclei which were snap-frozen immediately after extraction. They were carefully defrosted and incubated in a reaction containing [α-^{32}P]UTP for 30 min (see Material and methods). Only the transcripts that were initiated before freezing proceed and these transcripts incorporate the [α-^{32}P]UTP and can therefore be detected. Any mature transcripts present in the nuclei would not be detected. This technique is therefore suitable to compare rates of transcription between two genes or a gene under different conditions (Sambrook and Russell, 2001b). 7SL and Alu Jo transcript levels were examined using either 7SL and Alu Jo gel-purified fragments (Figure 3.4a) or using linearised pYES2int plasmid containing 7SL or Alu Jo inserts (Figure 3.4b). Equal loading of the two probes on the membrane is shown on an agarose gel on the right hand side of the figure. In the Figure 3.4b uncut pYES2int was also loaded on the agarose gel only as a control for the linearization. A clear transcript signal for 7SL is detected using both probes. However, for Alu Jo there is only trace signal detected in the nuclei when the fragment probe is used (Figure 3.4a) and no transcript detected when using the whole plasmid (Figure 3.4b). It is impossible to tell whether this weak signal is due to Alu Jo's transcription initiated by RNA polymerase III or RNA polymerase II. It may also be a background hybridisation signal that is non-specific. Run-on analysis with added α-amanitin was tried and led to complete loss of the Alu Jo signal, but it also resulted in reduced signal for 7SL making it impossible to draw any conclusion about the nature of the transcript (data not shown). In any case, Alu transcription in the HeLa nuclei was very low when compared to the 7SL.

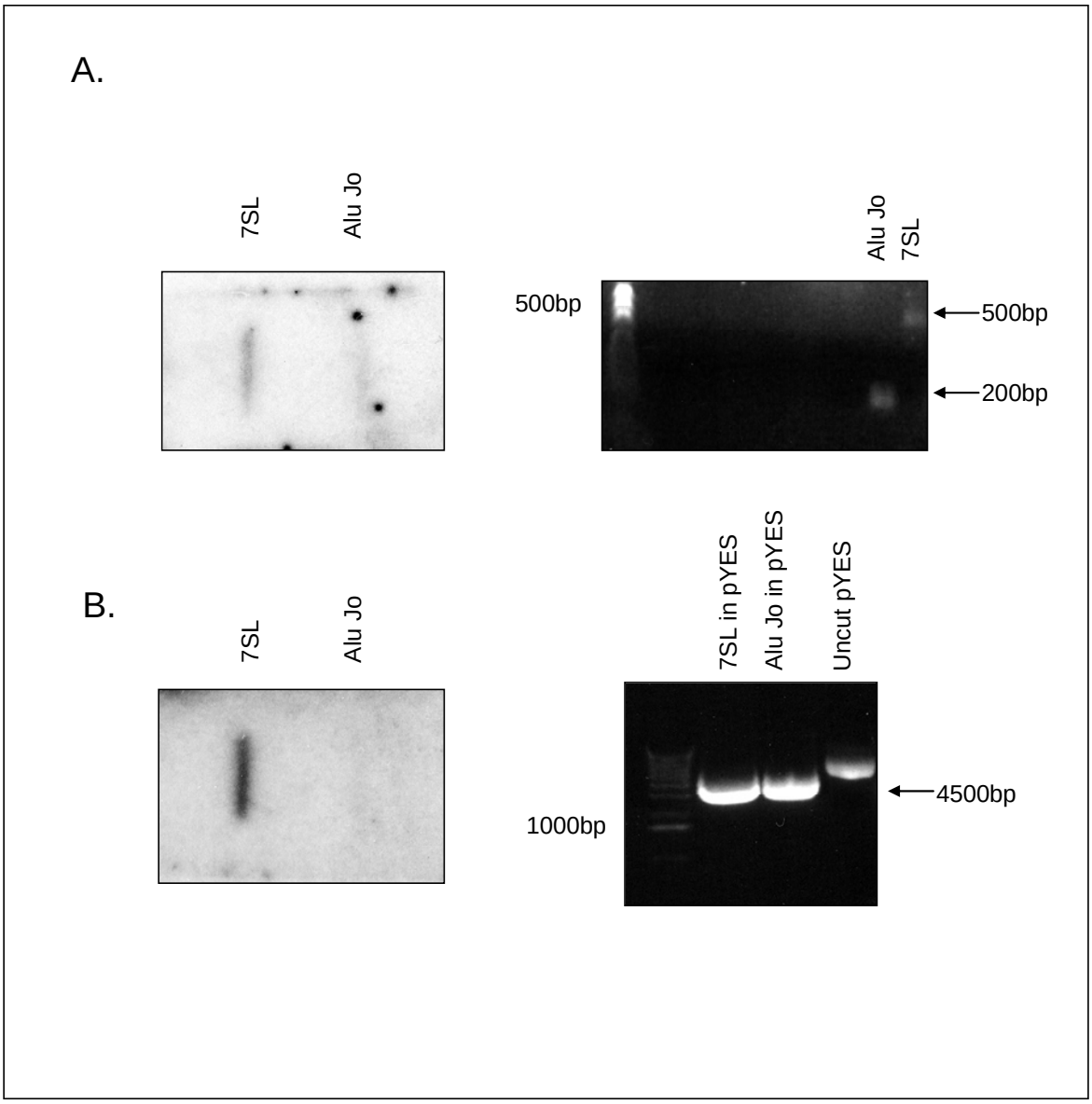


Figure 3-4 Nuclear run-on shows negligible levels of Alu Jo activity compared to 7SL in HeLa cells.
HeLa nuclei were defrosted and incubated in a reaction containing [α-^{32}P]UTP for 30 mins. Only the transcripts initiated before freezing can proceed and incorporate the radioactive UTP. This technique is therefore suitable for comparison of rates of transcription. (A) Slot blot showing nuclear run-on where the Alu Jo and the 7SL fragment were used as probes. (B) Slot blots showing nuclear run-on where linearised pYES2int vector containing Alu Jo or 7SL inserts were used as probes. Agarose gels on the right hand side show that equal amounts of probes were used. Marker sizes are indicated.

3.2.3 Despite the low transcript abundance, transcription machinery is present on Alus

Following analysis of Alu Jo's activity in transfected CCL cells, RT-PCR was performed to find whether there is indeed Alu Jo RNA present in HeLa cells. JoINF and PxuniqR primers were designed to ensure specific detection of the transcript. PxuniqR primer anneals to the tail of the left monomer of Alu Jo, which has a unique sequence. Two HeLa cDNAs were used and genomic DNA was used as a positive control for the PCR. Sample where no Superscript was added during cDNA synthesis was used as a control for genomic contamination. Figure 3.5 shows that very low Alu Jo transcript level was detected. Levels of the 7SL cDNA were approximately 1400 times higher than that of Alu Jo. Differences in the product levels can be partially explained by the fact that 7SL primers detect all 4 active genes. Also, annealing efficiency of the primer pair to each transcript could be different. However, the product from the genomic DNA templates suggests that JoINF/ PxuniqR primers anneal well and that a real difference between Alu Jo and 7SL RNA levels exists.

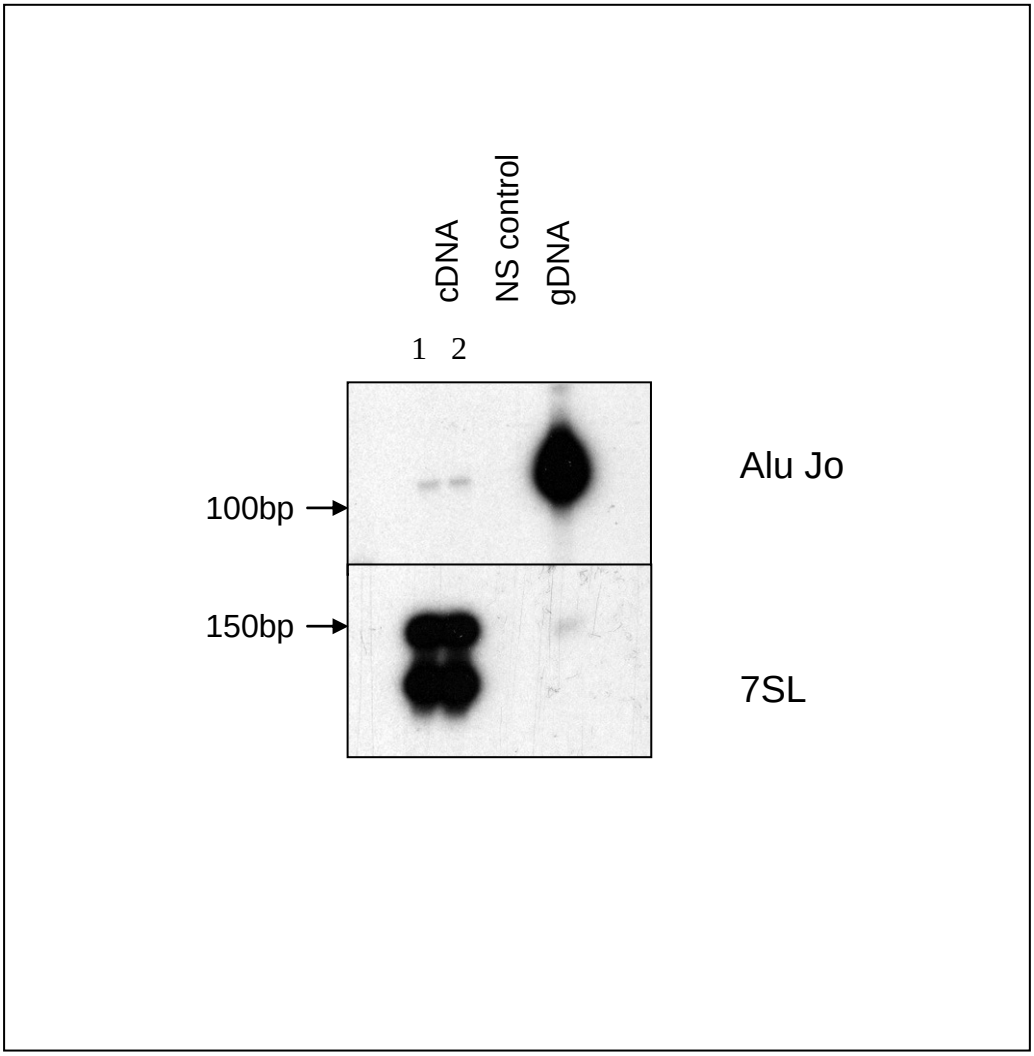


Figure 3-5 RT-PCR shows low level of Alu Jo RNA in HeLa cells.
JoinF and PxuniqR primers were used to detect Alu Jo transcript (lanes 1 and 2). Genomic DNA (gDNA) was used as a positive control. NS is a sample with no Superscript added during cDNA synthesis, used as a negative control for DNA contamination. 7SL was used as a control gene. Marker sizes are indicated. Autoradiograph.

JoINF/PxuniqR primers detected the left monomer of the transcript only, which could represent cytoplasmic processed Alu RNA - scAlu (Maraia et al., 1993; Matera et al., 1990). Further primer pairs were designed to detect the full length transcript and to determine whether the observed transcript is part of a longer transcript produced from a different promoter of another nearby Alu or a read-through transcript of RNA polymerase II (Figure 3.6). Localisation of each primer pair within or outside the Alu Jo sequence is shown on a diagram next to each PCR panel. HeLa cDNA and HeLa genomic DNA were used. Sample where no Superscript was added during cDNA synthesis was used as a control for genomic contamination. Top panel is RT-PCR using the JoINF/PxuniqR primers again as a control. The following panel shows the RT-PCR result using a forward primer designed for the adjacent 5' flanking sequence (Alu Jo genomicF) and PxuniqR primer. Next panel below shows the RT-PCR result using both primers placed outside of Alu Jo in the adjacent flanking sequence (Alu Jo genomicF/R). Last panel shows GAPDH product as additional positive control. Figure 3.6 shows that indeed full length transcript was detected in HeLa cells. However, RT-PCR using AluJo genomicF/R primers detected transcript containing the 5' and the 3' adjacent sequence (21 bp of the 5' end and 106 bp of the 3' end). This raises the possibility that Alu Jo is not transcribed from its own promoter, but the Alu Jo RNA is part of a longer, read-through transcript.

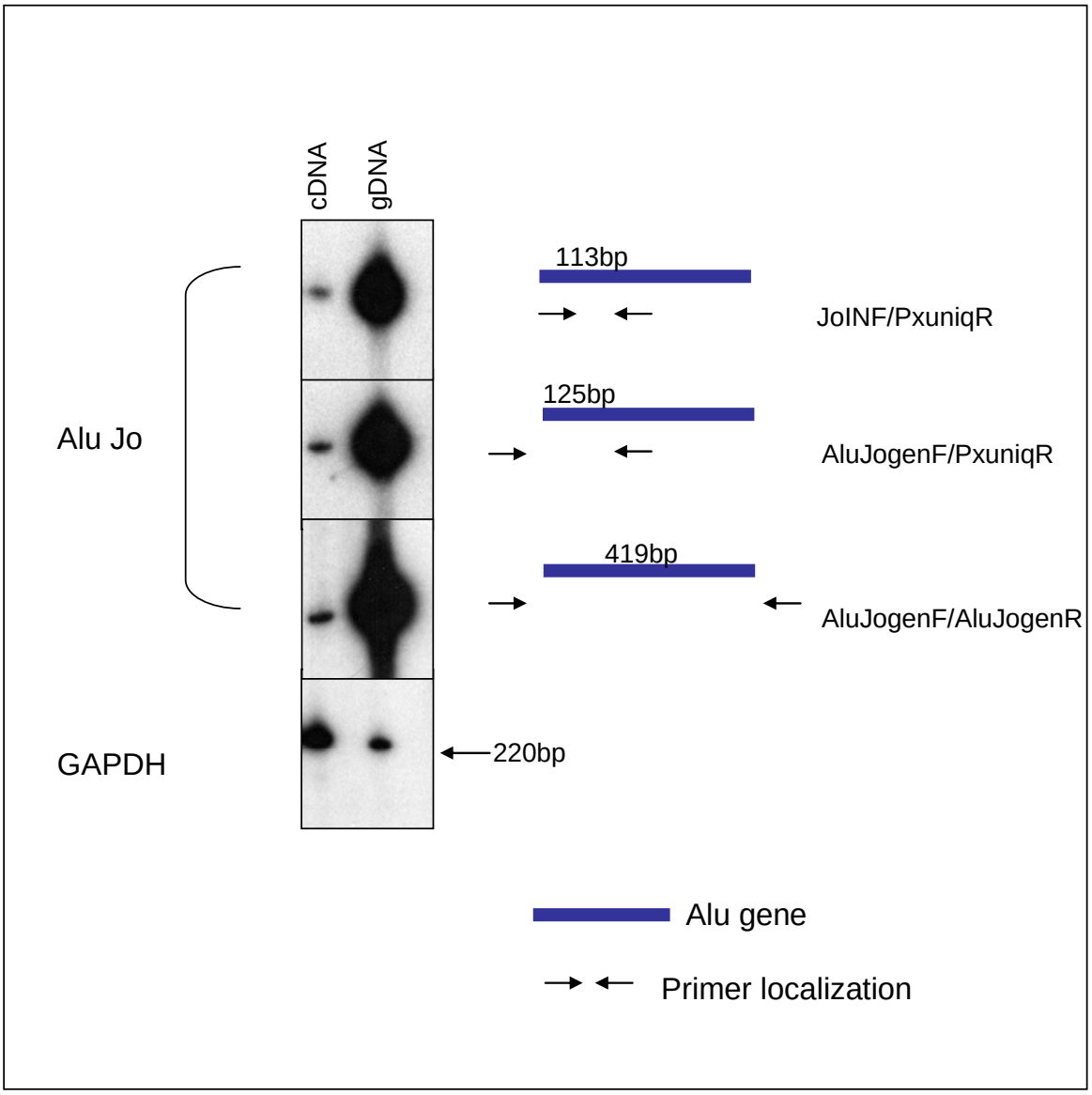


Figure 3-6 RT-PCR detects flanking sequence in the Alu Jo transcript.
Different sets of primers were used for RT-PCR of HeLa cDNA. Samples with no Superscript added during cDNA synthesis were used as a negative control for DNA contamination (data not shown). HeLa gDNA was used as a positive control for the PCR reaction. The diagrams next to the PCR panels show the localization and product sizes of each primer pair designed to detect transcript containing Alu Jo and its flanking sequence. GAPDH was used as a positive control. Autoradiograph.

It was further examined whether the observed Alu Jo transcript is RNA polymerase II- or RNA polymerase III-derived. HCT116 cells were subjected to ChIP analysis using antibodies against RNA polymerase III machinery components together with antibody for RNA polymerase II (Figure 3.7). Analysis included other Alus to check that the presence of RNA polymerase III machinery is not only a unique property of this Alu Jo. Primers were designed to look at individual Alus located on different human chromosomes (6, 10, 22). Alu19 is a primer set aimed at the middle part of a long stretch of tandem Alu repeats on another locus at chromosome 19. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. The U1 snRNA gene is a RNA polymerase II-transcribed gene and its promoter region was used as a positive control for RNA polymerase II antibody and a negative control for RNA polymerase III antibody. The 7SL gene was the reverse control, positive for the RNA polymerase III antibody and negative for the RNA polymerase II antibody.

RNA polymerase III machinery - transcription factor TFIIIB, transcription factor TFIIIC and RNA polymerase III - was detected at all studied Alus with very low levels at Alu19. On the other hand, RNA polymerase II and transcription factor TFIIB were only detected on the U1 snRNA gene promoter. The selective absence of RNA polymerase II suggested that the Alu Jo was not associated with RNA polymerase II and therefore the transcript is derived from either its own promoter or it may also be driven from a nearby RNA polymerase III-transcribed SINE. Both options occurring together are also possible.

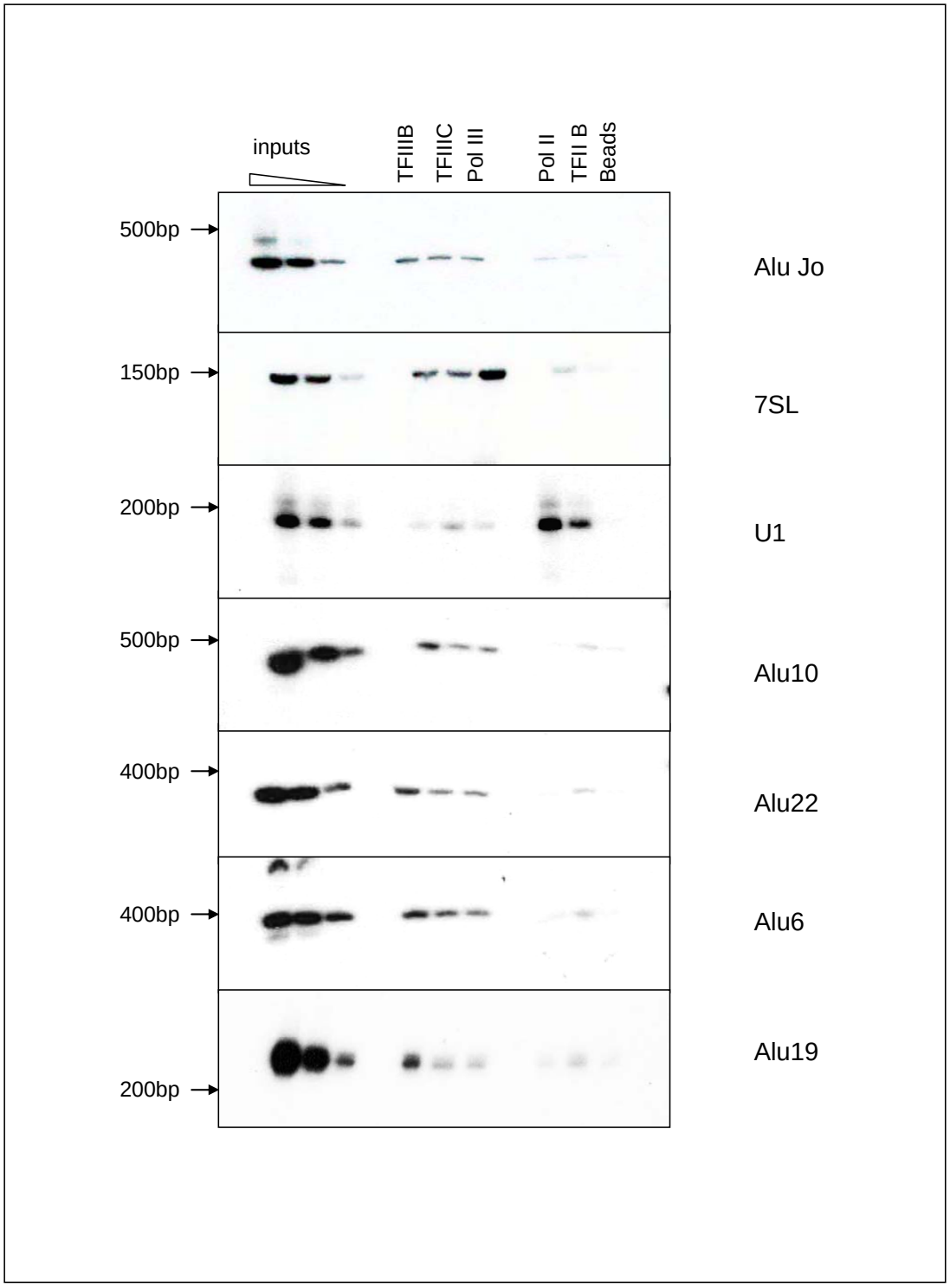


Figure 3-7 ChIP analysis shows the presence of the RNA polymerase III machinery but not the RNA polymerase II machinery on Alu genes in HCT116 cells.
ChIP analysis included Alu Jo together with Alus on other chromosomes and Alu19 (located in a cluster of SINEs on another location on chromosome 19). 7SL and U1 snRNA were used as reciprocal controls for RNA polymerase III and RNA polymerase II, respectively. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. Antibodies used in the ChIP analysis are listed at the top of the panel. Samples with beads-only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and H (human) in the third column of the table. Alu Jo was amplified using AluJogenomicF/R primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

It was surprising that all components of the RNA polymerase III transcription complex were found on Alu Jo given its low transcription rate. ChIP analysis was performed again using antibodies against TFIIIB, TFIIIC and RNA polymerase III, together with antibodies for acetylated histone H3 (directed against acetylated lysine 9 and 14) and H4 (directed against acetylated lysine 5, 8, 12, 16), both often associated with transcriptionally active genes (Kuo et al., 1996) (Figure 3.8). TFIIA was used as a negative control antibody. 7SL and tRNA ^{Sec} were used as positive control genes. As 7SL primers used here detect all 4 active genes and three pseudogenes, tRNA ^{Sec} was used because it is a single copy gene and therefore signal could be compared with the signal on a single Alu Jo. RNA polymerase III transcriptional machinery and both acetylated H3 and H4 were detected on both active genes and on the Alu Jo. TFIIIB on tRNA ^{Sec} was not detected (background signal only). This is expected because the tRNA ^{Sec} gene actually binds Brf2 (TFIIB-related factor 2) rather than Brf1 (TFIIB-related factor 1) present on other type 2 promoters (Fairley, unpublished data); TFIIIB antibody used here is directed against Brf1, hence the lack of signal. It was also noticed, that in both ChIP experiments, the occupancy of RNA polymerase III on Alus seems to be lower than on the active genes (7SL and tRNA ^{Sec}) relative to TFIIIC.

It has been shown so far that Alu Jo has a promoter that is active both *in vitro* and *in vivo*. Also, RNA polymerase III machinery was detected on the Alu Jo's promoter by ChIP analysis. Despite that, nuclear run-on analysis and RT-PCR analysis suggested that the rate of transcription of Alu Jo is very low when compared to 7SL. If the occupancy of the RNA polymerase III is indeed lower, this could mean that despite TFIIIB and TFIIIC being present, there may be a difficulty in the last step of the transcription complex assembly, recruitment of RNA polymerase III itself.

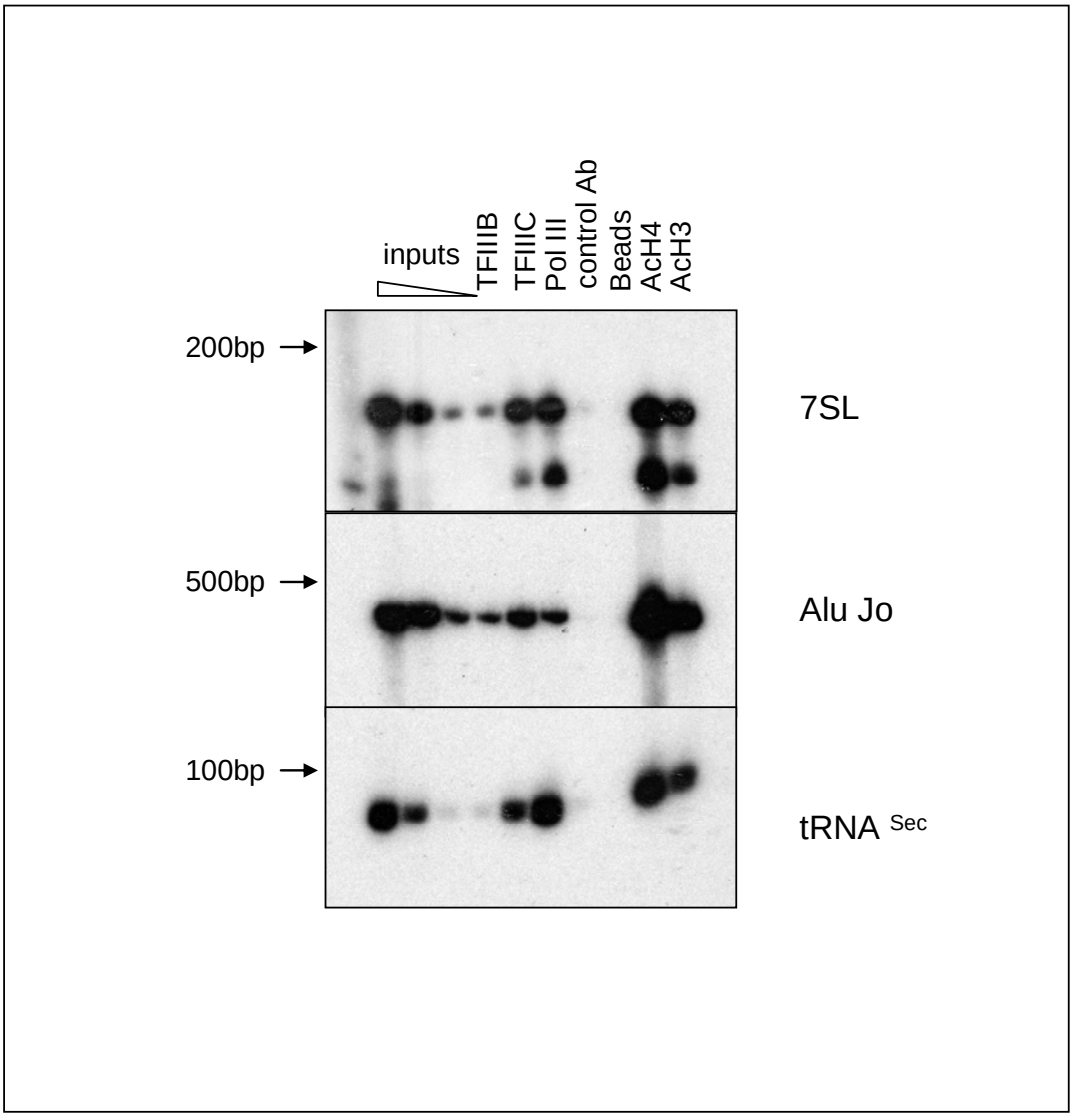


Figure 3-8 RNA polymerase III machinery and acetylated histones are present on Alu Jo in HeLa cells.
ChIP analysis of the RNA polymerase III machinery and acetylated H3 and acetylated H4 on Alu Jo. 7SL and $tRNA^{Sec}$ genes were used as positive controls. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. Antibodies used in the ChIP analysis are listed at the top of the panel. TFIIA antibody was used as a negative control antibody. Samples with beads-only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C in the second column of the table. Alu Jo was amplified using AluJogenomicF/R primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

HeLa and HCT116 cells were therefore subjected to more ChIP analyses and statistical analysis of the occupancy signals of the RNA polymerase III transcription complex (TFIIIC-TFIIIB- RNA polymerase III) was performed. The occupancy signals of TFIIIB, TFIIIC and RNA polymerase III at 7SL and different Alus (Alu Jo, Alu6, Alu22, Alu19) were quantified and the ratios of TFIIIB/TFIIIC, RNA polymerase III/TFIIIB and RNA polymerase III/TFIIIC were calculated. Images were quantified using ImageJ software according to ImageJ's instructions (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). ImageJ is a public domain Java image processing program. It quantifies gray values and the number of pixels found for each gray value for each band. It uses a simple graphical method that involves generating lane profile plots, drawing lines to enclose peaks of interest, and then measuring peak areas. Nine different ChIP analyses were used and 9 7SL gene analyses and 12 Alus were included. Both two-tailed t-test analysis and student t-test analysis were performed and showed that there is a significant ($p<0.05$) difference in the occupancy ratios between the two sets of samples (Table 3.1).

	Mean	St dev	T test P	Student t test P
7SL IIIB/IIIC	0.6623	0.3309	0.013	0.020
Alus IIIB/IIIC	1.1701	0.5245		
7SL RIII/IIIB	2.6134	1.7472	0.013	0.002
Alus RIII/IIIB	0.7518	0.3025		
7SL RIII/IIIC	1.3843	0.4341	0.008	0.007
Alus RIII/IIIC	0.8098	0.4364		

Table 3-1 Statistics of 7SL and Alu genes' occupancy by RNA polymerase III (RIII), TFIIIB (IIIB) and TFIIIC (IIIC).

Box and whisker plots show graphic representation of each sample sets' descriptive statistical values (Figure 3.9). As observed, there is a significantly higher level of RNA polymerase III to TFIIIB and RNA polymerase III to TFIIIC cross-linked on the 7SL gene than on Alus, showing that indeed, there is lower amount of cross-linked RNA polymerase III on Alus. It may mean that there is a problem with RNA polymerase III recruitment or its access to the Alu promoter. Alternatively, RNA polymerase III epitopes may be masked by other molecules present only on Alus, possibly also impeding its proper function. There is

surprisingly a significantly higher level of TFIIIB to TFIIIC detected on Alus. Possible explanation for this could be an epitope masking on TFIIIB (Brf1) when RNA polymerase III is present. That would lead to decreased signal on the 7SL gene.

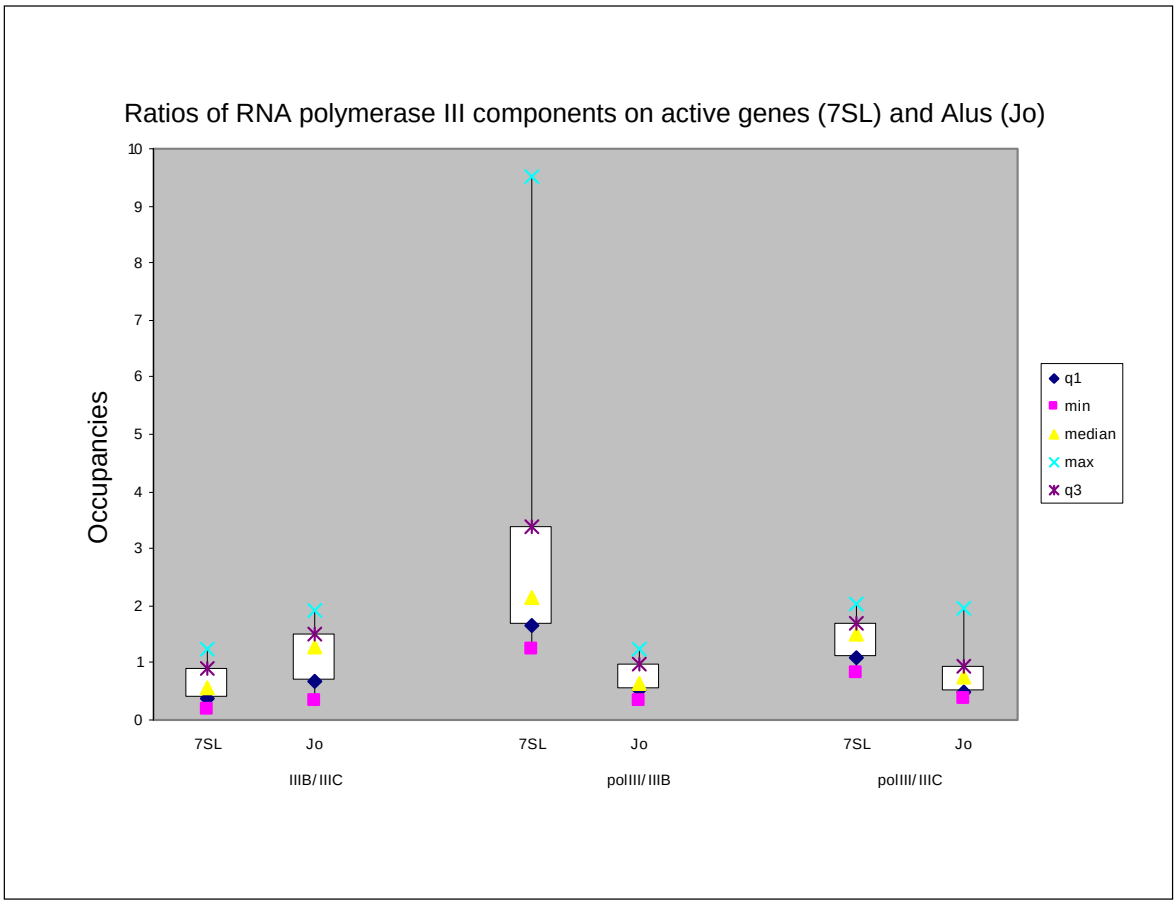


Figure 3-9 Ratios of RNA polymerase III machinery components on 7SL and Alus. Box and whisker plots chart shows graphic representations of descriptive statistical values found for each sample set. 9 ChIP analyses were included and 9 7SL gene and 12 Alus were analyzed. Ratios of TFIIIB/TFIIIC, RNA polymerase III/TFIIIB and RNA polymerase III/TFIIIC occupancies were measured by ImageJ.

3.3 Discussion

The PS subfamily of Alus comprises four old families called the Alu J, Sx, Sq and Sp families (Batzer et al., 1996). It was active 65 to 40 million years ago and accounts for approximately 85% of the total Alu elements in the human genome (Shen et al., 1991), but only 36% of the cDNAs representing full-length Alu transcripts belong to the PS subfamily (Shaikh et al., 1997).

In this chapter, Alu Jo was selected as a representative of this old Alu family in order to learn more about its transcriptional activity. It was characterised and studied *in vitro* and *in vivo*. It was active *in vitro,* but it gave a product longer than expected. Alu Jo, like other Alu elements, does not have a RNA polymerase III terminator sequence and RNA polymerase III therefore continues until it reaches a poly(T) signal in the adjacent sequence (Schmid and Maraia, 1992). Primer extension detected a product of Alu Jo-driven transcription. It was, however, difficult to detect Alu Jo's transcription in its genomic environment by primer extension. Its promoter is capable of driving transcription *in vivo,* as was shown by transient transfection, but even such activity needed to be amplified. These problems were encountered in similar studies before. Paulson and Schmid (1986) could only detect transcripts approximately corresponding to the expected Alu size at very high exposures. However, this band showed length heterogeneity, which was unexpected since Alus share a precise 5' end. Using more oligos derived from the Alu sequence, they found other products where at least one of them resulted from a strong stop for reverse transcription due to RNA secondary structure. After using more stringent conditions, which eliminated background bands, they saw a barely detectable product of expected size. In this chapter, RT-PCR proved to be more suitable than primer extension to study endogenous Alu transcripts. It is probable that due to the complicated secondary structure of CG-rich Alus, reverse transcriptase may have difficulty in traversing the stable secondary structure such that only very few molecules of cDNA were produced (Sambrook and Russell, 2001a). One solution would be to carry out the first strand synthesis at higher temperature using heat-resistant reverse transcriptase. However, random hexanucleotide primers could not be

used then, because some of the hybrids (RNA-DNA) will be unstable at temperatures at which the thermostable reverse transcriptase is active (Sambrook and Russell, 2001a).

There was clearly a scAlu transcript detected by RT-PCR. The level of this transcript was very low when compared to the 7SL. Paulson and Schmid also estimated the relative abundance of the Alu RNA compared with the 7SL RNA, as detected by primer extension, with 7SL RNA being at least 500-fold more abundant (Paulson and Schmid, 1986). This is less than the estimate in this study. 7SL RNA detected here was around 1400-fold more abundant. If 4 active 7SL genes (Ullu and Weiner, 1984) are detected by the primers and only one Alu Jo, each 7SL gene is expressed approximately 350-fold more than the Alu Jo. The ratio is not regarded as a precise number, but the inescapable conclusion is that there are very few copies of Alu Jo RNA in HeLa cells.

RT-PCR also detected the presence of the 5' upstream sequence in the Alu Jo's transcript as well as the 3' adjacent sequence. The 5' upstream sequence may be due to activity of a promoter somewhere upstream such as that the Alu RNA is then part of this read-through transcript. Jelinek et al. also detected 5' upstream sequences in Alu RNA (Jelinek et al., 1978). They reason that as there is Alu sequence present in hnRNA, Alu RNA may result from degradation products of hnRNA. Alu RNA forms duplex structures that are RNAse resistant. In this event, hnRNA degradation could result in Alu RNAs which are cleaved at sites near their duplex ends. Alu Jo could be transcribed as a part of the large NR1H2 RNA putatively derived from this genomic region (Figure 3.1). NR1H2 is a ubiquitously expressed nuclear receptor subfamily 1 group H member 2 (RefSeq NM_007121) encoding liver X receptor beta (LXR-β) transcription factor (Song et al., 1995). However, three other predictions for the NR1H2 start are almost 1.5 kb in the 3' end of Alu Jo and in general, NR1H2 is described in the UCSC browser to have genomic position chr19:55571497-55578079, again corresponding to the other three sequences. The putative NR1H2 sequence would need to be 50 kb compared to 7 kb of the other three NR1H2 sequences. It is therefore more likely to be a sequencing artefact. Due to the presence of RNA polymerase III on the Alu Jo promoter and little or no RNA polymerase II it is suggested here that the Alu Jo transcript may also be RNA polymerase III-derived. It can be derived from Alu Jo or from other nearby SINE. Alu Jo is

flanked in 5' sequence with AluSx, which would be transcribed in the Alu Jo direction. As it does not contain any T-run, RNA polymerase III could continue through to the adjacent sequence into Alu Jo. In the 3' end, however, Alu Jo is flanked by 3' end of LINE L1, which is RNA polymerase II-transcribed. The explanation for the presence of the 3' end sequence would be that Alu Jo, like other Alu elements, does not have a RNA polymerase III terminator sequence and RNA polymerase III therefore continues until it reaches a poly(T) signal in the adjacent sequence (Schmid and Maraia, 1992).

Several possible explanations for the low transcripts detected are discussed below. The presence of the 3' end in the Alu Jo's transcript could be a problem for the facilitated recycling pathway used for RNA polymerase III transcription (Dieci and Sentenac, 1996). It was shown that runoff transcription (without RNA polymerase III terminator) does not allow efficient recycling typical for active RNA polymerase III genes. Lack of this mechanism resulted in a much lower transcriptional rate. Future experiment would test this hypothesis by addition of a T-run into the Alu Jo's A-rich tail. In vitro transcription assay would compare levels of transcripts obtained using Alu Jo and Alu Jo/polyT in pGEM vector as templates.

Long tails of Alus also contribute to their instability (Li and Schmid, 2004) and this could be the case of Alu Jo too. The transcripts which are produced are unstable and quickly degraded and this could be the reason why only scAlu can be detected, as the left monomer is more stable (Li and Schmid, 2004). However, the nuclear run-on method is designed to detect newly initiated transcripts avoiding issues with RNA stability *in vivo*. Since transcripts are generated *in vitro* from RNA polymerase molecules that had initiated transcription when the nuclei were harvested, issues of transcript stability should be circumvented.

5' flanking sequence matching the first 37 nucleotides of the 7SL upstream sequence has been implicated as highly enhancing for *in vitro* transcription (Ullu and Weiner, 1985), but is not completely necessary (Dewannieux et al., 2003). It was not necessary in our hands, as Alu Jo inserted into the pGEM®-5Zf(+) plasmid does not contain flanking sequence and was still transcribed *in vivo* from this plasmid.

The presence of RNA polymerase III on Alu Jo and other studied Alus was surprising given the low level of its transcript. It was suggested previously that because Alus lack the appropriate upstream flanking sequences they cannot compete for RNA polymerase III transcription factors (Ullu and Weiner, 1985). However, this study showed that it is not the case for TFIIIB and TFIIIC. It is possible that having lower affinity for RNA polymerase III, Alus are out-competed by active RNA polymerase III transcribed genes. It was shown previously that transcription of a RNA polymerase III template can be lower if two templates were used in an *in vitro* assay and one of the two templates was added after the other (Bogenhagen et al., 1982). Detecting RNA polymerase on a promoter of a gene is regarded as a sign of transcriptional activity by some researchers (Alexiadis et al., 2007); however, given the low levels of transcripts detected normally in somatic cells, it suggests that the RNA polymerase III complex detected on Alus is very inefficient or poised. The conclusion from this work is that the problem may be in low occupancy of RNA polymerase III on Alu promoters. Lower amounts of cross-linked RNA polymerase III were detected on Alus by ChIP analysis, which could mean that RNA polymerase III has limited access to the promoter. It may also be that its epitopes are masked by some other proteins only present on Alus. In any case, RNA polymerase III is important for Alu's transcription. If RNA polymerase III is present in equal amount as on the 7SL genes, but its epitopes are masked, then its activity seems to be impaired compared to its activity on 7SL. What can be masking its epitopes or what can be impeding its proper access to the promoter or its function is unclear. However, DNA methylation could be the main candidate. It has been implicated in Alus' transcriptional repression before (Schmid, 1991). It is known in other genes to prevent access of the transcription machinery to the promoter. It also acts through transcriptional repression domains of the methyl-DNA-binding proteins or through the recruitment of repressor complexes. It will now be addressed in the following chapter in detail.

4 Chapter 4 – DNA methylation and Alu elements

4.1 Introduction

DNA methylation-mediated silencing and chromatin-derived silencing have long been investigated as possible causes of SINE's transcriptional repression. Alu sequences are CpG-rich and highly methylated (Schmid, 1991). In fact, more than 90% of methylated CpG dinucleotides in the human genome occur in retrotransposons (Bird, 2002). Association of H3K9 methylation, which is a marker of heterochromatin and is specifically associated with inactivation of gene expression (Lachner and Jenuwein, 2002), was demonstrated with Alu elements (Kondo and Issa, 2003), suggesting that H3K9 methylation may be related to suppression of Alu transciption through DNA methylation. There is evidence that methylation is connected with Alu transcriptional silencing both *in vitro* and *in vivo* (Li et al., 2000; Liu et al., 1994; Liu and Schmid, 1993).

DNA methylation was shown to inhibit Alu transcription *in vitro*, but only at low template concentrations (Liu and Schmid, 1993). At low template concentrations (10 to 50 ng template DNA used for the in vitro transcription assay), there was a 2.7- fold inhibition of APO Alu transcript expression due to methylation, but at high concentrations, inhibition was undetectable. This is probably because the methyl-DNA binding proteins are present in limiting concentration in the nuclear extracts. Flanking sequences did not alter the effect of methylation. The same research group then studied the effect of methylation on Alu transcription *in vivo* (Liu et al., 1994). HeLa cells were treated with 5-azacytidine to demethylate DNA and the DNA demethylation verified using enzymatic methyl-sensitive restriction digests. Alu transcripts were then detected using primer extension and northern blot. Treatment with 5-azacytidine resulted in a 5- to 8-fold increase in full length Alu transcript expression, although the effect on scAlu (left monomer only Alu) was much less pronounced.

Hela cells, 293 cells (human embryonic kidney cells) and K562 cells (an erythroleukemic line) were used for other *in vivo* studies of methylation-repressed transcription (Li et al., 2000). It was shown that naturally hypomethylated K562 cells have an unusually low level of Alu methylation compared to other somatic cells. Expression of Alu elements in all three cell

lines was investigated and it was found that K562 has extremely high levels of Alu RNAs compared to the other two. They contain Alu transcripts from different loci, indicating that many Alu loci were transcribed. Using actinomycin to block transcription, it was shown that these high levels are indeed due to transcription and not due to unusually stable transcripts. The three cell lines were further transfected with methylated and unmethylated Alu constructs. It turned out that in all the three cell lines, methylation inhibited expression of templates; in K562 cells, expression from unmethylated templates was 9- to 20-fold greater than from methylated templates.

The mechanism of methylation-induced repression could be either through direct repression of transcription due to exclusion of transcription factors which can not recognise a methylated version of their cognate DNA binding sites. Or transcription may be inhibited by the presence of the methyl-CpG binding domain proteins (MBD proteins), either directly, or through recruitment of transcription repressor complexes (see later). Alu elements were shown to bind to MBD columns when human genomic DNA fragments were examined (Brock et al., 1999; Shiraishi et al., 1999). Specifically, Alus were shown to be the main target sequences for MeCP2 binding *in vivo* (Koch and Stratling, 2004). However, MeCP2-mediated repression of Alu elements has not so far been demonstrated (Yu et al., 2001).

The role and the mechanism of DNA methylation in Alu silencing still needs to be clarified. Although DNA methylation is well documented to be connected with transcriptionally silenced genes and it was shown to be connected with Alu sequences and their repression, it is still not clear whether it is the primary silencing event or whether methylation occurs as a default mechanism, whereby a drop in the transcriptional potential of a gene leads to the spreading of DNA methylation to the promoter region to lock down gene transcription. For example, a stably transfected PV Alu repeat was transcriptionally silent in mouse cells, although unmethylated (Leeflang et al., 1992; Liu and Schmid, 1993). Alus demethylated by 5-azacytidine and transiently transfected into HeLa cells were inactive, whereas the identical templates were abundantly expressed in 293 cells (Liu et al., 1995). These studies show, that methylation is not necessary for repressing Alu transcription. In this chapter, the presence of methyl-DNA binding proteins on Alus was further investigated and DNA methylation-free systems

assayed to study the effect of removing DNA methylation on Alu transcriptional activity.

4.2 Results

4.2.1 Analysis of the Alu methylation status

As mentioned in the introduction Alu sequences are CpG-rich and have been repeatedly found to be highly methylated (Schmid, 1991). Moreover, majority of methylated CpG dinucleotides in the human genome occur in retrotransposons (Bird, 2002). To determine whether Alus used in this study are methylated, methylation sensitive restriction digest was performed with HeLa gDNA (Figure 4.1). HeLa cells were also treated with 8 μM 5-azacytidine (Sigma) for 8 days in an attempt to demethylate their DNA (Liu 1994). Three enzymes cut in Alu sequence, including the BstUI (only cuts when sequence is unmethylated), Tth111I and TaqI (Liu, 1994). Double digest with BstUI and Tth111I would give 251 bp product if BstUI site was unmethylated and no product if the site was methylated. TaqI is not methylation sensitive and double digest using TaqI and Tth111I releases 189 bp fragment that serves as a positive control. [α-^{32}P] dCTP-labelled Alu Jo or [γ-^{32}P]ATP end-labelled PV51 primer (data not shown) were used as probes for Southern blot. Alu Jo in pYES vector and Alu Jo fragment were loaded on the gel as controls for hybridization. Undigested HeLa gDNA served as additional control for the restriction digest.

Unfortunately, problems were encountered during the experiment, most likely in the restriction digest step (Figure 4.1). No product was detected in BstUI/Tth111I double digest in untreated HeLa. It could suggest that all Alus are methylated in HeLa cells. However, 5-azacytidine treatments did not result in any increase in the susceptibility of the Alu BstUI site to cleavage (lanes 4 and 6). Moreover, control TaqI/Tth111I double digest also did not result in any product (lanes 5 and 7). The difficulty was not likely to be at the hybridization step as the [α-^{32}P] dCTP-labelled Alu Jo probe hybridized to the control DNA samples (lanes 1, 2, and 3). The restriction digest was also repeated with MspI and HpaII (only cuts when sequence is unmethylated), but no products were obtained (data not shown). Bisulphite modification and methylation sensitive

PCR method using the CpGenome™ DNA Modification Kit (Chemicon® International) was tried next. HeLa gDNA was used first for the bisulphite reaction, where all unmethylated cytosines are deaminated and converted to uracils, while methylated cytosines remain unaltered. Primers were then designed using CpGWare™ Primer design Software (Millipore) to detect altered and unaltered Alu DNA. However, no products were detected in the PCR reaction (data not shown).

As difficulties were encountered it was not possible to establish experimentally whether studied Alus are methylated or not. Alu Jo was however one of human genomic fragments that bound strongly to a MBD column containing the MBD domain of MeCP2 (section 3.2.1), suggesting that it is methylated. For the other Alus it was necessary to rely on the evidence obtained from the literature and move on to establish whether Alus are bound by MBD proteins, which would also indicate whether they are methylated.

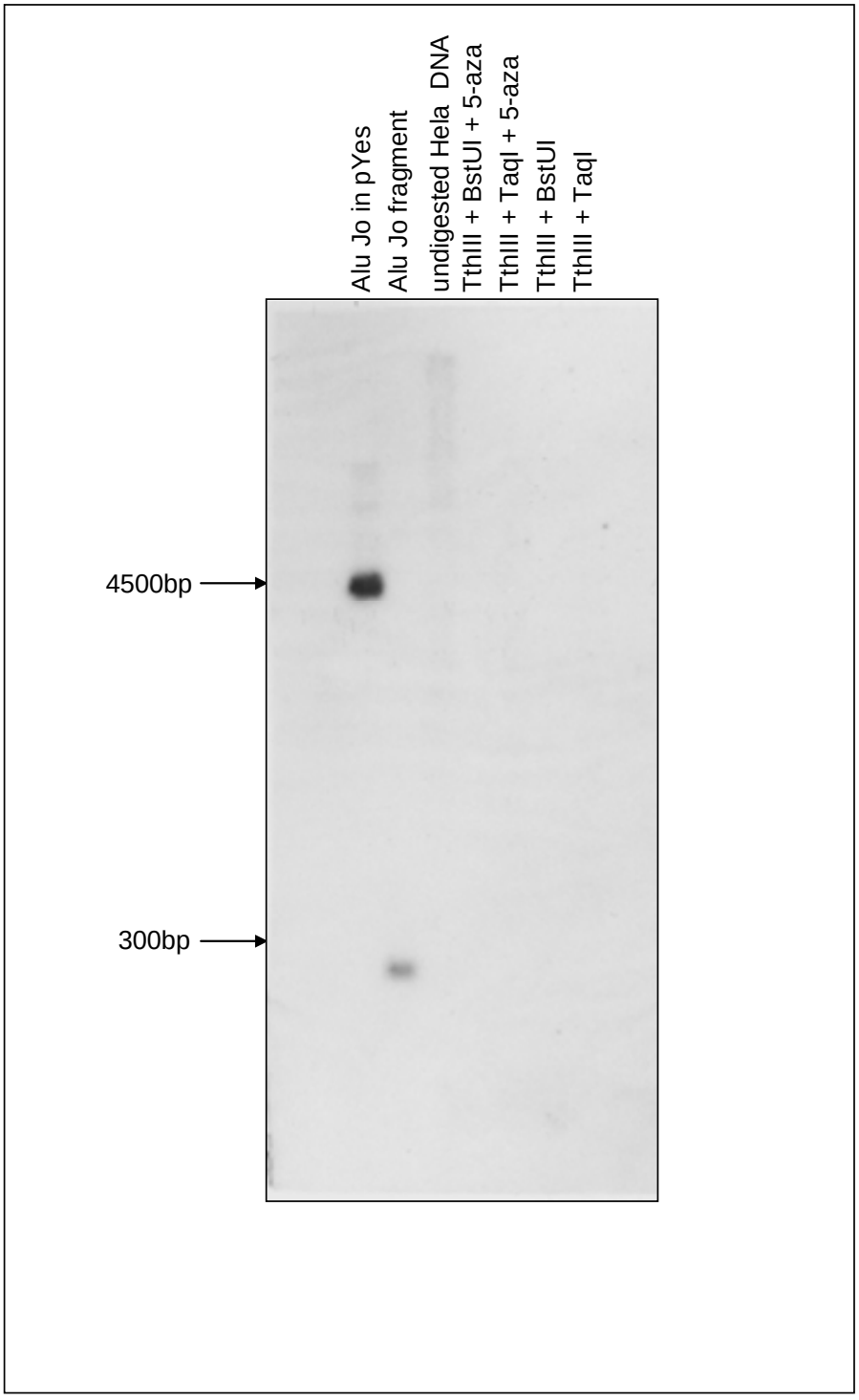


Figure 4-1 Southern blot analysis of Alu methylation.
Southern blot hybridization of genomic DNA extracted from 5-azacytidine treated and untreated HeLa cells was performed on DNAs digested with BstUI/Tth111I (lanes 4 and 6) and Taq1/Tth111I (lanes 5 and 7). Alu Jo in pYES vector, Alu Jo fragment and undigested HeLa gDNA were used as positive controls. [α-^{32}P] dCTP-labelled Alu Jo was used as a probe. Marker sizes are indicated.

4.2.2 Presence of MBD proteins on Alus

Methyl-CpG-binding domain proteins (MBD proteins) are the principle mediators of the repressive effect of DNA methylation (see Introduction). However, so far only MeCP2 has been shown to bind Alus and no repressive effect has been detected (Yu et al., 2001). The binding of MBD proteins was studied here using the chromatin immunoprecipitation assay, ChIP (Figure 4.2, 4.3). RNA polymerase III was used as a positive control. The previously described Alu Jo (see Chapter 3) and Alus on other chromosomes were examined. The 7SL gene was included as a negative control gene for MBD proteins and the Apolipoprotein E precursor (ApoE) gene, which is known to be bound by MBD1, MBD2 and MeCP2 and silenced by DNA methylation, was included as a positive control (Ballestar et al., 2003). ChIP analysis showed that MBD1 and MeCP2 (Figure 4.2) and MBD2 (Figure 4.3) were detected on Alus. MBD3 and Kaiso, other known contributors to DNA methylation-mediated silencing, were not tested here for lack of suitable antibodies. MeCP2 has been observed on Alus before and it indeed seemed to be giving the strongest signal on all the tested Alus. This is not due to a higher unspecific signal from the MeCP2 antibody, because it is not the strongest signal on the ApoE gene; ApoE seemed to be bound mainly by MBD1. MBD1 was also detected on Alu Jo and Alu 6. MBD2 was detected on all of the tested Alus.

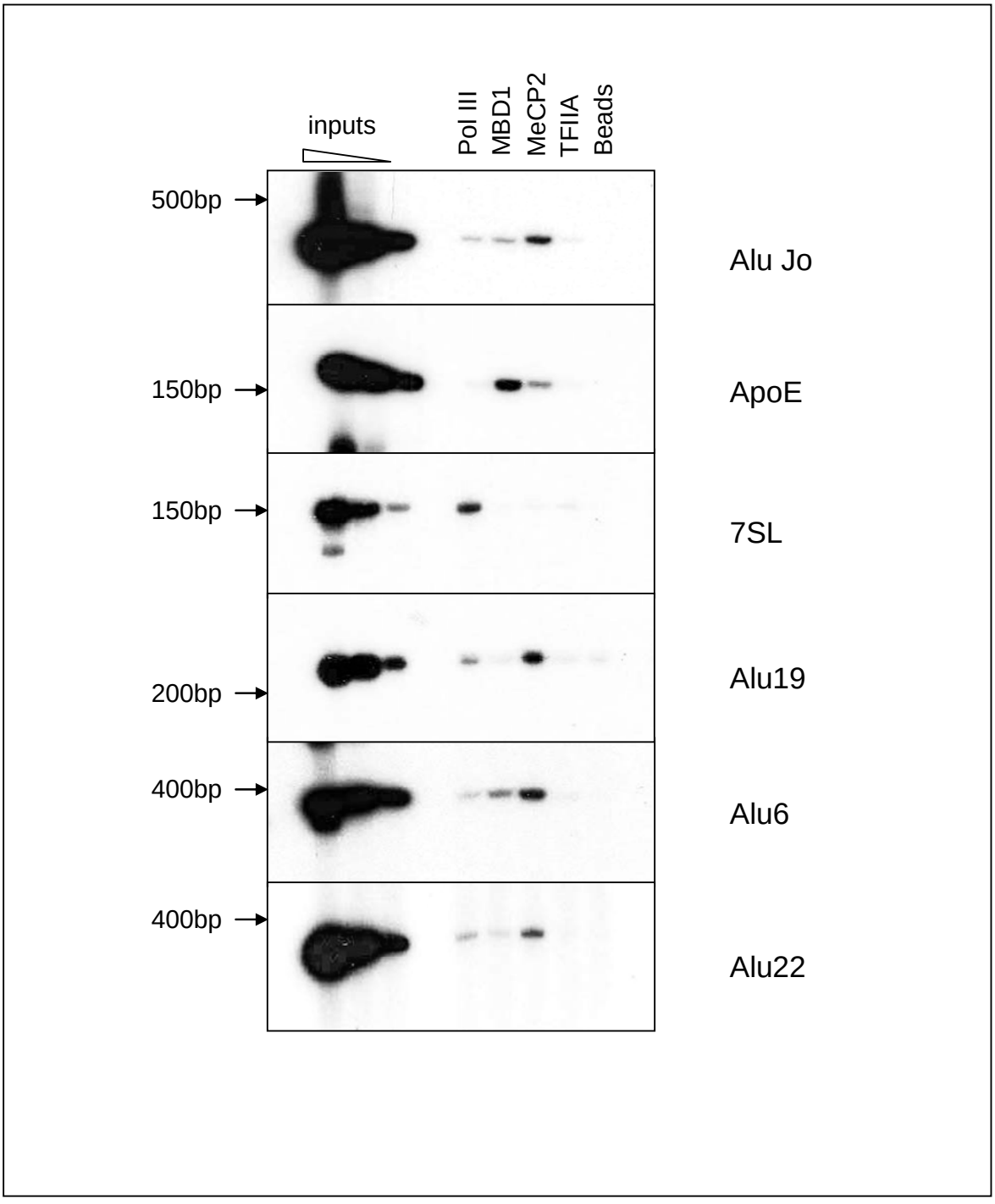


Figure 4-2 ChIP analysis shows the presence of MBD1 and MeCP2 on Alus in HeLa cells. ApoE gene was used as a positive control gene for MBD proteins. 7SL was used as a positive control for RNA polymerase III antibody. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA antibody was used as a negative control antibody. Samples with beads only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and H (human) in the third column of the table. Alu Jo was amplified using AluJogenomicF/R primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

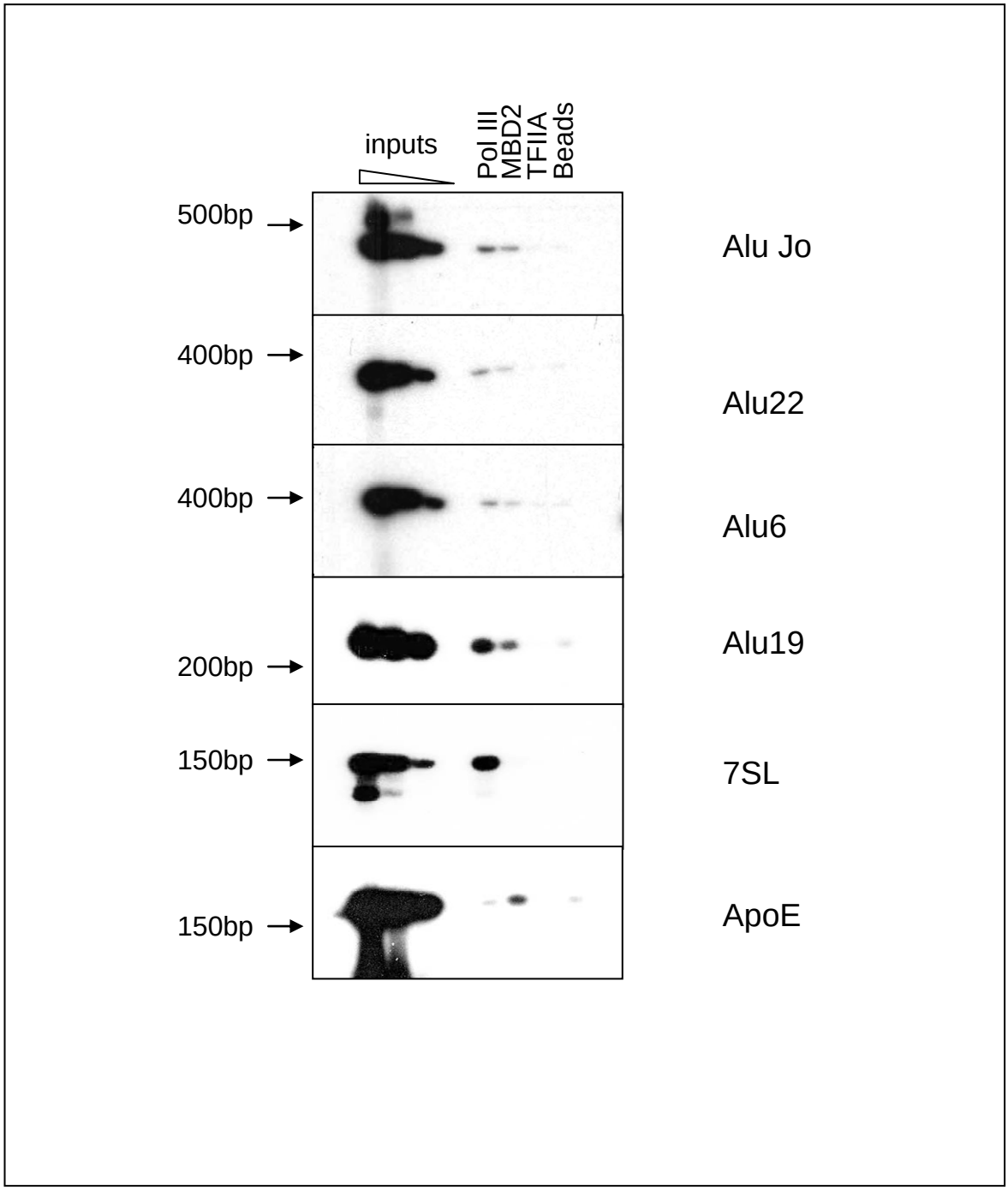


Figure 4-3 ChIP analysis shows the presence of MBD2 on Alus in HeLa cells.
ApoE gene was used as a positive control gene for MBD2 antibody. 7SL was used as a positive control for RNA polymerase III antibody. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA antibody was used as a negative control antibody. Samples with beads-only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and H (human) in the third column of the table. Alu Jo was amplified using AluJogenomicF/R primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

Two possible ways by which MBD proteins may repress transcription have been proposed. As they bind to methylated DNA they can directly prevent transcription factors from binding. In addition, they interact with repressor complexes which they recruit to the methylated DNA and establish transcriptional repression.

In chapter 3, it was discovered that Alus are bound by RNA polymerase III, TFIIIB and TFIIIC, but RNA polymerase III cross-linked significantly less to Alus than to the active genes, suggesting that something may be obstructing better binding. In the above experiment, MBD1, MBD2 and MeCP2 and RNA polymerase III seem to be present together on the Alus and their presence could explain the lower cross-linking of RNA polymerase III to the DNA. However, due to the nature of ChIP analysis it is necessary to rule out that RNA polymerase III and MBD proteins are in fact present on different copies of the same Alus. ChIP analysis uses populations of cells; it is therefore possible that although both RNA polymerase III and MBD proteins are present on a gene within the population, RNA polymerase III may be present on Alus in some cells and MBD proteins may be present on Alus in other cells, and not together. Such a situation would appear as co-occupancy in a single ChIP analysis. A modified ChIP assay, called the sequential ChIP, was used to determine whether RNA polymerase III and MBD proteins could be present at the same time on the same gene. Briefly, a ChIP assay was performed using 10 plates of cells per antibody (10 x more than in a normal ChIP assay) and the obtained supernatant was immmunoprecipitated with TFIIIC antibody (Ab7). TFIIIC-immunoprecipitated material was then diluted 1:10 and immunoprecipitated further with antibodies of interest. RNA polymerase III with TFIIIB were used here as positive controls as they co-occupy promoters together with TFIIIC. Signal from MBD proteins in the TFIIIC-immunoprecipitated material would mean that Alus are bound simultaneously by TFIIIC and MBD proteins.

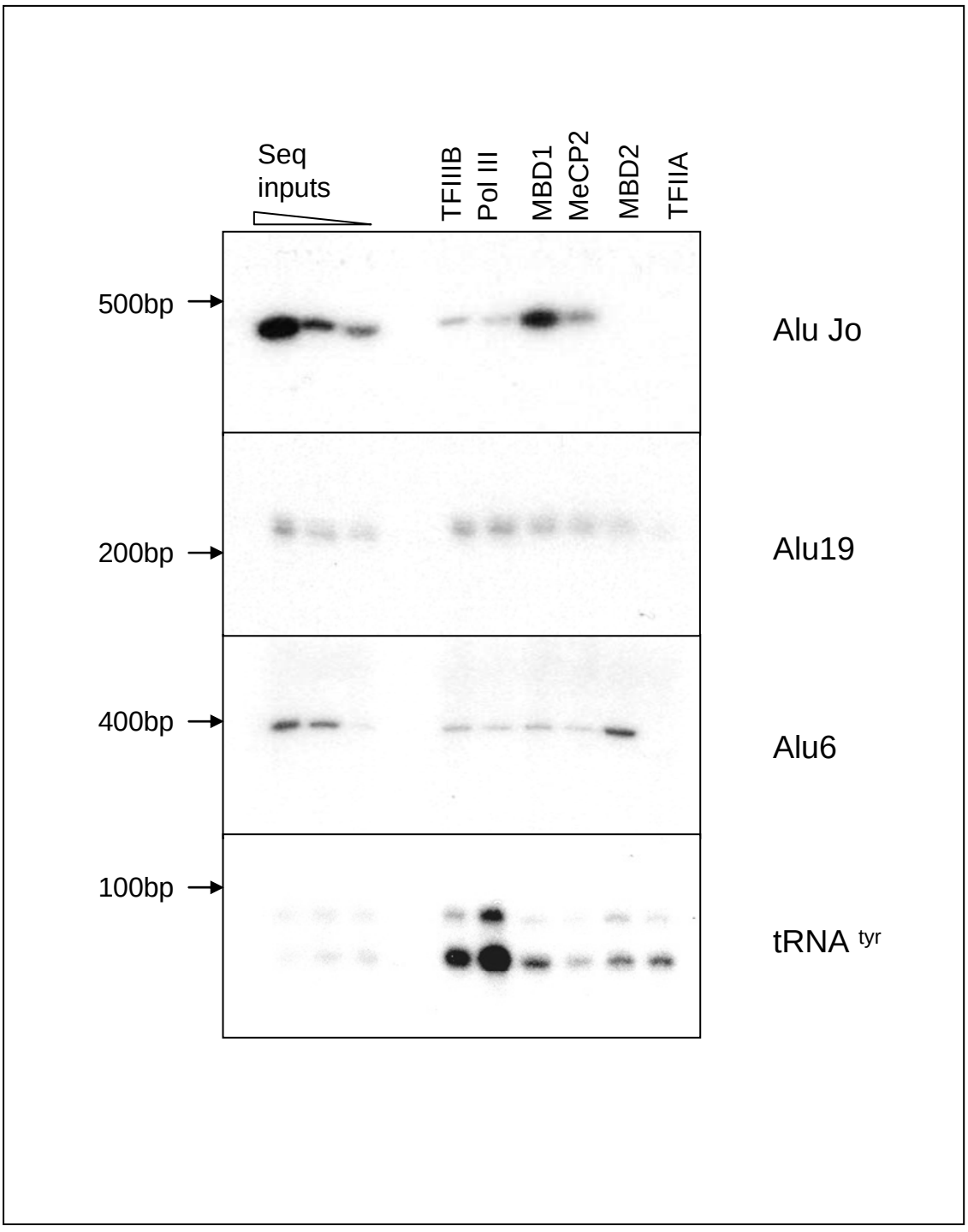


Figure 4-4 MBD proteins co-occupy Alu genes with transcription factor TFIIIC in HeLa cells. Sequential ChIP analysis. TFIIIC Ab (Ab7) was used in the first step (ChIP analysis). Precipitated material was diluted 1:10 in 1xNET buffer and further precipitated with the antibodies listed at the top of the panel (sequential ChIP). TFIIA was a negative control antibody. Input DNA was diluted to 10%, and precipitated. Sequential input was then again diluted to 20%, 10% and 5% to ensure quantitative PCR reactions. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and H (human) in the third column of the table. Alu Jo was amplified using AluJogenomicF/R primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

Figure 4.4 shows sequential ChIP performed in HeLa cells. Three distinct Alus, Alu Jo, Alu19 and Alu 6 were tested. tRNA ^{Tyr} is shown as a RNA polymerase III-transcribed active gene. Higher exposure was needed to detect its signal from sequential ChIP inputs, but it resulted in a background signal from MBD proteins showing at this exposure. It is considered a background signal as TFIIA (negative control antibody) is also showing at this exposure. Shorter exposure did not detect this signal (data not shown). As expected, TFIIIB and RNA polymerase III co-precipitated with TFIIIC on all tested genes, although the levels of TFIIIB and RNA polymerase III on Alus are lower than that on tRNA ^{Tyr}. As discussed in Chapter 3, there is significantly lower occupancy of RNA polymerase III on Alus then on actively transcribed genes (Figure 3.7 and 3.8). On contrary, higher occupancy of TFIIIB were observed on Alus than on actively transcribed genes. This was thought to be due to an epitope masking on TFIIIB when RNA polymerase III is present. This may not be the same if TFIIIB is co-precipitated by TFIIIC.

All tested MBD proteins also precipitated from the TFIIIC-bound material. Interestingly, some differences from standard ChIP analysis were detected in MBD proteins' occupancy. MBD1 rather than MeCP2 gave the strongest signal on Alu Jo and no MBD2 was detected. On Alu 6, MBD2 gave the strongest signal rather than MeCP2 (Figure 4.2, 4.3). This suggests selective occupancy of MBD proteins on Alus which are co-bound by TFIIIC.

In conclusion, MBD proteins were detected at the same Alus as transcription factor TFIIIC; therefore, its recognition of Alu promoters and binding is not obstructed by the presence of MBD proteins. This is in agreement with the previous statistical analysis of TFIIIC occupancy (Chapter 3), where the TFIIIC was comparable at Alus and at active genes. Binding of TFIIIC is the first step in RNA polymerase III transcription complex assembly. Further sequential ChIP using TFIIIB and RNA polymerase III in the first ChIP step will be needed to assess the co-occupancy of these proteins with MBD proteins and to assess whether MBD proteins may prevent TFIIIB and RNA polymerase III from proper binding.

Apart from direct inhibition, another major mechanism of repression by MBD proteins is through their cooperation with various repressor complexes. MBD

proteins were shown to be part of different chromatin remodelling and corepressor complexes. MeCP2 was shown to be associated with mSin3a and HDACs (Jones et al., 1998; Nan et al., 1998), but also with normally a transcriptional activator complex SWI/SNF via Brahma (Harikrishnan et al., 2005), although that was challenged by another study (Hu et al., 2006). MBD2 was shown to be associated with mSin3a and HDACs alone or as part of the MeCP1 complex (Boeke et al., 2000; Ng et al., 1999) and to interact with the HDAC1/2-containing NuRD complex (Zhang et al., 1999). MBD1 was shown to interact with the histone H3K9 methylase SETDB1 (Hu et al., 2006), but recruitment of SETDB1 was not tested here. In figure 4.5, the possibility that MBD proteins also bring these complexes to Alus was investigated using ChIP analysis. Three distinct Alus were studied and additional genes were used as controls. Apolipoprotein E precursor (ApoE) is a methylated gene and although it is not known whether it is silenced by any of these protein complexes or whether they are present, it was used here for a comparison of different occupancies of the complexes between an inactive gene known to be bound by MBD1, MBD2 and MeCP2 and the active genes 7SL and tRNA ^{Sec}. tRNA ^{Sec} is a single copy gene and therefore it also ensures looking at occupancies at a single gene, comparable for each Alu, where primers used for the PCR analysis were designed to look at a single sequence. 7SL is also an active gene, but there are inactive 7SL pseudogenes also detected with the primers used. MeCP2 antibody was used as a positive control. Beads and TFIIA are negative control antibodies. Brm, Brahma, represents a component of a SWI/SNF2 complex (Wang et al., 1996), Mi2 is a component of the NuRD complex (Zhang et al., 1998) and mSin3a is a component of the SIN3A/HDAC complex (Zhang et al., 1997). HDAC2 is a component of both NuRD and SIN3A complexes.

Figure 4.5 shows that of the proteins studied, Brm seems to be the protein only enriched on the genes associated with MBD proteins. It was not associated with the active genes. None of the other studied proteins – mi2, mSin3a and HDACs- were specifically associated with MBD proteins-bound genes. Mi-2 seems to be mainly enriched on the 7SL and Alu 6, with traces on all other studied genes. mSin3A was again mainly enriched on 7SL, with some signal detected on the Alu Jo, Alu 6 and ApoE. Although the occupancies of the proteins appear very low, it is important to regard them as present as they are all above the levels of negative control antibodies (TFIIA and beads). HDAC2 (and HDAC1, data not

shown) were present on all tested genes, repressed or active. Given the acetylated levels of histones H3 and H4 on RNA polymerase III-transcribed genes and Alu Jo (Figure 3.8), presence of HDACs is surprising. The role of HDACs at acetylated RNA polymerase III-transcribed genes and Alus remains unknown. In general, HDACs are usually associated with corepressor complexes and proteins (reviewed in (Knoepfler and Eisenman, 1999; Ng and Bird, 2000), but new evidence suggests that they may also act as activators of transcription (Nusinzon and Horvath, 2005; Sakamoto et al., 2004; Zupkovitz et al., 2006). The mechanisms of such transcriptional activation of HDACs are also not known.

The mSin3a and mi2 proteins were detected on the 7SL. However, they may be in fact associated with the 7SL pseudogenes, as they were not particularly enriched on the tRNA ^{Sec} gene.

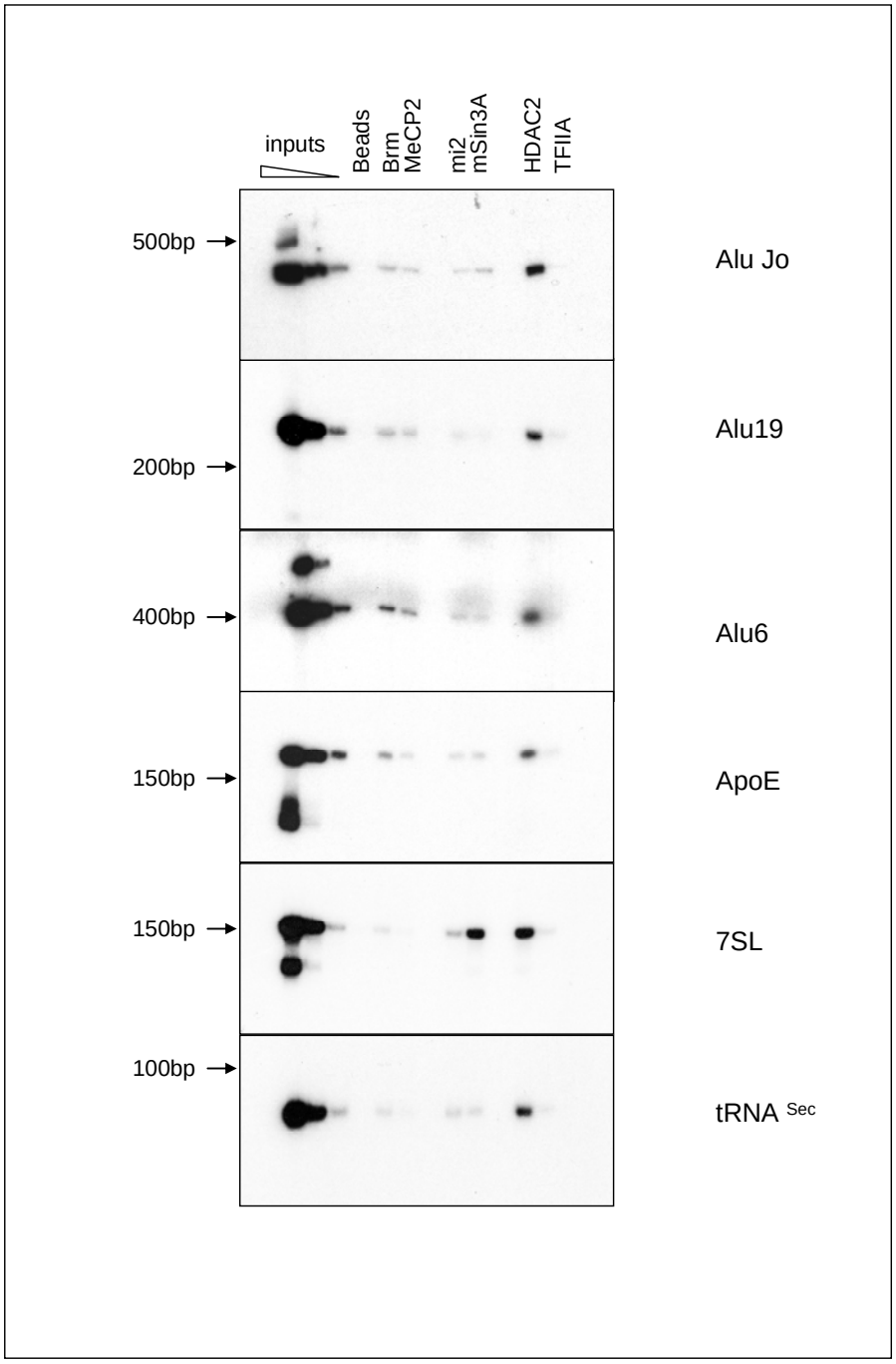


Figure 4-5 The occupancy of SWI/SNF2 and NuRD repressor complexes on Alus in HeLa cells.
ChIP analysis shows that Brm is the only protein that is specifically enriched on the genes associated with MBD proteins. Other tested proteins do not show specific occupancy on Alus. All antibodies used for the ChIP analysis are listed on the top of the first panel. Brm=Brahma, mi-2=helicase/ATP-ase, NuRD complex. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA antibody was used as a negative control antibody. Samples with beads-only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and H (human) in the third column of the table. Alu Jo was amplified using AluJogenomicF/R primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

In conclusion, Brahma (Brm) seems to be specifically associated with methylated genes. It is plausible that it is recruited via MeCP2, because Brahma has been shown to associate with MeCP2 before (Harikrishnan et al., 2005). It is a component of SWI/SNF2 chromatin remodelling complexes, which have been regarded for a long time as activators. New evidence shows that they can also be recruited as a part of repressive mechanisms to some genes (Watanabe et al., 2006). Other complexes may contribute to the repression of methylated Alus, whether recruited via MeCP2 (SIN3A and HDACs) or MBD2 (NuRD and HDACs), or independently as in the case of RNA polymerase III-transcribed genes 7SL (SIN3A and NuRD) and tRNA ^{Sec} (HDACs). So, methylated Alus may be regulated via MBD proteins, which potentially directly inhibit access or assembly of RNA polymerase III and TFIIIB (after TFIIIC binding), or may inhibit via their association with corepressors, where a Brahma-containing SWI/SNF complex may be specific to MBD proteins-directed Alu repression.

4.2.3 Alu activity in Saccharomyces cerevisiae

Another approach for analysing the effect of methylation on Alu silencing is to remove or inhibit methylation and study Alu activity in a methylation-free environment. Previous studies used 5-azacytidine to reduce DNA methylation (Liu et al., 1994) or K562 cells with naturally hypomethylated DNA (Li et al., 2000). 5-azacytidine treatment of HeLa cells was also tried here in order to demethylate DNA, but complete demethylation was never successfully achieved (tested with enzymatic methyl-sensitive restriction digests, data not shown). As no methylation-free human or mammalian cells were available at the time, an alternative *in vivo* system was searched for. It was known that yeasts as well as other fungi do not have endogenous DNA methylation (Antequera et al., 1984). Because yeasts have been routinely used as model systems to study gene transcription and its regulation, integrating Alu Jo into the yeast genome and studying its transcription in the yeast genomic environment was considered. This would bring a novel *in vivo* system for studying Alu element biology and it could be potentially very interesting for the following reasons. Not only do both *S. cerevisiae* and *Schizosaccharomyces pombe* have no detectable DNA methylation

(Antequera et al., 1984; Hendrich and Tweedie, 2003), but *S. cerevisiae* also has no histone tail methylation connected with chromatin-mediated silencing (Sims et al., 2003). Also, the genome of *S. cerevisiae* is well characterised (Goffeau et al., 1996). Yeasts have no endogenous Alu elements. There are 5 retroelements in *S. cerevisiae*, Ty1-Ty5, but these are transcribed by RNA polymerase II (Bolton and Boeke, 2003). The yeast 7SL gene shows just 12% homology with human 7SL and this fact, together with no polymerase II-derived Alu RNAs normally present in the mammalian cells, avoids common cross hybridisation of probes with 7SL and RNA polymerase II-derived Alu transcripts. Also the RNA polymerase III transcriptome has been well studied (Roberts et al., 2003). It makes yeasts an interesting model system for studying Alu elements in a methylation-free system.

The budding yeast *S. cerevisiae* strain used in this study was the GGBY 62 strain, his4-912Δ lys2-128Δ ura3-52 (collection of Dr McInerny). Cells were grown on a complete media with depleted uracil (Formedium LTD, England). Alu Jo was integrated using pYES2int vector (Invitrogen) which has the 2μORI fragment removed. GGBY 62 cells were transformed using the lithium acetate method, as described in Materials and Methods. Inserting the vector into the *ura3* locus in budding yeast was facilitated by digestion with *Stu*I, which linearised the vector within the *ura3* gene. Homologous recombination between the URA3 gene in the pYES2int vector and mutated *ura3* (*ura3-52*) on chromosome V replaced the deficient copy of this gene and enabled cells to grow on media with depleted uracil. This recombination process also integrated the Alu Jo element into the *S. cerevisiae* genome. It was further verified by PCR analysis using JoINF/JoINR primers designed to detect full-length Alu Jo (figure 4.6a). Untransformed GGBY cells (wt) and GGBY cells transformed with pYES2int only (empty pYES) were used as negative controls. Purified Alu Jo in pYES2int was used as a positive control for the PCR. Three different cell clones were obtained, Jo11, Jo13 and Jo15. Jo13 cells did not grow very well on media with depleted uracil and although some Alu Jo was detected by PCR, it is possible that it was an incomplete integration or it acquired significant mutation in the ura3 locus.

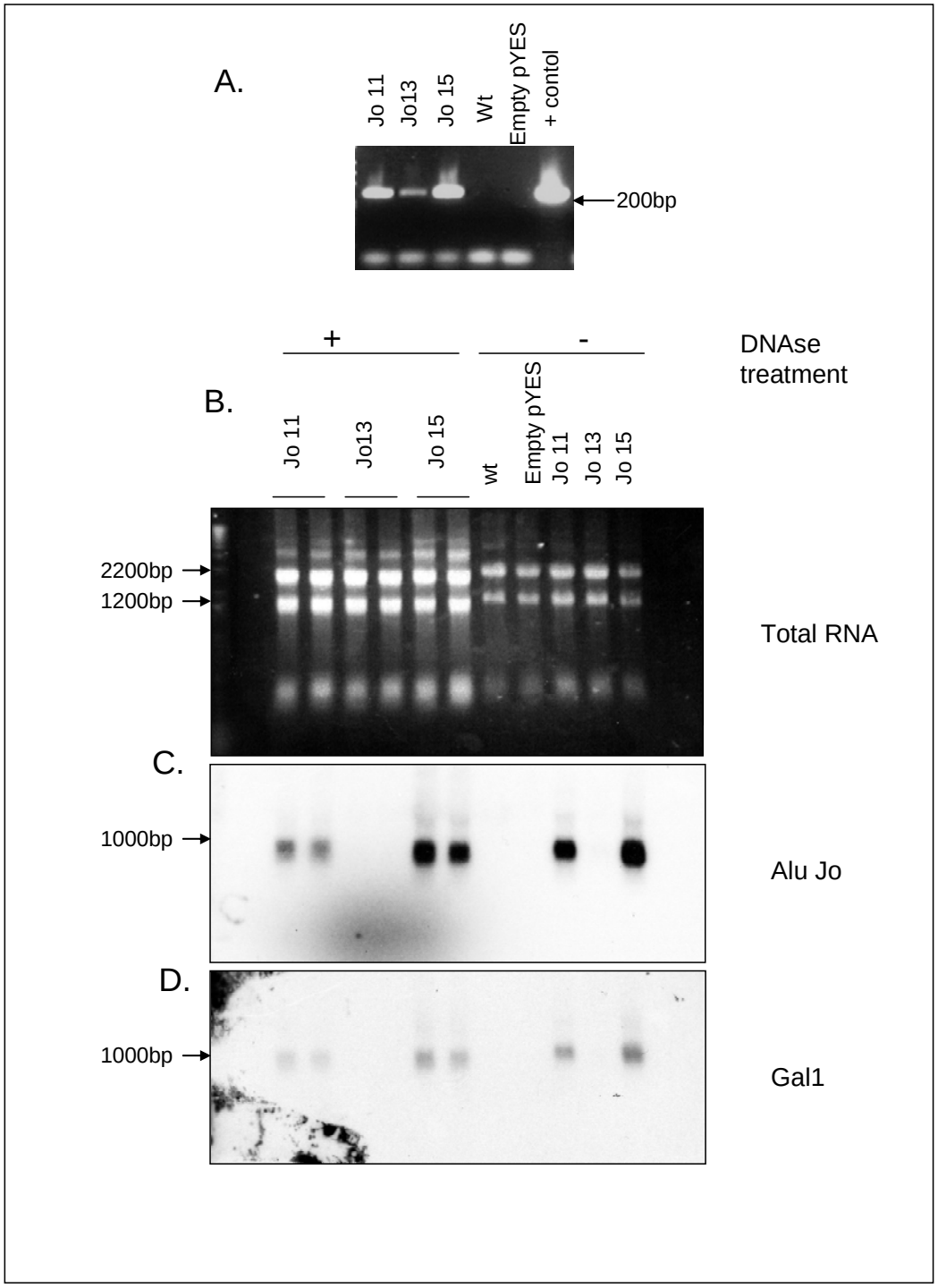


Figure 4-6 Alu Jo integrated into the S. cerevisiae genome is active.
(A) Agarose gel showing stable integration of Alu Jo into three *S.cerevisiae* lines. PCR used gDNA of each transformed cell line. gDNA of GGBY 62 wt and cells transformed with empty pYES were used as negative controls. Purified Alu Jo in pYES was used as a positive control. (B) Agarose gel shows equal loading of RNA used for Northern blot. Total RNA was extracted from each transformed *S.cerevisiae* line. Right hand side of the panel shows RNA treated with DNAseI to prevent contamination with gDNA (C) The Alu Jo transcript detected using Alu Jo fragment as probe in two yeast lines. Northern blot. (D) The Gal1 probe detects transcript driven by upstream Gal1 promoter. Northern blot. The right hand side of the panel shows RNA treated with DNaseI to prevent contamination with gDNA. GGBY 62 wt and cells transformed with empty pYES were used as negative controls. Marker sizes are indicated.

Total RNA was then extracted from the cells and separated on a formamide gel and figure 4.6b shows equal loading of samples. The activity of integrated Alu Jo was examined by Northern blot (figure 4.6c). Samples were treated with DNase I (Ambion) to ensure that signal is not due to DNA contamination. Jo11, Jo13 and Jo15 samples after DNAse I treatment are shown in duplicates. Samples not treated with DNAse I were also loaded and examined, as these included RNA from untransformed GGBY cells (wt) and cells transformed with empty pYES2int (empty pYES) as negative controls. Labelled purified Alu Jo fragment was used as a probe. Figure 4.6c shows that Alu Jo is expressed in both Jo11 and Jo15 cells, but not in Jo13 cells. Weak signal detected in PCR reaction in figure 4.6a and lack of signal in the Northern blot analysis further suggests that pYES2int plasmid may not integrated properly in Jo13 cells. The possibility that it is present in these cells as a circular plasmid is unlikely, as the removed 2μORI fragment prevents it from being replicated.

However, it was also noticed that when the Alu Jo signal was stripped (verified by exposure of film on the blot for several days) and reprobed with Gal1 probe, there was a weak signal. The Gal1 promoter is upstream of Alu Jo on the pYES2int plasmid (figure 4.6d). It is normally inactive in the presence of 2% glucose or absence of galactose (Flick and Johnston, 1990). Interestingly, Gal1 promoter is only active where Alu Jo is active and it is inactive if inactive Alu Jo is near it (Jo13) or if present on empty pYES2int. It is therefore likely that Alu Jo is stimulating the activity of the Gal1 promoter, even in the absence of galactose. Such effects have been described previously for various RNA polymerase III-transcribed genes, such as tRNAs (see Discussion). In this experimental system it is, however, undesirable as it would complicate the experiment.

Alu Jo activity in yeast cells was also verified by RT-PCR analysis (figure 4.7). Yeast actin was used as a loading control and full length Alu Jo was assayed using JoINF/JoINR primers. Samples where reverse transcriptase was omitted during cDNA synthesis were used as controls for genomic contamination. Figure 4.7 again confirmed that Alu Jo was actively transcribed in Jo11 and Jo15 GGBY

transformed cells. Due to the unusual activity of the Gal1 promoter, this experiment was not pursued any further.

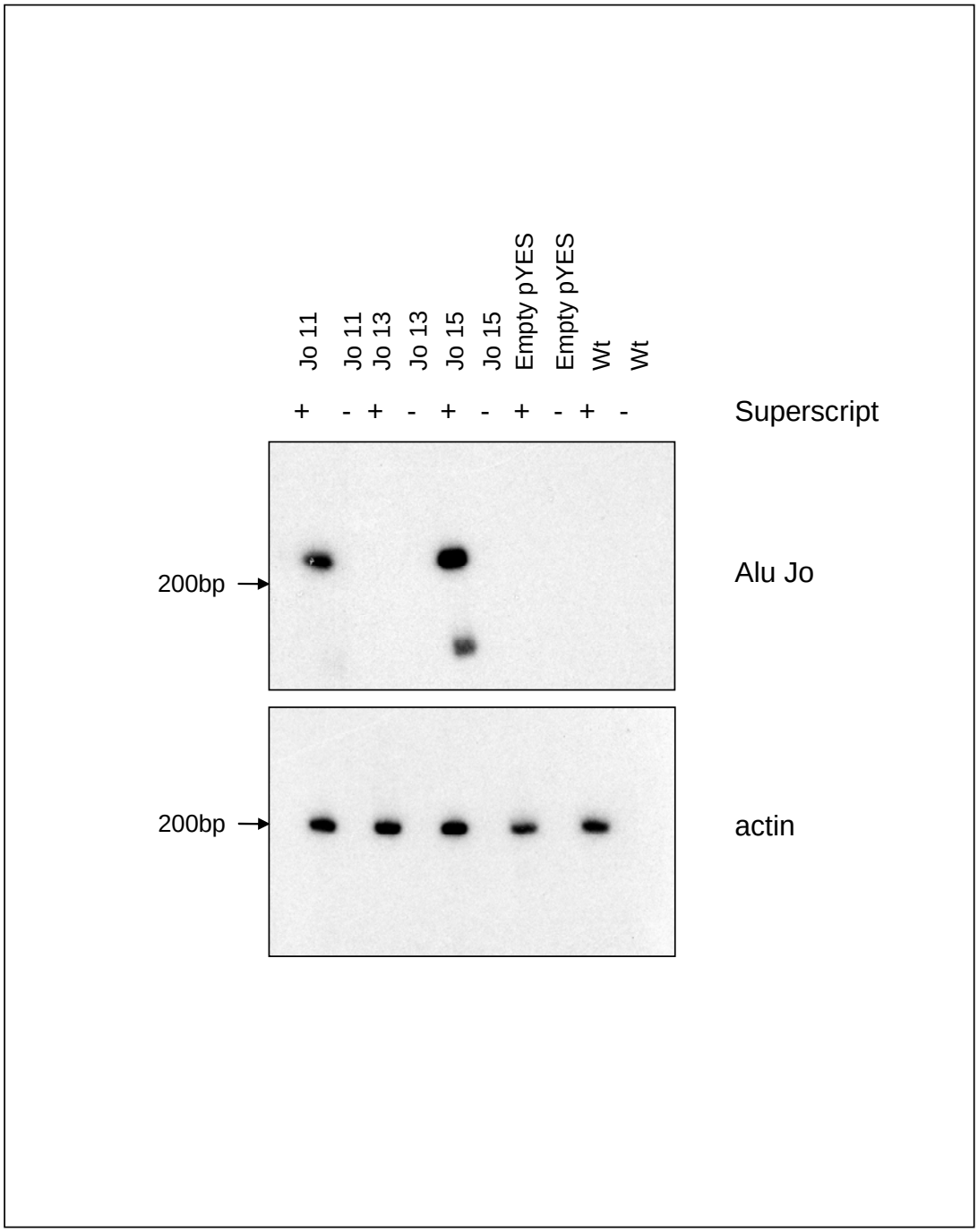


Figure 4-7 RT-PCR confirmed Alu Jo activity in S. cerevisiae.
RT-PCR using gDNA of each transformed cell line. gDNA of GGBY 62 wt and cells transformed with empty pYES were used as negative controls. Samples where no reverse transcriptase (- Superscript) was added served as negative controls. Endogenous actin was used as a control for the RT-PCR. Alu Jo was amplified using AluJoINF/R primers. Actin was amplified using actin S.C. primers listed in Table 2-3. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

4.2.4 HCT 116 DKO cells to study the effect of methylation on Alu silencing

Later during the course of this study, the colorectal cancer cell line HCT-116, in which two major DNA methyltransferases, DNMT1 and DNMT3b have been genetically disrupted (DKO cells: HCTII6 Dnmt1-/Dnmt3b-), was obtained as a gift from Prof Bird and Dr Stancheva (Rhee et al., 2002). Global cytosine methylation patterns in mammalian genomes seem to be established by a complex interplay of at least three independently encoded DNA methyltransferases: Dnmt1, Dnmt3a and Dnmt3b. DNA methyltransferases are commonly classified as *de novo* (DNMT3a and DNMT3b) and maintenance (DNMT1) enzymes (Bestor, 2000). While the lack of each individual enzyme has little effect on the DNA methylation patterns in human cells (Rhee et al., 2002; Rhee et al., 2000), in the DKO cells DNA methyltransferase activity is almost abolished and there is a 95% reduction in 5-methylcytosine content, including at repetitive sequences (Rhee et al., 2002). Figure 4.8 shows a map of the human Dnmt1 and Dnmt3b locus and target constructs. Deletions were verified by PCR (data not shown). These HCT-II6 DKO cells seemed to be the perfect system to study transcriptional activity of Alus in a 'methylation-free' environment.

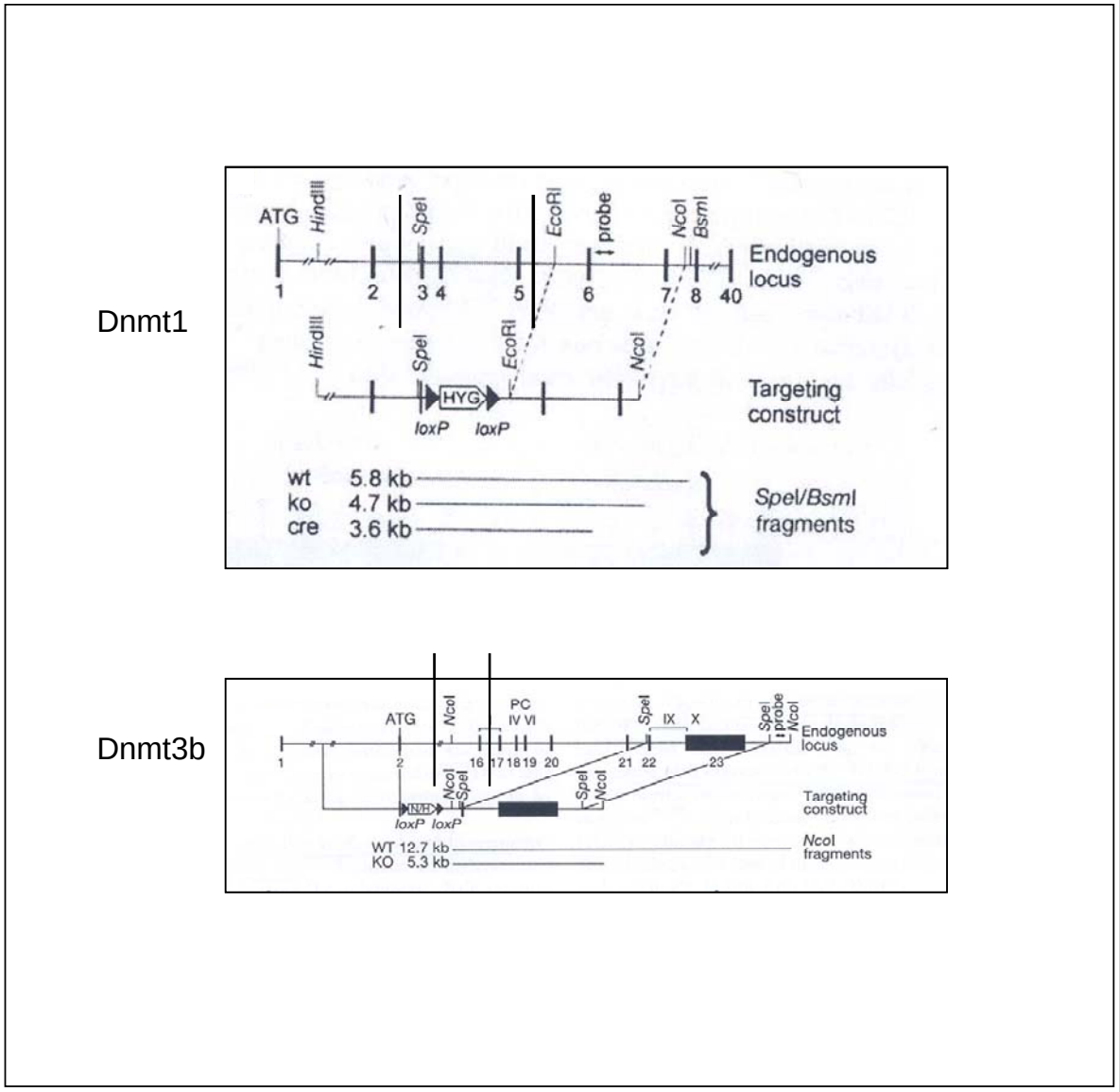


Figure 4-8 HCT 116 cells – Map of the human Dnmt1 and Dnmt3b locus and target constructs.
(adapted from Rhee et al, 2000 and Rhee et al, 2002).

Total RNA was extracted from both wt and DKO cells and Alu activity was studied by RT-PCR (figure 4.9). Duplicates of each RT-PCR are shown. In this experiment, consensus primers were used for the RT-PCR in order to look at activation of many closely related Alus. Other RNA polymerase III-transcribed genes were used as controls. 5S rRNA is a type1 promoter gene and 7SL and tRNAs are type2 RNA polymerase III-transcribed genes. GAPDH mRNA was used as a loading control, as it was shown previously that it is not affected in DKO cells (Paz et al., 2003). ARPPPO mRNA, which is used commonly as a loading control in our laboratory, was also included and it was shown to be negatively affected by loss of CpG methylation. Figure 4.9 shows an increase in Alu expression in the DKO cells. However, all other RNA polymerase III-transcribed genes were upregulated too. This was unexpected, because 5S rRNA, which is clearly affected here, was shown previously not to be influenced by CpG methylation (Besser et al., 1990).

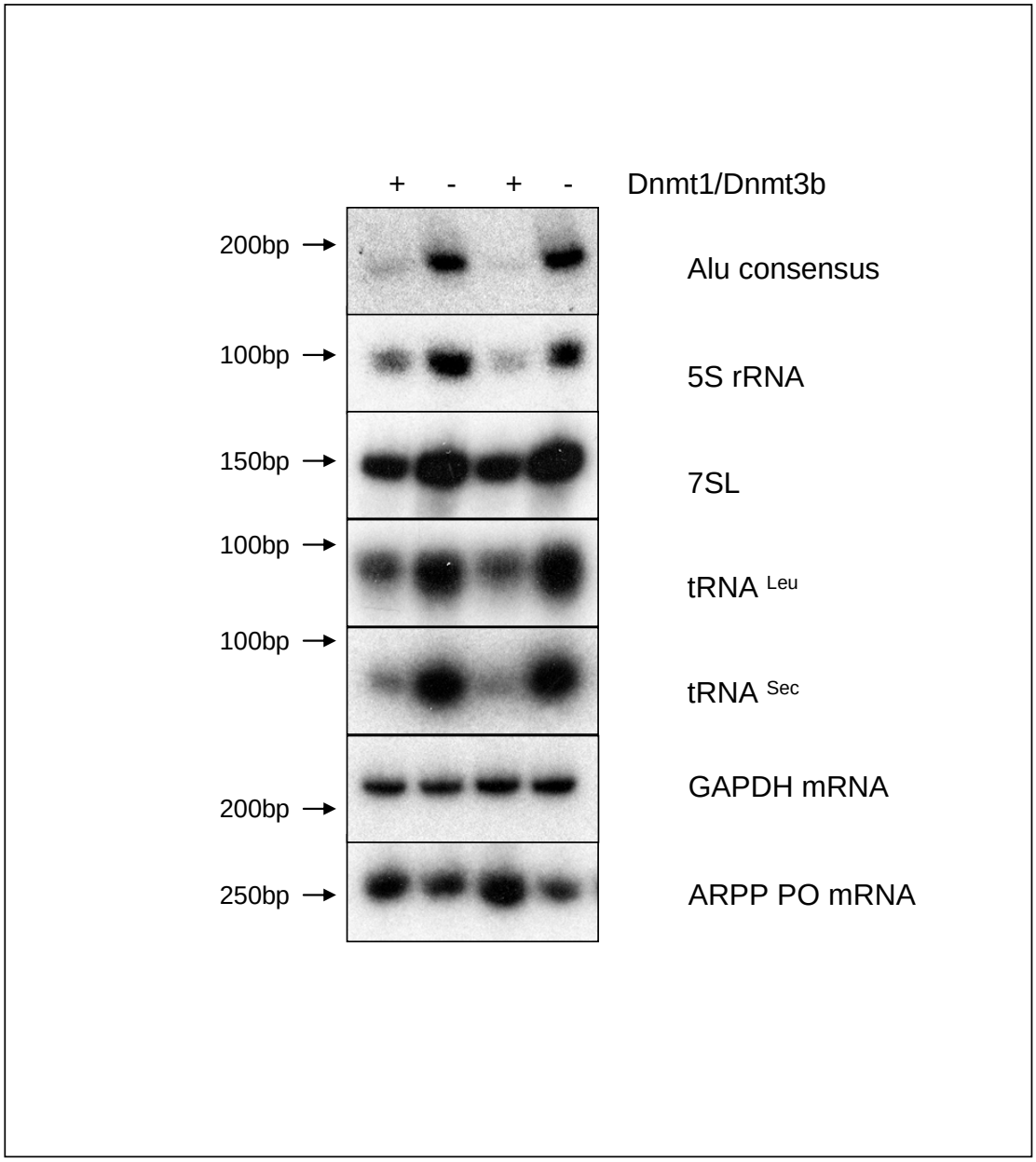


Figure 4-9 RT-PCR shows an increase in transcription of Alus in DKO HCT116.
RT-PCR shows that in Dnmt1/Dnmt3b KO cells (DKO HCT116) there is an increase in Alu expression. There is also an increase in expression of all other tested RNA polymerase III-transcribed genes. Samples are shown in duplicates. GAPDH was used as a loading control. Level of ARPP PO mRNA is decreased in HCTII6 DKO cells. Samples with no Superscript added during cDNA synthesis were used as a negative control for DNA contamination (data not shown). Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter R (ChIP) in the second column and H (human) in the third column of the table. The Alu cons PCR used PV51/JoINR consensus primers. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

We therefore wanted to verify that some of the regulators of RNA polymerase III transcription were not affected in the DKO cells. An obvious candidate was p53, as it was shown to be a general repressor of RNA polymerase III activity (Cairns and White, 1998) and its inactivation was necessary in order to create the Dnmt1-/- viable mouse fibroblasts used in Chapter 5, which have similar DNA methylation levels as DKO HCT116 cells (Jackson-Grusby et al., 2001; Jorgensen et al., 2004).Total protein extracts were made from both wt and DKO HCT116 cells and levels of p53 were verified using western blot analysis (figure 4.10). Actin was used here as a loading control. Samples were loaded in duplicates on a 12% SDS gel. As figure 4.10 shows, levels of p53 protein are significantly reduced in DKO cells. HCT116 DKO cells are widely used in experiments studying effect of DNA methylation and it was surprising to find that the fact that p53 levels are reduced in the DKO cells has not been previously noticed. It could have been expected as the survival of the Dnmt1n/n KO mouse fibroblast requires presence of a homozygous p53 mutation (Jackson-Grusby et al., 2001).

This poses an unfortunate problem for interpreting the data about Alu elements in these cells. Elevated levels of all RNA polymerase III transcripts in the DKO cells could be DNA methylation-dependent or, more likely, due to reduced levels of p53 (Cairns and White, 1998; Chesnokov et al., 1996). These cells therefore could not be used to study the effect of DNA methylation on Alus.

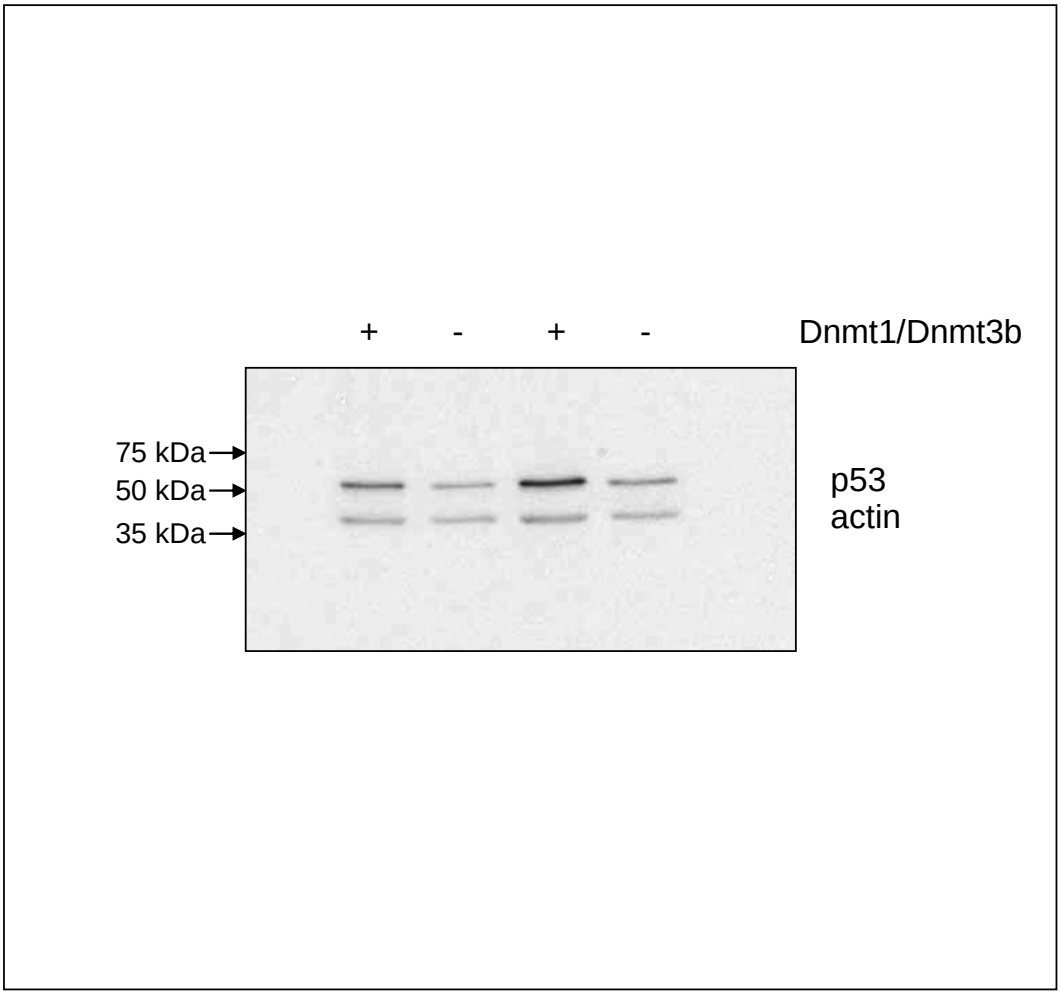


Figure 4-10 Level of the p53 protein is decreased in DKO cells.
Western blot. Total cell extracts of wt HCT116 and Dnmt1/Dnmt3b KO HCT116 (DKO) were used. Samples were loaded in duplicates. Actin used as a loading control. 12% SDS gel. Marker sizes are indicated on the left.

4.3 Discussion

There are two main mechanisms by which DNA methylation inhibits gene transcription. Methylated CpGs can directly inhibit binding of transcription factors to their cognate sequences (Watt and Molloy, 1988). Secondly, DNA methylation can inhibit gene activity indirectly via binding of methyl-CpG-binding domain proteins (MBD proteins). MBD proteins recognise methylated DNA and bind to the sequences and can repress transcription via their transcriptional repression domains (TRDs) and also occlude target sequences and prevent transcription factors from binding (Nan et al., 1997; Ng et al., 2000). However, the main effect of MBD proteins is through recruitment of corepressor and chromatin remodelling complexes to silence gene expression (Boeke et al., 2000; Jones et al., 1998; Nan et al., 1998; Ng et al., 1999; Sarraf and Stancheva, 2004; Zhang et al., 1999). Apart from these mechanisms, it was recently discovered that also DNA methyltransferases themselves can establish silencing on genes via interaction with HDACs (Fuks et al., 2000; Fuks et al., 2001) or the histone methyltransferase SUV39H1 (Geiman et al., 2004).

In this chapter, the effect of MBD proteins and MBD proteins-mediated silencing on Alu expression was studied. It was shown before that Alus are highly methylated and that removing methylation can increase their transcription *in vitro* and *in vivo* (Li et al., 2000; Liu et al., 1994; Liu and Schmid, 1993). The mechanisms of DNA methylation-mediated silencing in the case of Alus have not been studied in great detail. It was found that Alu sequences are amongst the preferential binding sites of MeCP2 (Koch and Stratling, 2004). The effect of MeCP2 on Alu silencing was investigated using a Gal4-linked transcription repression domain (TRD) of MeCP2, but it had no effect on transcription of an Alu reporter. In transient transfection assays, MeCP2 had no inhibiting effect on the AluSx reporter construct, although it was shown to inhibit an L1 (member of the long interspersed repeat elements) reporter construct in the same assay (Yu et al., 2001).

Here, the occupancy of MBD proteins on Alus was investigated using ChIP analysis. Apolipoprotein E precursor was used as a positive control gene. Binding

of MeCP2 to Alus was confirmed (Figure 4.2), together with MBD1 and MBD2 (Figure 4.2, 4.3). MeCP2 seemed to be giving the strongest signal on all the tested Alus. MBD2 was detected on all of the tested Alus and MBD1 was detected on Alu Jo and Alu 6. To answer whether MBD proteins act alone or they act via recruitment of corepressors and chromatin remodelling complexes, another ChIP analysis was performed (Figure 4.5). The presence of SIN3A, NuRD, Brm-containing SWI/SNF corepressor and chromatin remodelling complexes and HDACs alone were examined.

Interestingly, Brm component of the SWI/SNF2 complex seemed to be associated specifically with methylated genes. Brm-containing SWI/SNF2 complex is usually associated with positive effect on genes' activity (Muchardt and Yaniv, 1993). But it has also been reported to be involved in repression (Watanabe et al., 2006). It has been shown to be recruited to methylated and repressed genes by MeCP2 via its component Brahma (Harikrishnan et al., 2005). Brahma was shown to associate with MeCP2 *in vivo*. Binding patterns of both proteins to methylated genes were similar and both of them were released from methylated genes upon treatment with methylation inhibitor. In agreement, this study confirms enrichment of SWI/SNF2 on the methylated genes, potentially recruited specifically by MeCP2.

The association of HDACs, SIN3A or NuRD was not specific to methylated or active genes. HDACs were found on all tested genes. HDACs are associated with many corepressor complexes (reviewed in (Knoepfler and Eisenman, 1999; Ng and Bird, 2000), therefore it is not surprising to find them on all the repressed genes, regardless of whether SIN3A or NuRD components were detected. HDACs are known to repress RNA polymerase III-derived transcription (Sutcliffe et al., 2000). HDACs could be recruited to methylated RNA polymerase III-transcribed genes via MeCP2 directly (Jones et al., 1998; Nan et al., 1998) or via SWI/SNF and mSin3a (Sif et al., 2001; Tong et al., 1998; Zhang et al., 1997), but they can be also recruited through mSin3a via p53 which was shown to interact with it (Murphy et al., 1999). P53 is a known repressor of RNA polymerase III-transcribed genes, including Alus (Cairns and White, 1998; Chesnokov et al., 1996). In the case of 7SL, both SIN3A and HDACs may in fact be present on the 7SL pseudogenes rather than the four active 7SL genes. The presence of HDACs at tRNA ^{Sec} is more surprising. Despite of HDACs being best known for their

participation in transcriptional repression, HDACs were recently also shown to enhance activation of transcription of certain genes (Zupkovitz et al., 2006). A genome-wide study using HDAC1-deficient cells showed that the transcription of only 7% of genes is deregulated (partially due to the compensatory effect of HDAC2). Expression of some of the genes (about two fifths) was downregulated in the absence of HDACs. Some of them increased after TSA treatment, suggesting that they are repressed by compensating HDACs. Some of them, however, such as interferon responsive genes, displayed a negative response to TSA treatment, supporting previous studies (Nusinzon and Horvath, 2005; Sakamoto et al., 2004) Such a positive effect has not yet been shown for any RNA polymerase III transcribed gene. There is also an obvious discrepancy between HDACs occupancy and acetylated H3 and H4 on both RNA polymerase III-transcribed genes and Alus. Acetylated histones H3 and H4 were observed on Alus in other studies (Hakimi et al., 2002; Johnson et al., 1998), but the presence of HDACs have never been described before. Significance of the co-occupancy of acetylated histones and HDACs on Alus remains to be determined. Despite the low transcriptional activity of Alus, histone acetylation may be linked to the transcriptional activation potential of Alus, such as the rapid activation of Alus expression after exposure to cell stress (Liu et al., 1995).

NuRD complex seemed to be present on both methylated and active genes. Its specificity is not clear. It was shown to interact with MBD2 and be recruited to methyl DNA *in vitro* (Zhang et al., 1999); however, in itself, mi2/NuRD does not seem to have a significant affinity for methylated DNA *in vitro* (Hendrich and Bird, 1998; Zhang et al., 1999). In the case of 7SL, it may be in fact present on 7SL pseudogenes rather than active 7SL genes.

In summary, SWI/SNF2 complex seems to be specifically enriched on methylated genes, however, other complexes may also contribute to the repression of methylated Alus, whether recruited via MeCP2 (SIN3A and HDACs) or MBD2 (NuRD and HDACs), or independently.

MBD1, detected on Alus here, is not known to recruit the studied complexes. It is, however, known to form a stable complex with the histone H3K9 methylase SETDB1 (Sarraf and Stancheva, 2004), which may be maintaining H3K9 methylation present on Alus (Kondo and Issa, 2003).

MBD proteins could also silence transcription via their TRD domains or by obstructing transcription factors from binding to Alus. It was shown in the previous chapter that although TFIIIB and TFIIIC occupancy levels seem to be indistinguishable on Alus and active RNA polymerase III genes, RNA polymerase III levels were significantly lower suggesting that something may prevent RNA polymerase III from better binding. MBD proteins were potential candidates. Sequential ChIP analysis was performed to verify that MBD proteins and RNA polymerase III machinery are indeed present on the same Alus at the same time. TFIIIC, which is the component associating first with promoters during RNA polymerase III complex assembly, was used in the first step of sequential ChIP analysis. As figure 4.4 shows, MBD proteins indeed co-precipitate with TFIIIC, suggesting that TFIIIC binding is not prevented by their presence. However, whether TFIIIB or RNA polymerase III binding may be inhibited remains to be established. Using TFIIIB and RNA polymerase III in the first step of sequential ChIP analysis would address that. Interestingly, some differences from standard ChIP analysis were detected in MBD proteins' occupancy. MBD1 rather than MeCP2 gave the strongest signal on Alu Jo and no MBD2 was detected. On Alu 6, MBD2 gave the strongest signal rather than MeCP2 (compare figure 4.4 and figure 4.2, 4.3). A possible explanation could be that sequential ChIP analysis only detects Alus bound simultaneously by TFIIIC and MBD proteins, and not the Alu copies only bound by MBD proteins. Therefore, there could be different occupancy of MBD proteins present on the Alu Jo and Alu 6 when these are simultaneously bound by TFIIIC or possible MBD epitope masking may occur in the presence of TFIIIC.

Overall, the detected MBD proteins may act both via inhibition of RNA polymerase III machinery from proper binding (namely RNA polymerase III) and recruitment of corepressor complexes, specifically SWI/SNF.

DNA methylation is regarded as a global repressor of Alus. On the other hand, treatment of cells with adenovirus type 2 (Russanova et al., 1995) or heat shock or cycloheximide (Li et al., 2000) leads to an increase in Alu RNA without altering methylation of their DNA. Despite DNA methylation and DNA methylation-mediated silencing being widely recognised, in certain genes and promoters it is established as a secondary 'lock' on sequences which first became transcriptionally inactive (reviewed in (Clark and Melki, 2002; Turker,

2002) resulting in further and deeper repression. Transfected PV Alu remains transcriptionally silenced without becoming methylated (Leeflang et al., 1992). So, perhaps Alu sequences are only co-silenced by DNA methylation and their silencing depends on individual circumstances, such as promoter structure, flanking sequences and chromatin situation, that can supersede global regulation (Li and Schmid, 2001).

The effect of DNA methylation on Alu transcription has so far been studied using 5-azacytidine treatment (Liu et al., 1994) or in naturally hypomethylated K562 cells (Li et al., 2000). Here, an attempt was made to study 'methylation-free' environments.

The budding yeast *S.cerevisiae* was first used as a model system to study Alu expression as the organism has no endogenous methylation (Antequera et al., 1984). An Alu Jo element was integrated into the yeast genome and its activity assayed using Northern blot analysis. Alu Jo was expressed in two different cell lines (figure 4.6, 4.7). However, it was also revealed that the Gal1 promoter, which is normally inactive in the presence of glucose and absence of galactose, was active. Since the Gal1 promoter is only active where Alu Jo is active (figure 4.6), it is possible that Alu Jo is stimulating the activity of the Gal1 promoter even in the absence of galactose. The ability to influence transcription of nearby genes has been described previously for tRNAs and Ty1 elements in budding yeast (Bolton and Boeke, 2003; Hull et al., 1994). tRNA genes have been shown to strongly inhibit transcription from nearby RNA polymerase II promoters and this inhibition was dependent on the active transcription of the tRNA gene (Hull et al., 1994). Ty1 element, on the other hand, was shown to stimulate transcription of nearby tRNAs (Bolton and Boeke, 2003). Despite such a capacity being an interesting property of Alu Jo, it was undesirable in this experimental system. Future experiments would require using a different integrating vector, where no other promoter is present.

HCT 116 cells, where deletion of Dnmt1 and Dnmt3b led to loss of more than 95% of genomic methylation, were used next to study Alu elements in a virtually 'methylation-free' environment. Alu transcriptional activity was increased in the DKO cells upon CpG methylation removal (figure 4.9). However, all other RNA polymerase III-transcribed genes were affected too. An upstream regulator of

RNA polymerase III activity was suspected and, indeed, levels of p53 protein were shown to be decreased in DKO cells (figure 4.10). Alus are known targets of p53 *in vitro* and *in vivo* (Chesnokov et al., 1996) and due to this fact, this system could not be used for further analysis.

Because of difficulties finding a good DNA methylation-free system, the significance of MBD proteins that were found to associate with tested Alus and their repressive effect could not be further studied. Alus, however, have homologous sequences in rodent genomes, the B1 SINEs (see Chapter5) and the next focus was on them. The closely related B2 family (another RNA polymerase III-transcribed SINE family) was also studied and mouse fibroblasts with Dnmt1 deletion resulting in 95% reduction in 5-methylcytosine content (Jackson-Grusby et al., 2001; Jorgensen et al., 2004) revealed interesting data.

5 Chapter 5 - B1 and B2 transcription is not silenced by DNA methylation

5.1 Introduction

Except for rodents and primates, SINEs from all other animals examined are unrelated to the 7SL RNA but are instead homologous to tRNAs. Rodents have both- the 7SL-derived B1 family and tRNA-derived B2 family (Kramerov et al., 1979). Sequence analysis indicates that B1 is a homologue to the Alu left monomer. B1s and B2s are expressed at very low levels under normal circumstances, although B2s' expression levels appear somewhat higher than that of B1s (Carey et al., 1986). B1 and B2 elements often respond in a similar manner to a number of activating stimuli such as cell stress (Fornace et al., 1989; Kalkkila et al., 2004; Li et al., 1999; Liu et al., 1995; Price and Calderwood, 1992), DNA-damage (Rudin and Thompson, 2001), cell growth (Lania et al., 1987; Singh et al., 1985) and viral infections (Carey et al., 1986; Lania et al., 1987; Scott et al., 1983; Singh et al., 1985). On the other hand, their activity decreases during differentiation of embryonal carcinoma cells (White et al., 1989).

Both B2 and B1 elements contain an RNA polymerase III promoter and are transcribed by RNA polymerase III (Carey et al., 1986; Krayev et al., 1982; Singh et al., 1985). Like Alus, they are common in hnRNA as part of RNA polymerase II derived mRNAs (Ryskov et al., 1983). Given the number of copies, 564 000 of B1 and 348 000 of B2 in the mouse genome (Waterston et al., 2002), their very low transcription rate signifies that they are subject to repression. While methylation was thought to be the main repressive mechanism for human Alus it has never been studied for B1 and B2 sequences, which are also methylated (Jeong and Lee, 2005; Yates et al., 1999). For B2, unlike Alus, global repression was alleviated by depleting linker histone H1 from chromatin (Carey and Singh, 1988; Russanova et al., 1995).

In this chapter, the hypothesis that DNA methylation-mediated silencing or chromatin silencing inhibit transcription of B1 and B2 was investigated.

5.2 Results

5.2.1 RNA polymerase III components are present on B1 and B2

Alus and B1 and B2 respond to stimuli in a similar manner. Previous studies proposed that Alu promoters get masked by chromatin proteins and are inaccessible to the RNA polymerase III transcriptional machinery (Kim et al., 2001; Russanova et al., 1995), suggesting that B1s and B2s may be similarly affected. To find out whether the repressive mechanism acting on B1s and B2s inhibits access of the RNA polymerase III machinery, the occupancy of RNA polymerase III components at B1 and B2 promoters was investigated *in vivo*. The A31 mouse fibroblast cell line was used for the *in vivo* analysis. RNA polymerase III, TFIIIB and TFIIIC were tested as they were shown to be necessary and sufficient for transcription of SINEs *in vitro* (Singh et al., 1985). Prior to the analysis it was necessary to verify B1 and B2 expression levels in these cells. Total RNA was extracted and cDNA made. Samples where no reverse transcriptase was used during cDNA preparing were used as controls for genomic DNA contamination. RT-PCR was then performed and levels of B1 and B2 activity compared to the levels of 7SL as a RNA polymerase III transcribed active gene (Figure 5.1). Consensus primers were used for both B1 and B2 RT-PCR. Figure 5.1 shows that both B1 and B2 RNA were detected. Although direct comparison with 7SL is not possible here (the strength of the signal depends on number of cycles of amplification and the annealing capacity of each primer), it can be estimated that RNA levels from four active 7SL genes are higher than RNA levels produced from approximately a hundred B1 and B2 sequences detected by these primers when similar number of amplification cycles was employed.

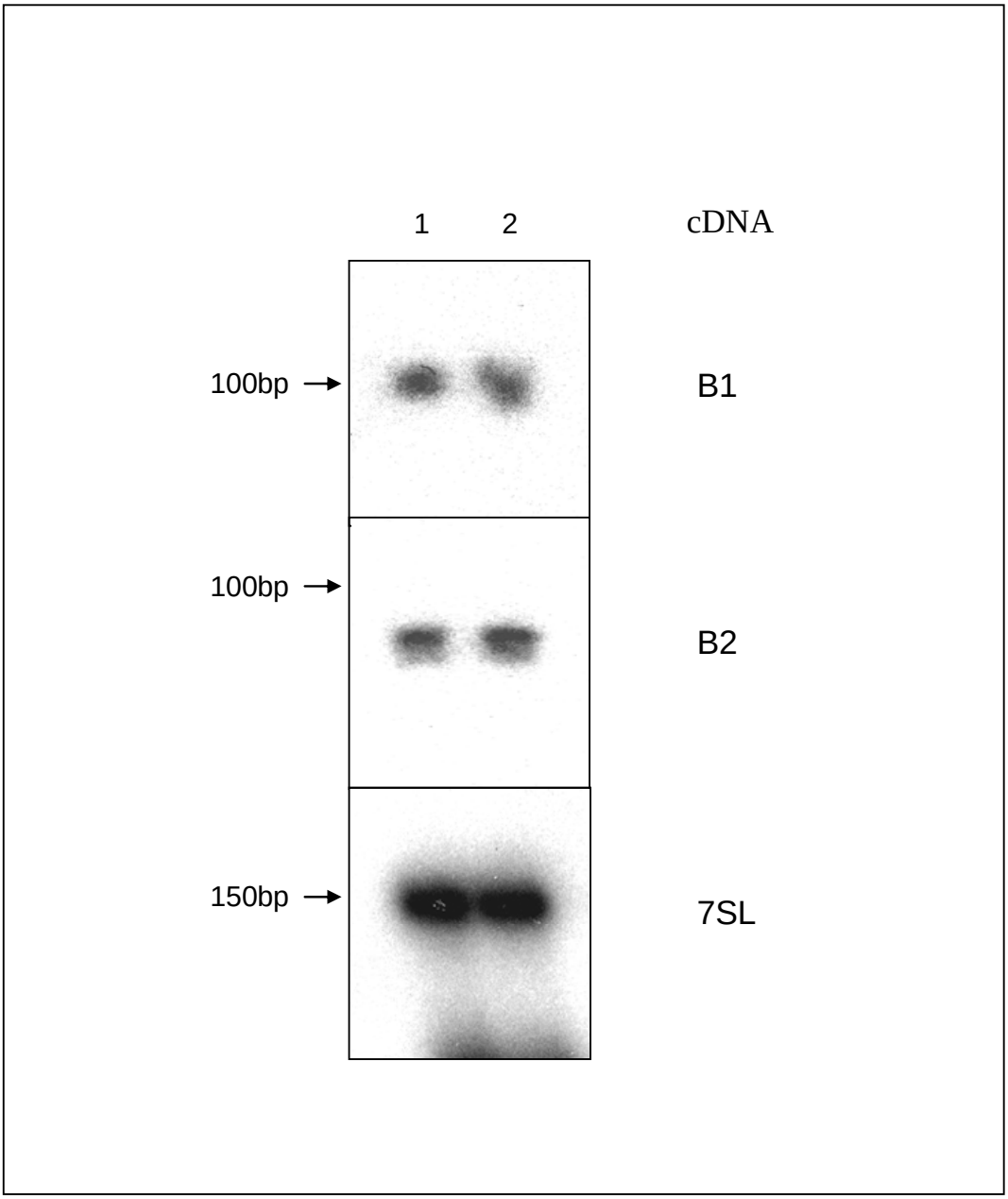


Figure 5-1 Low levels of B1 and B2 transcripts were detected in A31 cells.
RT-PCR. B1 and B2 are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each. 7SL is a positive control gene. The primers used for RT-PCR reaction are listed in Table 2-3, with letter R (RT-PCR) in the second column and letter M (mouse) in the third column of the table. Samples are shown in duplicates. NS is a sample with no Superscript added during cDNA synthesis, used as a negative control for DNA contamination (data not shown). Marker sizes are indicated. Autoradiograph.

Having verified that both families are indeed expressed at low levels in A31 cells, chromatin immunoprecipitation assay was performed in order to determine RNA polymerase III machinery levels on both SINEs families (Figure 5.2). Antibodies against TFIIIB, TFIIIC and RNA polymerase III were used, together with antibodies for acetylated histone H3 (directed against acetylated lysine 9 and 14) and H4 (directed against acetylated lysine 5, 8, 12, 16), both often associated with transcriptionally active genes (Kuo et al., 1996). TFIIA was used as a negative control antibody. Beads were used as an additional negative control. Set1 and Set2 were genomic primers for B1 and B2 SINEs on chromosome 9, designed to detect two sites of this unique region, which is surrounded within 3 kb distance by other SINEs only. B1 and B2 are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each. tRNA ^{Leu} was used as a positive control gene. H19 is a RNA polymerase II-transcribed gene known to be repressed by DNA methylation in the mouse genome (Fuks et al., 2003) and it was used here as a negative control. As figure 5.2 shows, RNA polymerase III transcriptional machinery and both acetylated H3 and H4 were detected on all B1 and B2 genes studied, whether present scattered within RNA polymerase II-transcribed regions (B1 and B2) or in the middle of a cluster of other B1 and B2s (Set1 and Set2).

Lower level of TFIIIC was also detected on the H19 gene. There are two possible explanations. Firstly, there may be a TFIIIC binding site in the H19 sequence. As described in sections 1.2.1 and 1.2.2, TFIIIC is the first step of RNA polymerase III transcription machinery assembly and it is the main DNA binding protein. Secondly, it is possible that the H19 sequence contains SINEs or their remnants and TFIIIC binds to those sequences. SINEs are located throughout the genome, and they tend to be enriched in gene-rich regions (Korenberg and Rykowski, 1988). In the past, SINEs inserted and became part of many genes, spreading RNA polymerase II promoters (Ferrigno et al., 2001) and alternative splice sites (Sorek et al., 2002; Sorek et al., 2004). H19 gene was used here as a negative control gene as it is RNA polymerase II-transcribed. It is an imprinted gene that is expressed, although exclusively from the maternal allele (Bartolomei et al., 1991). Acetylated histones H3 and H4 may therefore be present on the maternal

allele and their association with H19 gene was observed before (Huang et al., 2004).

It is surprising to find that many B1s and B2s are occupied by RNA polymerase III machinery in A31 cells, considering that the level of expression of both families is very low. It seems that there is no obvious problem with RNA polymerase III machinery assembly- TFIIIC binding followed by TFIIIB recruitment and recruitment of RNA polymerase III. However, the RNA polymerase III occupancy appears to be lower on all B1/B2 SINEs when compared to $tRNA^{Leu}$ or 7SL (figure 5.2 and see later).

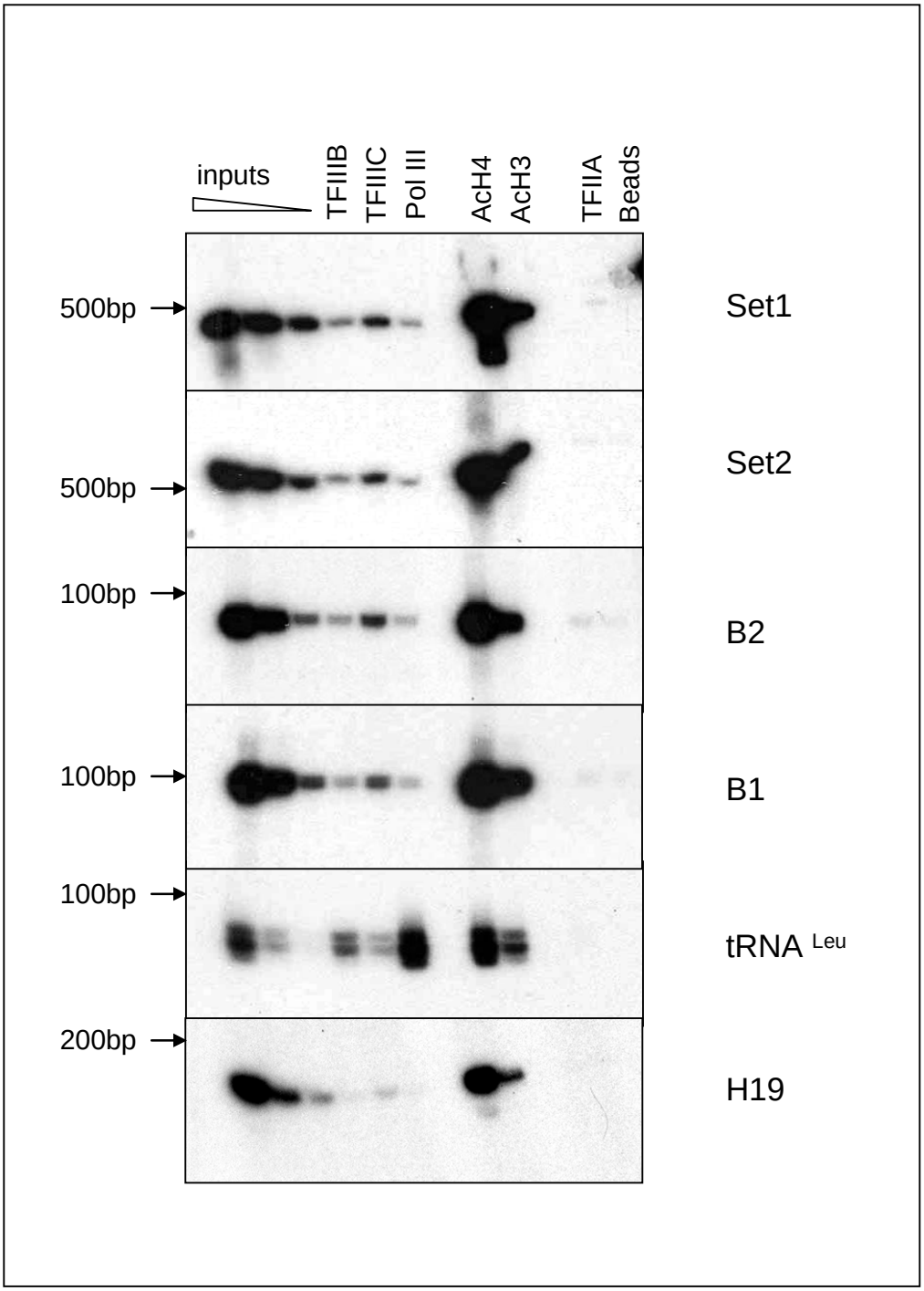


Figure 5-2 ChIP analysis shows the occupancy of the RNA polymerase III machinery and acetylated histones on B1 and B2 in A31 cells.
Set1 and Set2 were genomic primers for B1 and B2 SINEs on chromosome 9, designed to detect two sites of this unique region, which is surrounded within a 3 kb distance by other SINEs only. B1 and B2 are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each. All antibodies used for the ChIP analysis are listed on top of the first panel. TFIIA was used as a control antibody. Samples with beads-only were used as a general negative control. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

To determine whether levels of RNA polymerase III on B1 and B2 families are significantly different from active RNA polymerase III-transcribed genes, mouse fibroblasts were subjected to more ChIP assays and statistical analysis of the occupancy signals of the RNA polymerase III transcription complex (TFIIIC-TFIIIB-RNA polymerase III) was performed. The occupancy signals of TFIIIB, TFIIIC and RNA polymerase III at 7SL and B1 and B2 families were quantified and the ratios of TFIIIB/TFIIIC, RNA polymerase III/TFIIIB and RNA polymerase III/TFIIIC were calculated. Six different ChIP analyses were used and 7SL, B1 and B2 were tested. Both two-tailed t-test analysis and student t-test analysis were performed to correct the analysis for small sample sets (Table 5.1).

As table 5.1 shows, both B1 and B2 families are co-ordinately affected. There was no significant difference in occupancy ratio of IIIB/IIIC between 7SL and B1 or B2. However, there was significant difference ($p<0.05$) of occupancy ratios of RIII/IIIB and RIII/IIIC between B1 and B2 and 7SL (see table).

	Mean	St dev	T test P	Student t test P
7SL IIIB/IIIC	0.9862	0.5181	0.274	0.270
B1 IIIB/IIIC	0.6896	0.3477		
7SL IIIB/IIIC	0.9862	0.5181	0.174	0.150
B2 IIIB/IIIC	0.6409	0.1813		
7SL RIII/IIIB	3.1287	1.3859	0.015	0.008
B1 RIII/IIIB	1.1029	0.5384		
7SL RIII/IIIB	3.1287	1.3859	0.017	0.012
B2 RIII/IIIB	1.2352	0.5823		
7SL RIII/IIIC	2.6594	1.1585	0.008	0.002
B1 RIII/IIIC	0.6414	0.1800		
7SL RIII/IIIC	2.6594	1.1585	0.007	0.003
B2 RIII/IIIC	0.727	0.2760		

Table 5-1 Statistics of 7SL and B1 and B2 genes' occupancy by RNA polymerase III (RIII), TFIIIB (IIIB) and TFIIIC (IIIC).

Box and whisker plots show graphic representation of the samples' descriptive statistical values (Figure 5.3). Chart shows that there are higher occupancy ratios of all RNA polymerase III components on the 7SL. While for TFIIIB to TFIIIC it is not significantly higher, the ratios of RNA polymerase III to TFIIIB and RNA polymerase III to TFIIIC are significantly higher on the 7SL gene then on B1 or B2,

showing that there is lower amount of cross-linked RNA polymerase III on both B1 and B2. This implies that there is a problem with RNA polymerase III recruitment or its access to the SINE promoter, which would then result in lower transcription or lack of transcription from the majority of the B1s and B2s. Or perhaps, RNA polymerase III is present but its epitopes are masked by other molecules present on B1 and B2, possibly impeding its proper function.

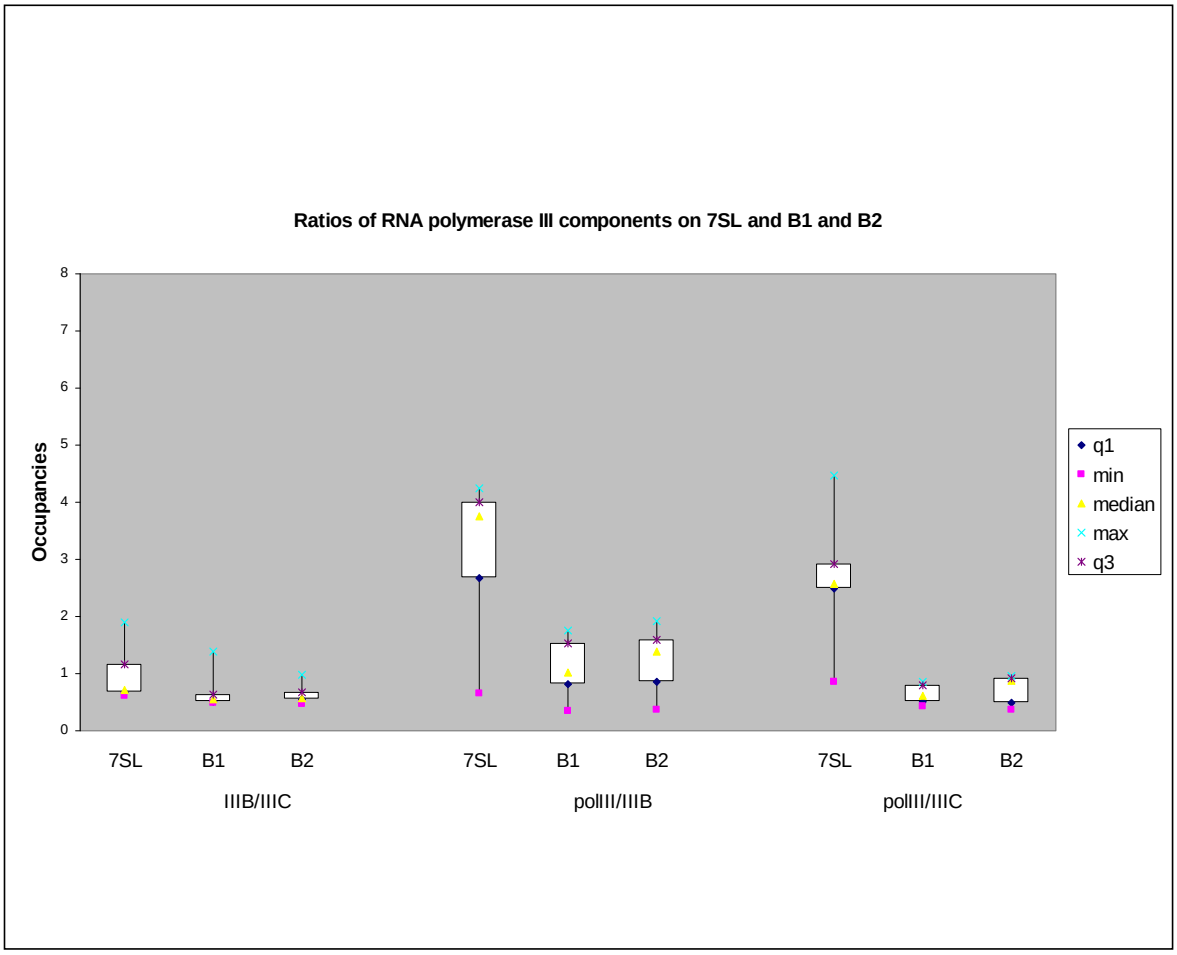


Figure 5-3 Ratios of RNA polymerase III machinery components on 7SL and B1 and B2. Box and whisker plots chart shows graphic representations of descriptive statistical values found for each sample set. Six ChIP analyses were included. Ratios of TFIIIB/TFIIIC, RNA polymerase III/TFIIIB and RNA polymerase III/TFIIIC occupancies were measured by ImageJ.

5.2.2 B1 and B2 and methylation – presence of MBD proteins

There is not much evidence about B1 or B2 and methylation and its effect on B1 and B2 activity. It is known that they are methylated (Jeong and Lee, 2005; Yates et al., 1999). Also, B1 and B2 were amongst mouse genomic fragments that bound strongly to the MBD column containing MBD domain of MeCP2 (A Hever, M Bailey, personal communication). Because of difficulties determining Alu methylation in section 4.2.1, B1 and B2 methylation status was not determined. Establishing whether B1 and B2 are bound by MBD proteins would therefore indicate whether B1 and B2 are methylated.

To find out whether MBD proteins bind to B1 and B2 and could therefore inhibit RNA polymerase III access to their promoters or its function, ChIP analysis was performed to look at their occupancy. Antibodies against MBD1, MeCP2 and MBD2 were used. Acetylated H4 was used as a positive control antibody. H3K9me3 is also known to associate with methylated DNA and so it was also studied here using antibody against it. tRNA ^{Leu} and 7SL are encoded by actively transcribed RNA polymerase III genes and were used here as negative controls. Apolipoprotein E precursor (ApoE) is methylated in different cells at different levels, heavily in rat liver, less in other tested tissues (Driscoll and Getz, 1984). It was used as it was shown in human cells to be bound by all MBD proteins. Whether that is the case in rodent fibroblasts was unknown.

ChIP analysis revealed that B1 and B2 genes are indeed bound by all MBD proteins studied here, MBD2, MeCP2, and MBD1 (figure 5.4, 5.5). ApoE is also bound by all MBD proteins, as is its human homologue (Ballestar et al., 2003). Both B1 and B2 were also bound by trimethylated H3K9 (figure 5.4), which has previously been reported for human Alus (Kondo and Issa, 2003). This is despite the fact that B1 and B2 are also occupied by AcH4. It is possible that acetylated H4 and H3K9me3 are in fact present on different copies of B1 and B2. B1 and B2 primers are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each, some of which could have H3K9me3 and other AcH4. Also, ChIP analysis uses populations of cells; it is therefore possible that although both H3K9me3 and AcH4 are present on a gene within the population, H3K9me3 may be present on B1 and B2 in some cells and AcH4 may be present on B1 and B2 in

other cells, and not together. Such a situation would appear as co-occupancy in a single ChIP analysis.

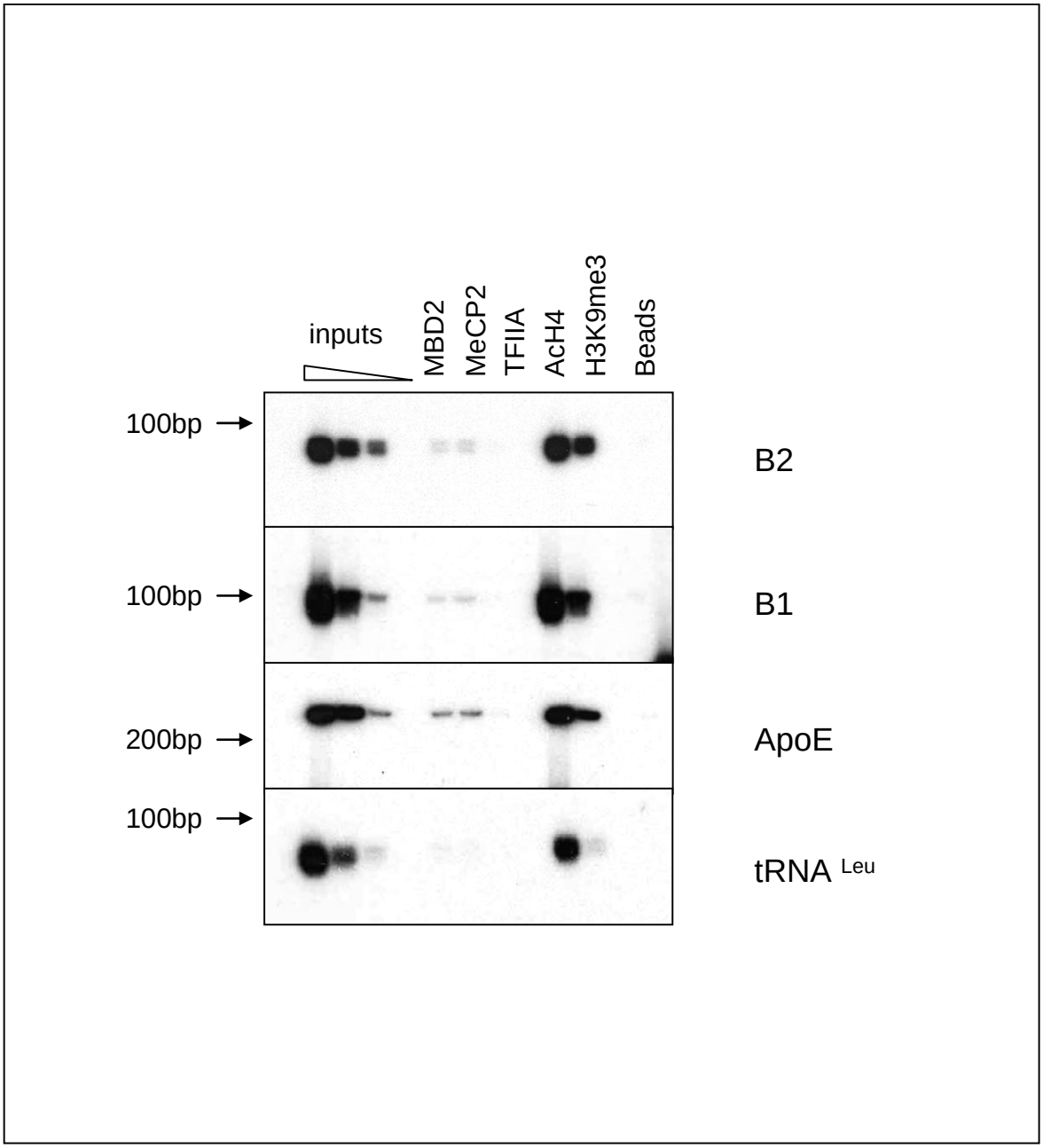


Figure 5-4 MBD2, MeCP2 and H3K9me3 were detected on B1 and B2 genes in A31 cells. ChIP analysis. B1 and B2 are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each. ApoE gene was used as a positive control gene for MBD proteins. tRNA Leu was used as a negative control gene for MBD proteins. All antibodies used for the ChIP analysis are listed on top of the first panel. TFIIA was used as a control antibody. Samples with beads-only were used as a general negative control for the ChIP. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-32P] dCTP. Autoradiograph. Marker sizes are indicated.

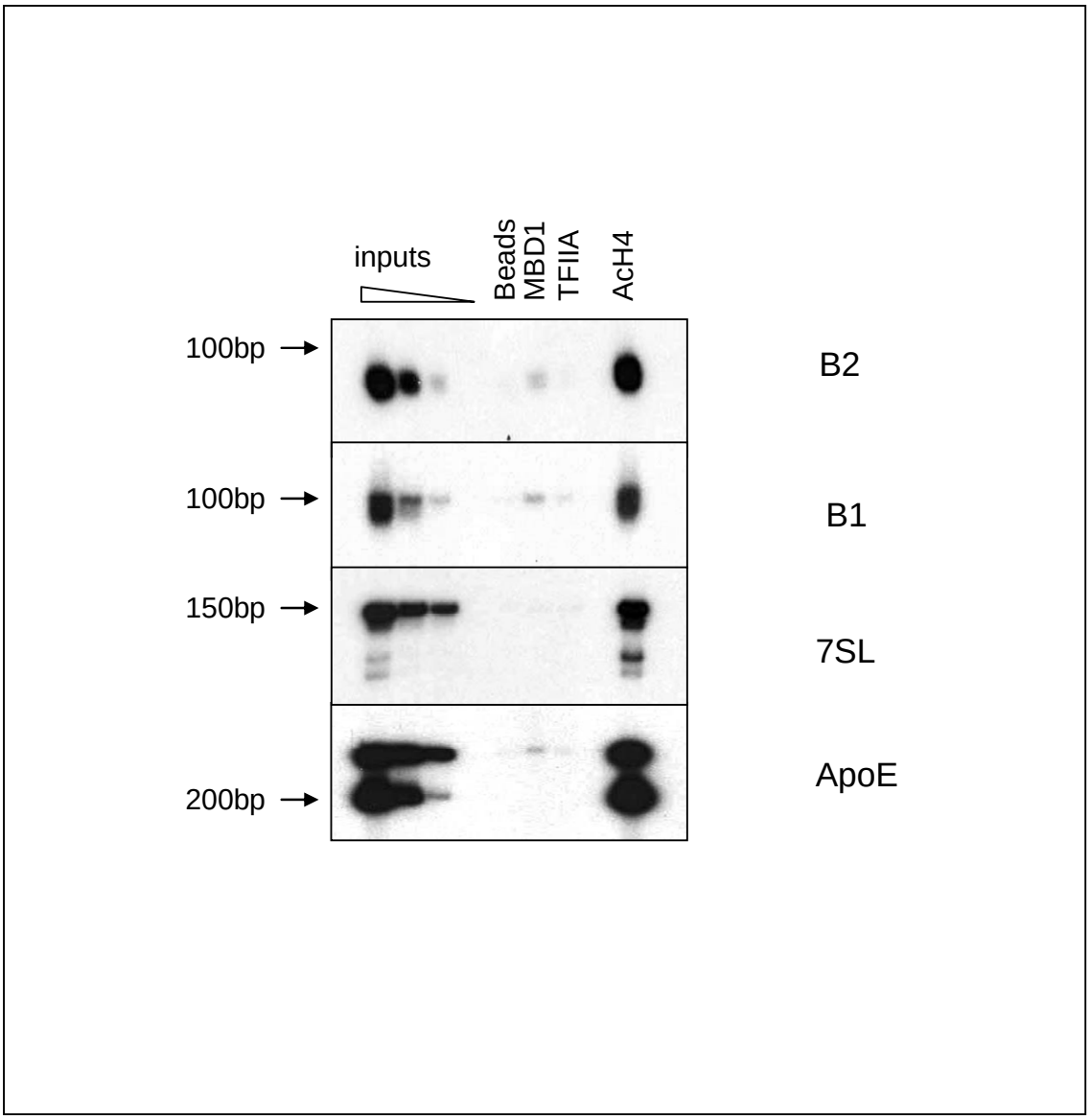


Figure 5-5 MBD1 occupies B1 and B2 genes in A31 cells.
ChIP analysis. B1 and B2 are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each. The ApoE gene was used as a positive control gene for MBD1. 7SL was used as a negative control gene for MBD1. TFIIA and beads were used as control antibodies. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

These data confirmed that MBD proteins are associated with B1 and B2 sequences. A sequential ChIP was used to determine whether RNA polymerase III and MBD proteins could be present at the same time on the same gene. Briefly, a ChIP assay was performed using 10 plates of cells per antibody (10 x more than in a normal ChIP assay) and the obtained supernatant was immmunoprecipitated with RNA polymerase III antibody (1900). RNA polymerase III-immunoprecipitated material was then diluted 1:10 and immunoprecipitated further with antibodies of interest. TFIIIC with TFIIIB were used here as positive controls as they co-occupy promoters together with RNA polymerase III. If MBD proteins are detected on B1 and B2 in RNA polymerase III-immunoprecipitated material, it is considered that they co-occupy the same B1 and B2 sequences. Figure 5.6 shows sequential ChIP performed in A31 cells. Antibodies against TFIIIB, TFIIIC together with MBD1, MBD2 and MeCP2 were used. TFIIA was a negative control antibody. B1, B2, Set1 and Set2 are the same sequences used in previous simple ChIP analysis. tRNA ^{Tyr} is shown as an active RNA polymerase III-transcribed active gene. TFIIIB and TFIIIC co-precipitated on all tested genes. MBD2 and MeCP2 were found on all tested B1 and B2 sequences. MBD1 was found on B1 and B2 when detected with consensus primers, providing evidence that some B1s and B2s are indeed targets of MBD1. It was not detected on Set1 and Set2 suggesting that MBD1 does not co-occupy these sequences with RNA polymerase III machinery. MBD proteins were also detected on the tRNA ^{Tyr} gene. Higher exposure was needed to detect its signal from sequential ChIP inputs, but it resulted in some signal from MBD proteins, not seen in a shorter exposure.

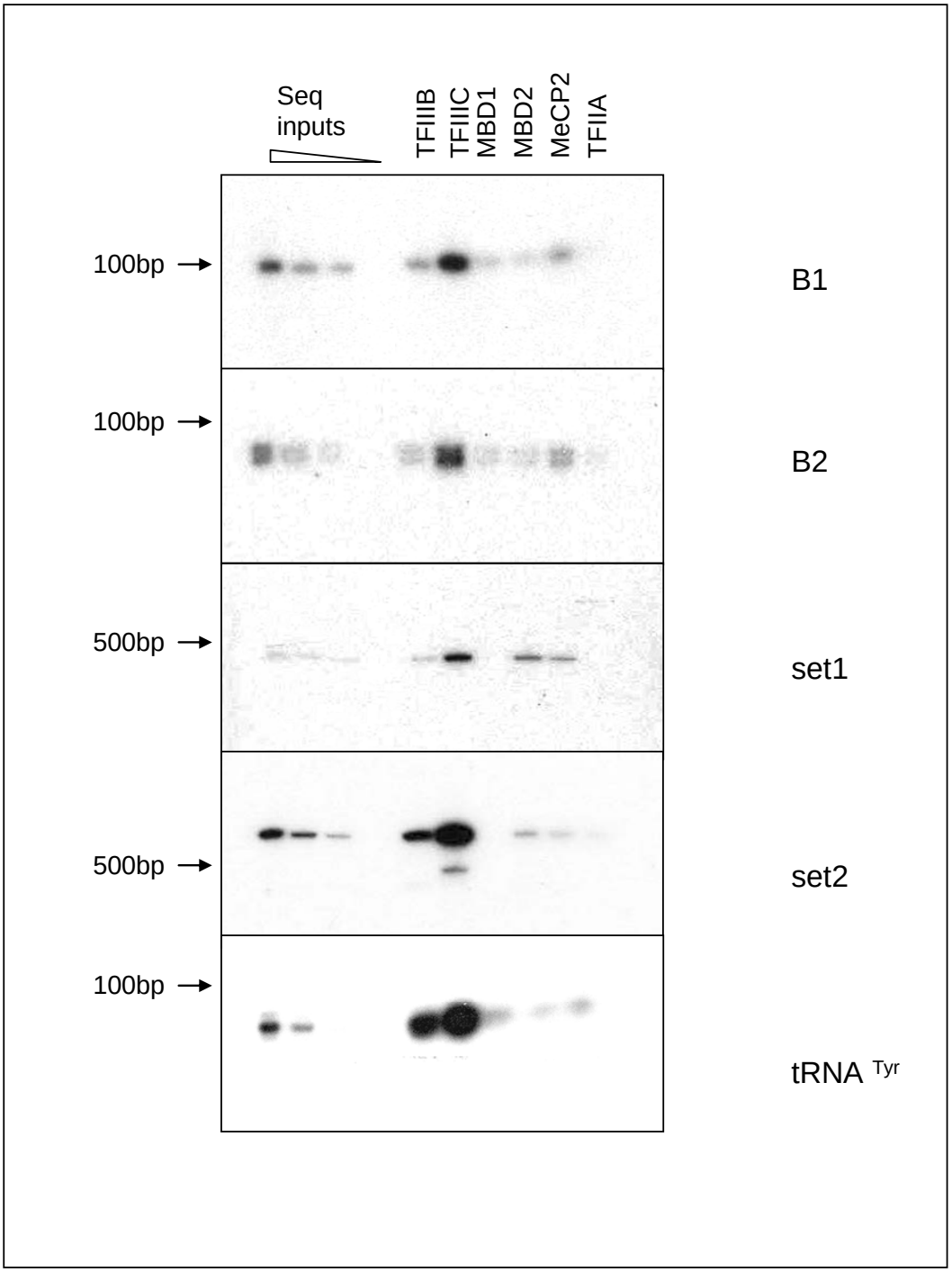


Figure 5-6 Sequential ChIP shows the co-occupancy of the RNA polymerase III and MBD proteins on B2 and B1 in A31 cells.
RNA polymerase III antibody (1900) was used in the first step (ChIP analysis). Precipitated material was diluted 1:10 in 1xNET buffer and further precipitated with the antibodies listed at the top of the panel (sequential ChIP). TFIIA was a negative control antibody. Input DNA was diluted to 10%, and precipitated. Sequential input was then again diluted to 20%, 10% and 5% to ensure quantitative PCR reactions. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and M (mouse) in the third column of the table. Set1 and Set2 were genomic primers for B1 and B2 SINEs on chromosome 9, designed to detect two sites of this unique region, which is surrounded within 3 kb distance by other SINEs only. B1 and B2 are consensus primers detecting a subgroup of about a hundred B1 and B2 sequences each. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

In the above experiment, it was shown that RNA polymerase III and MBD proteins occupy B1s and B2s together. It is now a question whether MBD proteins inhibit B1s and B2s' transcription and whether they inhibit it directly or via recruitment of corepressors and chromatin remodelling complexes. MBD2 and MeCP2 studied here are known to interact with corepressor and chromatin remodelling complexes. MeCP2 has been the most studied in mice. While other MBD proteins seem to be partially redundant in their action, MeCP2 is essential for normal post-natal neurological development in mice (Chen et al., 2001; Guy et al., 2001). It has been shown to interact with a number of cofactors, including mSin3a and HDACs (Jones et al., 1998; Nan et al., 1998) and the SWI/SNF2 complex via Brahma (Harikrishnan et al., 2005). In mice, MeCP2 is a known repressor of the H19 gene (Drewell et al., 2002; Fuks et al., 2003). It represses H19 through its TRD domain and this repression is almost entirely (>95%) dependent on recruitment of HDACs (Drewell et al., 2002). MBD2 was also shown to interact with HDACs (Ng et al., 1999; Zhang et al., 1999). MBD1 is not known to interact with HDACs or SWI/SNF2 and its partners were not tested here. ChIP analysis was performed to establish whether HDACs are present, perhaps recruited via MeCP2 or possibly other MBD proteins. Brahma (Brm) was also studied, as it was shown to interact with MeCP2 (Harikrishnan et al., 2005) and HDACs (Watanabe et al., 2006). The MeCP2 antibody was used as a positive control. ApoE was used here as it was shown in previous experiment to be bound by MeCP2. tRNA ^{Sec} is an actively-transcribed RNA polymerase III gene. As figure 5.7 shows, MeCP2 is enriched on methylated genes compared to the tRNA ^{Sec} gene. HDAC1 and HDAC2 are, however, present on all these genes, suggesting that they can be recruited independently of MBD proteins. Brm was only enriched on B1, B2 and ApoE genes. Brm is recruited to methylated genes by MeCP2 in some cell lines (Harikrishnan et al., 2005). These and our finding suggest that Brm could be recruited to B1 and B2 and ApoE via its interaction with MeCP2. However, direct protein-protein interactions have not been addressed here.

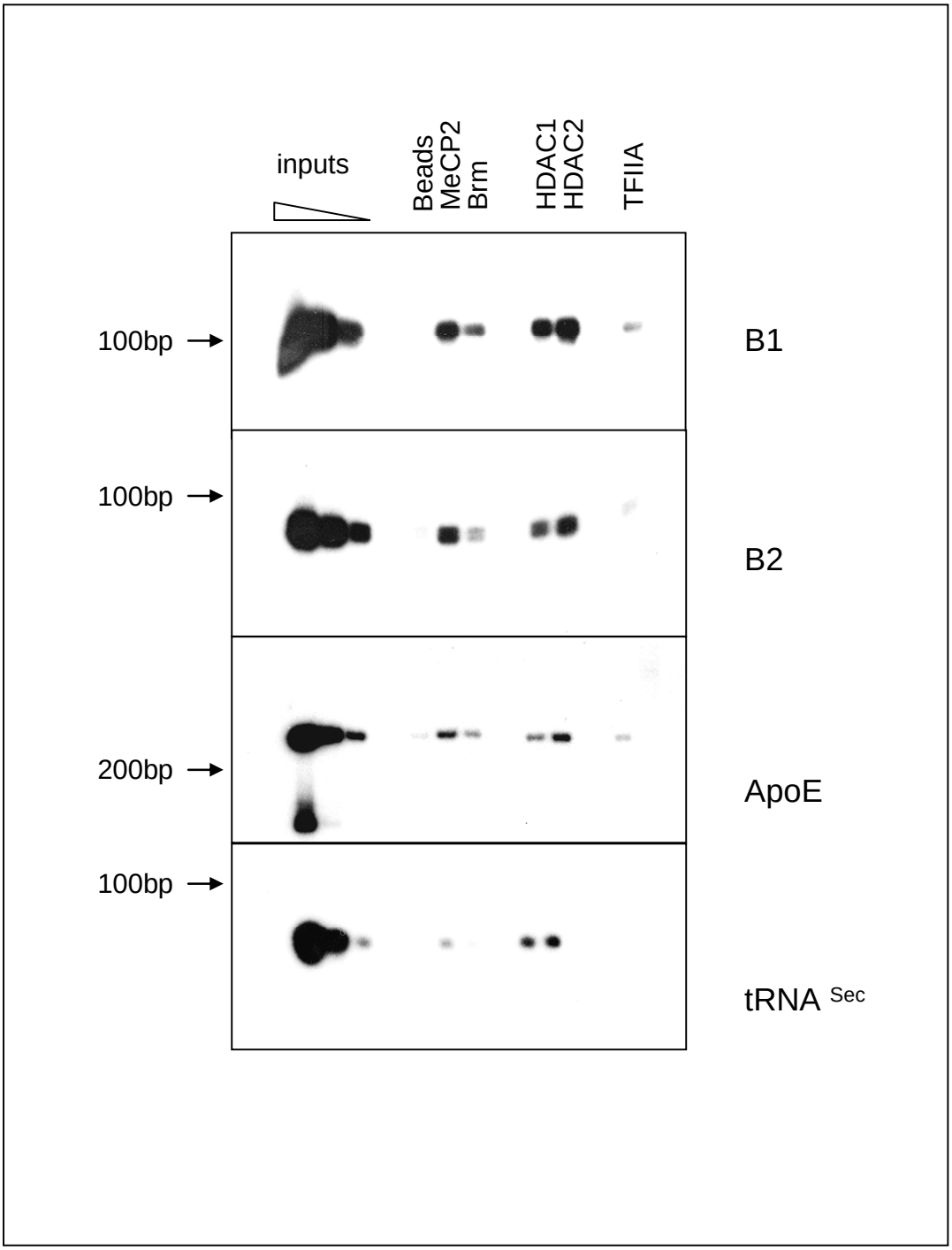


Figure 5-7 ChIP analysis shows that Brm is specifically enriched on genes associated with MBD proteins.
Other tested proteins, namely HDAC1, HDAC2 do not show specific occupancy on B1, B2. All antibodies used for the ChIP analysis are listed on the top of the first panel. Brm=Brahma. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA antibody was used as a negative control antibody. Samples with beads-only were used as a general negative control for ChIP. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

5.2.3 B1 and B2 RNA levels are not upregulated in MeCP2 null mice

The above experiment suggests that HDACs and Brm, which is part of the SWI/SNF2 remodelling complex, are present at B1 and B2 sequences. It is not clear whether these are recruited via MeCP2 and whether MeCP2 directly or via these proteins inhibits B1 and B2 activity. One way to establish MeCP2 importance is to study B1 and B2 expression upon MeCP2 removal. MeCP2 null mice were generated by cre/lox deletion of exons 3 and 4 of the MeCP2 gene (Guy et al., 2001). MeCP2 null males and females are viable and appear normal until about 6 weeks of age. A period of rapid regression follows, when they develop a number of defects including hind limb clasping, irregular breathing, uneven wearing of teeth and various behavioural changes. Progression of symptoms leads to weight loss and death at approximately 8 weeks (Guy et al., 2001). $MeCP2^{+/-}$ heterozygotes did not exhibit this rapid deteriorating effect. Instead, symptoms appeared at about 9 months, suggesting that the condition was long-term stable.

Frozen kidneys of two matching pairs of mice were obtained from M. Bailey (gift from A. Bird). Two months old MeCP2 null male C57/Balb/6 (b13) and age matched wild type C57/Balb/6 (b14) and nine months old MeCP2 heterozygote female (672) and aged matched wild type female (671). Another wild type male is also included (771). All of the mutants were symptomatic (Guy, personal communication). Total RNA was extracted from the kidney tissue and cDNA made. Samples where no reverse transcriptase was added during cDNA synthesis were used as negative control for genomic contamination (data not shown). If MeCP2 is critical in repression of B1 and B2, their expression should increase in mice tissue lacking MeCP2. Samples were normalised for ARPPPO mRNA expression and expression of B1 and B2 in those samples assayed (Figure 5.8). Samples from both kidneys of each mouse are shown. Samples were also quantified and the values are displayed in the chart below. Comparison of MeCP2 -/y male (b13) and MeCP2 +/y male (b14) shows no increase in B1 and B2 expression in MeCP2 null mouse kidneys. As expected from the result of the

MeCP2 null male, there is also no increase in B1 and B2 expression in the MeCP2 heterozygote female (672) relative to the wild type female (671). There seem to be quite significant variation between the two kidneys of each animal. It is also interesting that the additional MeCP2 wild type male expresses more B1 and B2 in its kidneys than the studied mutants. It is clear from this experiment that removing MeCP2 from B1 and B2 does not result in increased levels of B1 and B2. MeCP2 binds to B1s and B2s, but, as found in case of Alus (Yu et al., 2001), it does not seem to repress them.

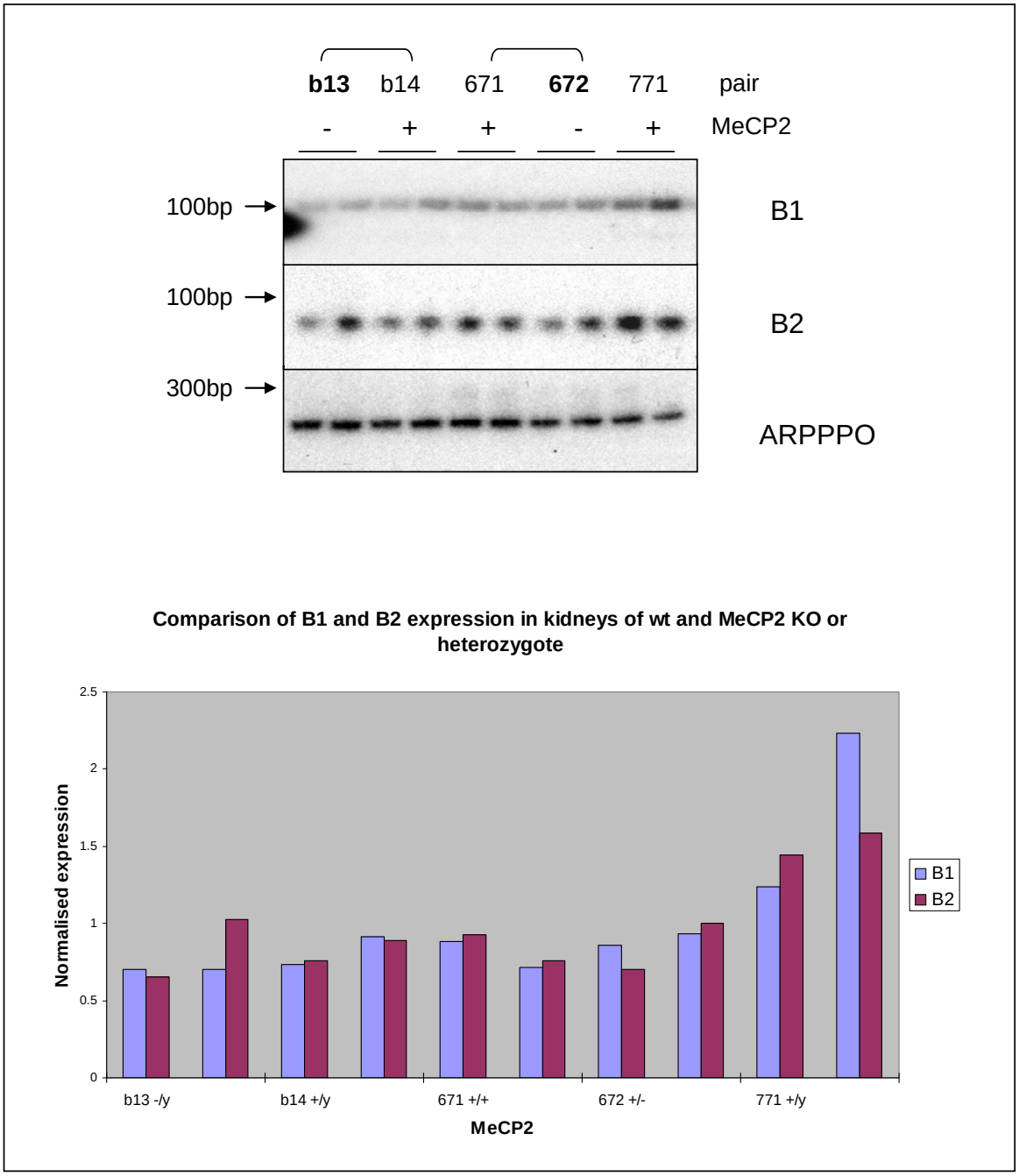


Figure 5-8 RT-PCR shows that the B1 and B2 RNA is not upregulated in kidneys of the MeCP2 KO mouse.
B13 KO and b14 wt are a matched pair, and 671 wt and 672 KO are a matched pair, whilst 771 is an additional wt mouse. The two cDNAs were isolated separately from each kidney. B1 and B2 expression was quantified for both kidneys of each animal and plotted in the bar graph. Auradiographs. Marker sizes are indicated.

5.2.4 Effect of removing DNA methylation on B1 and B2 expression

MBD proteins were shown to bind to methylated B1 and B2 genes, but their role in regulation of these sequences is not clear. B1 and B2 expression did not increase upon MeCP2 removal, suggesting that MeCP2 is not critical for B1 and B2 repression. It remains possible that other MBD proteins compensate for lack of MeCP2 in these mice. A different approach was therefore adopted. Instead of removing MBD proteins one by one, removing DNA methylation would result in releasing MBD proteins from B1 and B2 promoters and alleviating DNA methylation-mediated silencing if it acts on B1 and B2 sequences.

Li et al. (1992) made Dnmt1 $^{-/-}$ mouse embryonic stem cells (Dnmt1 $^{n/n}$) by deleting the first exon of the Dnmt1 gene with *neo* and *hyg* expression cassettes in two rounds of targeting. Deleting of Dnmt1 led to a substantial demethylation of about 80% of total cytosines (Li et al., 1992). This is mainly because these cells retained *de novo* methylation activity (Lei et al., 1996), which is performed by Dnmt3a and Dnmt3b (Okano et al., 1999). Studies of deletions of DNA methyltransferases showed that only Dnmt1 mutants manifest marked loss of genomic cytosine methylation (Li et al., 1992; Okano et al., 1999; Okano et al., 1998).

In Dnmt1 $^{n/n}$ mouse embryonic stem cells, methylation of B1 was reduced to 43% of the levels of wt ES cells (Yates et al., 1999). This compares with the H19 region, which had less than 1.4% of its original methylation. Dnmt1 is a maintaining DNA methyltransferase and these cells lack the enzymatic activity responsible for spreading and maintaining methylation, however *de novo* methylation still remains in these cells (Lei et al., 1996). This suggested that B1s are target of *de novo* methylation.

The cells used here were mouse embryonic fibroblasts derived from 9.5 days old embryos (I.Ben-Porath and H.Cedar, unpublished data). They were Dnmt1 $^{n/n}$ knock out, as described (Li et al., 1992), with introduced mutational inactivation of *Trp53*, resulting in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells and control *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ cells. The

presence of a homozygous p53 mutation allows survival of this somatic cell line, which otherwise succumbs to apoptotic death (Jackson-Grusby et al., 2001). These cells have less than 5% of the normal level of DNA methylation (Jorgensen et al., 2004).

To study the effect of methylation on MBD proteins' binding to B1 and B2, binding of MBD2 and MeCP2 in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblast was compared to binding in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ fibroblasts using ChIP analysis (figure 5.9). MBD1 was not studied here due to lack of a suitable antibody. The MBD1 (Imgenex) antibody used previously in A31 cells did not detect MBD1 in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ cells in ChIP or Western blot (data not shown). Immunoprecipitated DNA was first normalised using input DNA ensuring equal input of material from each cell line. Apart from MBD2 and MeCP2 antibodies, acetylated H4 antibody was used as a positive control and TFIIA antibody and beads as negative controls. Levels of trimethylated H3K9 in the two cell lines were also studied using H3K9me3 antibody. B1 and B2 families were studied using consensus primers, and Set1 primers were used to look at individual B1 and B2 genes on chromosome 9, remote from any RNA polymerase II-transcribed genes. ApoE was used here as a positive control gene, as it was shown previously to be bound by both MBD2 and MeCP2. tRNA ^{Leu} was used as a negative control gene. As figure 5.9a shows, MBD2 and MeCP2 occupy all tested methylated genes in control fibroblasts. In *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ embryonic fibroblasts, where levels of DNA methylation are significantly reduced, there is a reduction in both MBD2 and MeCP2 binding to the methylated promoters. Levels of both proteins were not reduced in the *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts (figure 5.9b), suggesting that lower occupancy on the B1 and B2 promoters is due to a lower affinity of MBD proteins. In fact, the abundance of both MBD2 and MeCP2 is increased in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cell extracts, possibly due to their release from chromatin and consequent higher extractability. Further to the ChIP analysis, it was also interesting that levels of trimethylated H3K9 were only mildly reduced on the ApoE gene, with no reduction on B1 or B2. Unfortunately, due to lack of suitable antibody, MBD1 was not included in these studies. As it binds to both methylated and unmethylated CpG (Jorgensen et al., 2004), it is possible that it remains bound to B1 and B2 and through its interaction with Suv39h1 or SETDB1 maintains histone methylation (Fujita et al., 2003b; Sarraf and Stancheva, 2004).

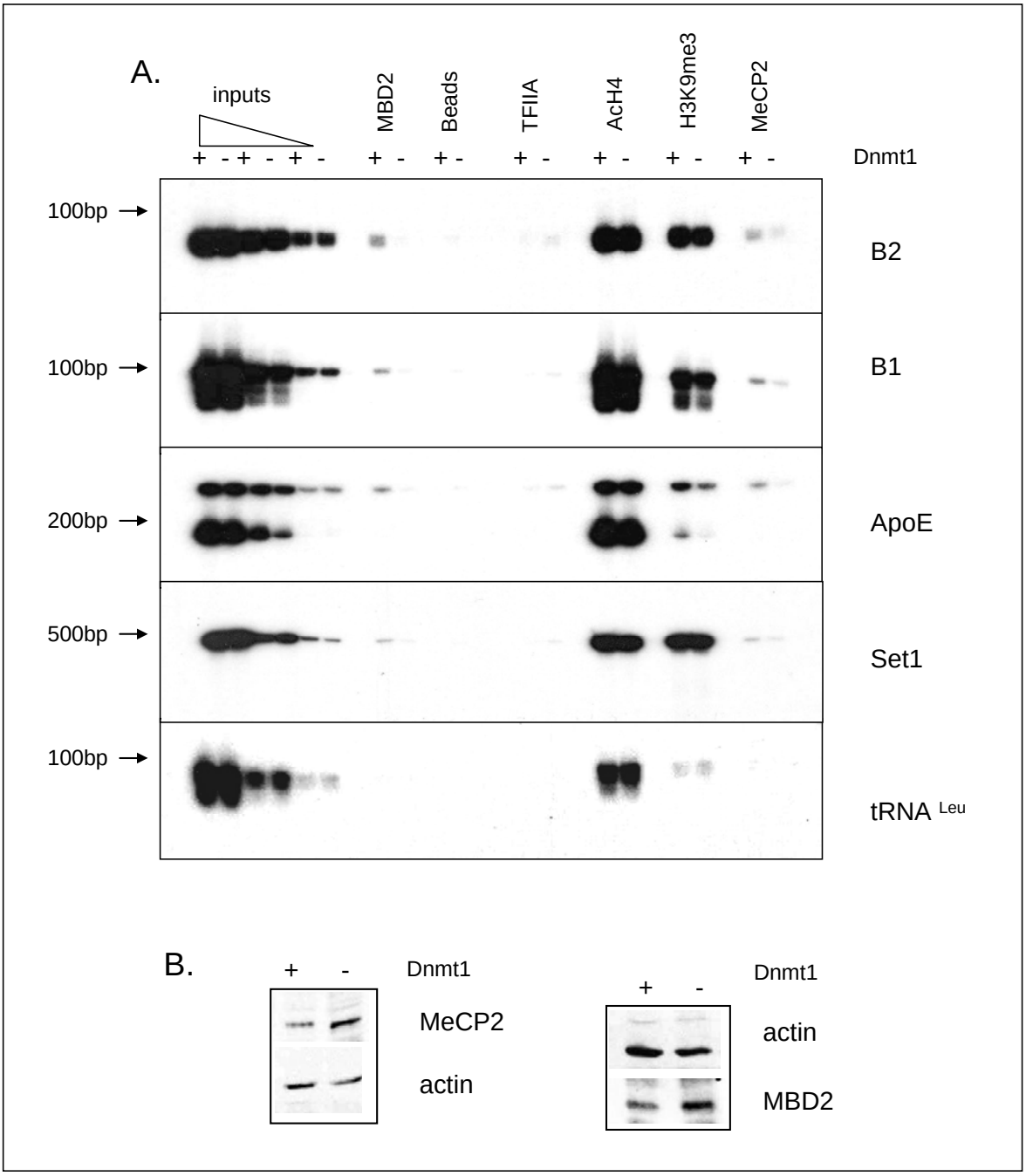


Figure 5-9 MBD2 and MeCP2 occupy methylated genes in DNA methylation-dependent manner.
(A) ChIP analysis of MBD proteins on B1 and B2 promoters in *Dnmt1$^{n/n}$ p53$^{-/-}$* and *Dnmt1$^{+/+}$ p53$^{-/-}$* cells. All antibodies used for the ChIP analysis are listed on the top of the first panel. Input DNA was diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA antibody was used as a negative control antibody. Samples with beads-only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated. (B) Western analysis of MBD2 and MeCP2 shows that levels of both proteins were not reduced in the *Dnmt1$^{n/n}$ p53$^{-/-}$* fibroblasts. Actin was used as a loading control. Total cell extracts were used.

Cells were than examined for expression of B1 and B2 in order to find whether reducing DNA methylation and removing MBD proteins from B1 and B2 genes leads to an increase in their expression. Total RNA was extracted and cDNA made from both *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts (figure 5.10). Samples where no reverse transcriptase was added during cDNA synthesis were used as a negative control for genomic DNA contamination (data not shown). Consensus B1 and B2 primers were used to detect B1 and B2 RNAs. 5S and 7SL are RNA polymerase III-transcribed genes and their RNA levels serve as controls for RNA polymerase III activity in the two cell lines. The p53BP2 gene encodes p53 binding protein 2 and was identified as a target of MBD1. In HeLa cells, it has a methylated promoter and it is silenced both by DNA methylation and methylated H3K9 (Sarraf and Stancheva, 2004). Demethylation of DNA with 5-azacytidine led to an increase of its activity. It was therefore used here as a positive control, together with ApoE. GAPDH was a loading control for equal cDNA levels. Figure 5.10 shows that there was no increase in RNA polymerase III transcript expression in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. Notably, there was no increase in B1 or B2 expression. In contrast, expression of the two DNA methylation-silenced genes, p53BP2 and ApoE, increases in the *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts.

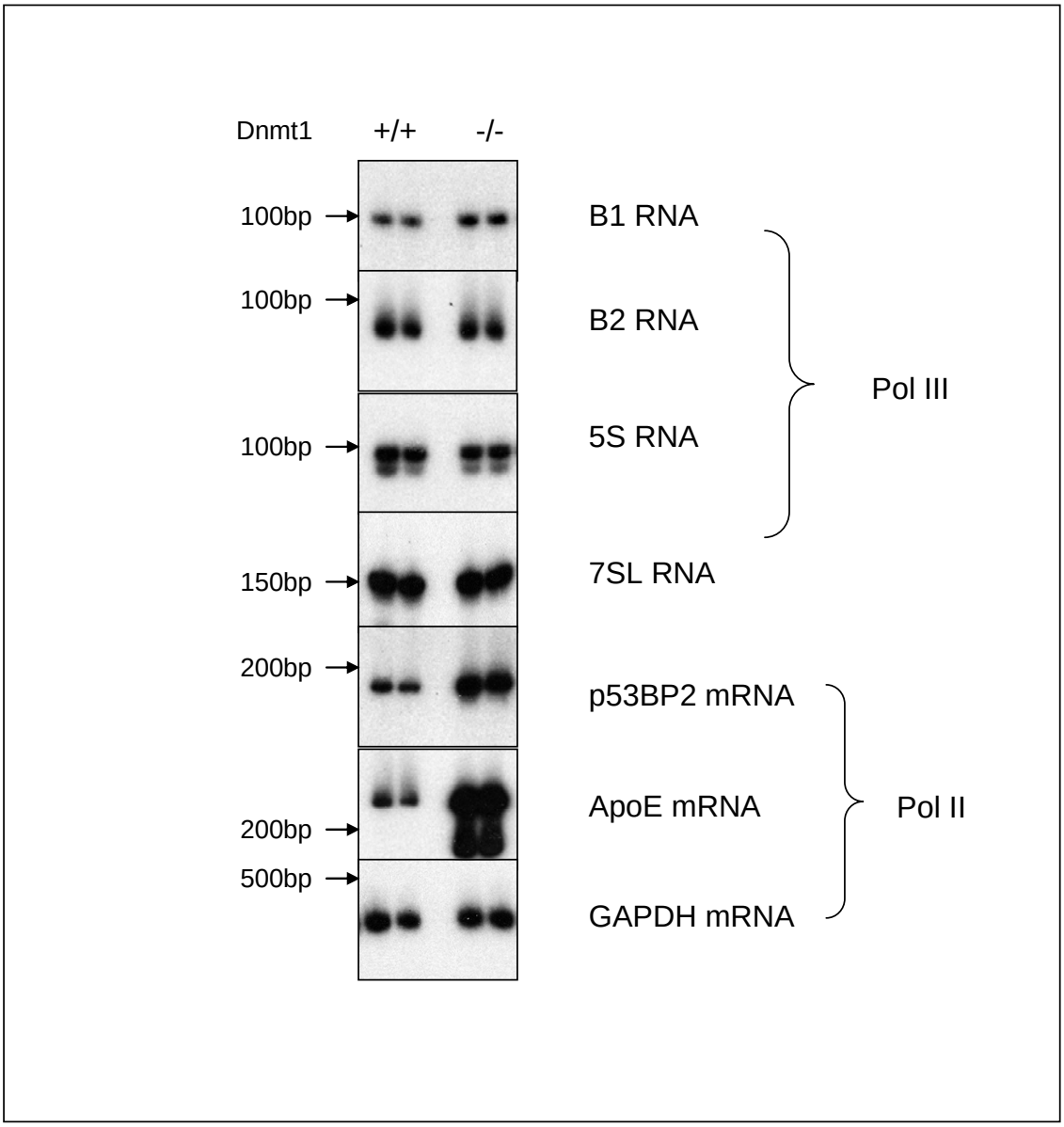


Figure 5-10 RT-PCR shows no increase in the transcription of B1 and B2 in Dnmt1$^{n/n}$ p53$^{-/-}$ cells.
Consensus B1 and B2 primers were used to detect B1 and B2 RNAs. 5S and 7SL are RNA polymerase III-transcribed genes and their RNA levels serve as controls for RNA polymerase III activity in the two cell lines. The p53BP2 is silenced by DNA methylation. Level of its mRNA together with ApoE mRNAs were used as positive controls. Pol II – genes transcribed by RNA polymerase II; Pol III - genes transcribed by RNA polymerase III. Samples are shown in duplicates. GAPDH was used as a loading control. Samples with no Superscript added during cDNA synthesis were used as a negative control for DNA contamination (data not shown). Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter R (RT-PCR) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-32P] dCTP. Autoradiograph. Marker sizes are indicated.

RT-PCR cannot distinguish between newly transcribed RNA and steady state levels. It cannot be excluded that some minor changes happen at the transcriptional levels. However, if methylation-mediated silencing was the main mechanism for controlling expression, an increase in overall B1 and B2 RNA levels would be expected.

One way to gain a further insight into changes in transcriptional activity is to look at promoter occupancy by the RNA polymerase III transcriptional machinery. In previous experiments, it was established that RNA polymerase III levels on B1 and B2 genes are reduced when compared to actively transcribed genes (figure 5.2; figure 5.3). If MBD proteins prevent RNA polymerase III from access to B1 and B2 promoters, removing MBD proteins from the promoters should lead to an increase in occupancy of RNA polymerase III on B1 and B2. ChIP analysis was performed in the *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cell lines (figure 5.11). Input material was first normalised. Antibodies against TFIIIB, TFIIIC, RNA polymerase III and acetylated H3 and acetylated H4 were used to investigate differences in occupancy of transcription machinery and marks of active genes. TFIIA and beads were used as negative controls. 7SL was a positive control gene; the RNA polymerase II-transcribed H19 gene was used as a negative control. Figure 5.11 shows that, as in A31 fibroblasts, transcription machinery is present on B1 and B2 families in both *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ embryonic fibroblasts. Notably, the levels of occupancy remain constant; there is no increase in the occupancies of individual components in the *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. This is consistent with the absence of change in B1 and B2 expression.

There were traces of RNA polymerase III machinery also detected on the H19 gene. It is possible that the H19 sequence contains SINEs or their remnants.

Overall, this experiment argues against the possibility that an increase in transcription was compensated by increased degradation of B1 and B2 RNA and suggests that the unchanged levels of B1 and B2 RNA in the RT-PCR analysis truly reflect a lack of increase at the level of transcription. The data also suggests that DNA methylation-dependent MBD proteins are not responsible for preventing (or not exclusively) RNA polymerase III recruitment to B1 and B2 genes.

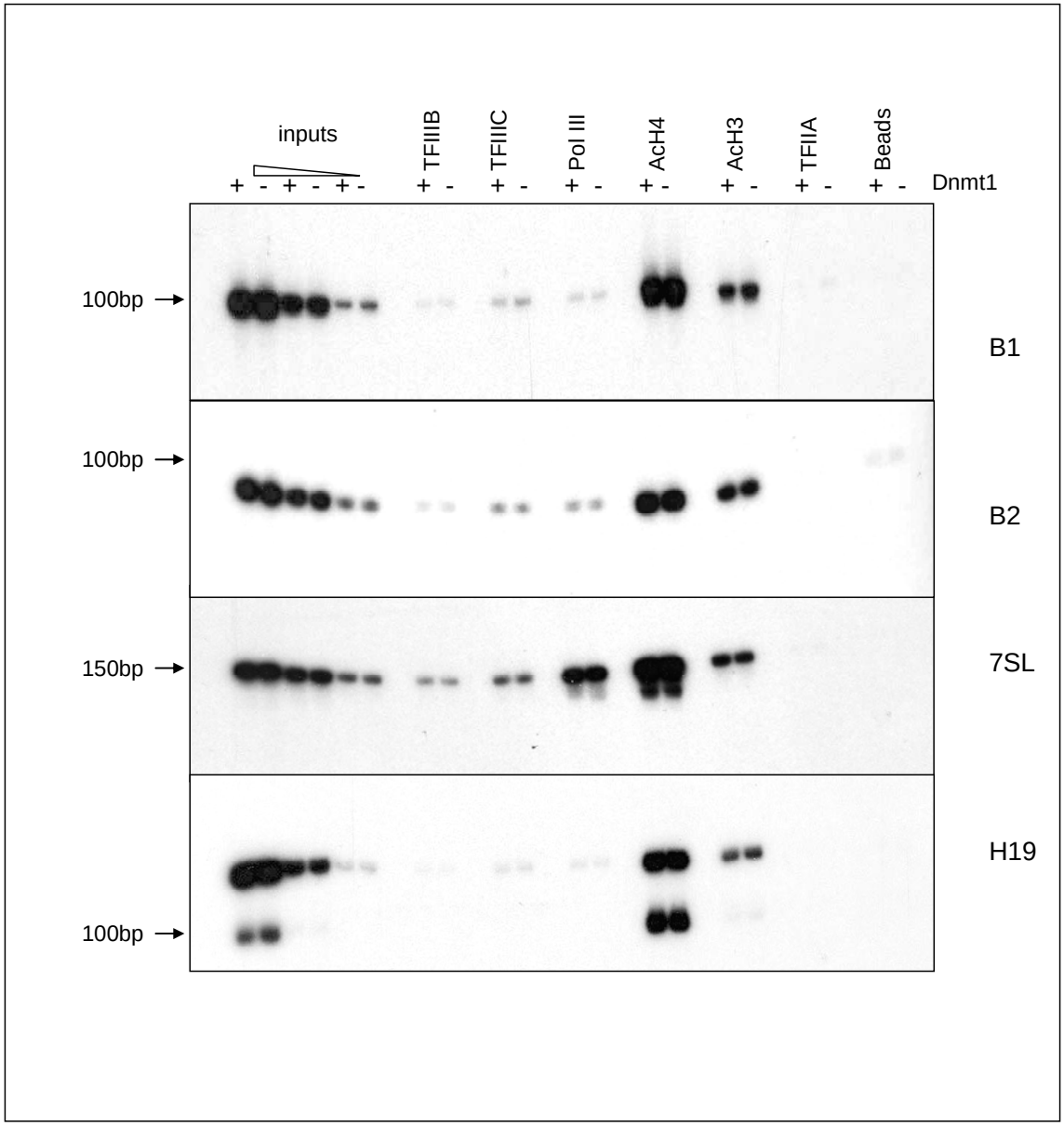


Figure 5-11 ChIP analysis shows no increase in the occupancy of RNA polymerase III machinery in Dnmt1$^{n/n}$ p53$^{-/-}$ cells.
ChIP analysis was performed in the *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cell lines. Antibodies against TFIIIB, TFIIIC, RNA polymerase III and acetylated H3 and H4 were used to investigate differences in the occupancy of the transcription machinery and the marks of active genes. Input material was first normalised to allow direct comparison between the two sets. It was then diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA and beads were used as negative controls. 7SL was a positive control gene; the RNA polymerase II-transcribed H19 gene was used as a negative control. Autoradiograph. Marker sizes are indicated.

MBD proteins associate with corepressor complexes. To investigate their recruitment to B1s and B2s in these cells and whether there is a change upon methylation removal, ChIP analysis was performed. The occupancy of Brm, HDAC1 and HDAC2 proteins in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts was compared (figure 5.12). B1, B2 and set1 were studied together with methylated ApoE and the active RNA polymerase III gene tRNA ^{Sec}. Samples were first normalised for equal inputs. As an additional control, acetylated H4 was used, as it was shown in the previous experiment that its occupancy is not changing. TFIIA and beads were negative controls.

MeCP2 showed reduction in occupancy in the *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts, as before (figure 5.9). Surprisingly, no other proteins were detected in the *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ fibroblasts, apart from HDAC1 at tRNA^{Sec} (figure 5.12). There is also an increase in occupancy of HDAC2 on B1 and B2 and ApoE in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. Unfortunately, it is not known whether ApoE gene (used here as a positive control) is occupied by the studied complexes in these cells.

This result was unexpected, as it is in striking contrast to the ChIP analysis performed in A31 cells (figure 5.7), where these proteins were detected on all tested active and silent genes. This discrepancy could be due to differences between the two cell lines. However, it is more likely to be related to inactivation of p53. p53 is a strong regulator of transcription and its deletion can result in a number of changes. It can affect levels of B1 and B2 expression in these cells. Since it is deleted in both *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts, it is not affecting the relative change in B1 and B2 activities. Any increase in B1s and B2s due to lack of p53 would be constant in the two cell lines. It is likely to affect the binding of corepressors with which it is known to interact. P53 was shown to interact both with HDACs and mSin3a (Murphy et al., 1999) and SWI/SNF (Lee et al., 2002). Better positive control used in future experiment needs to determine this discrepancy.

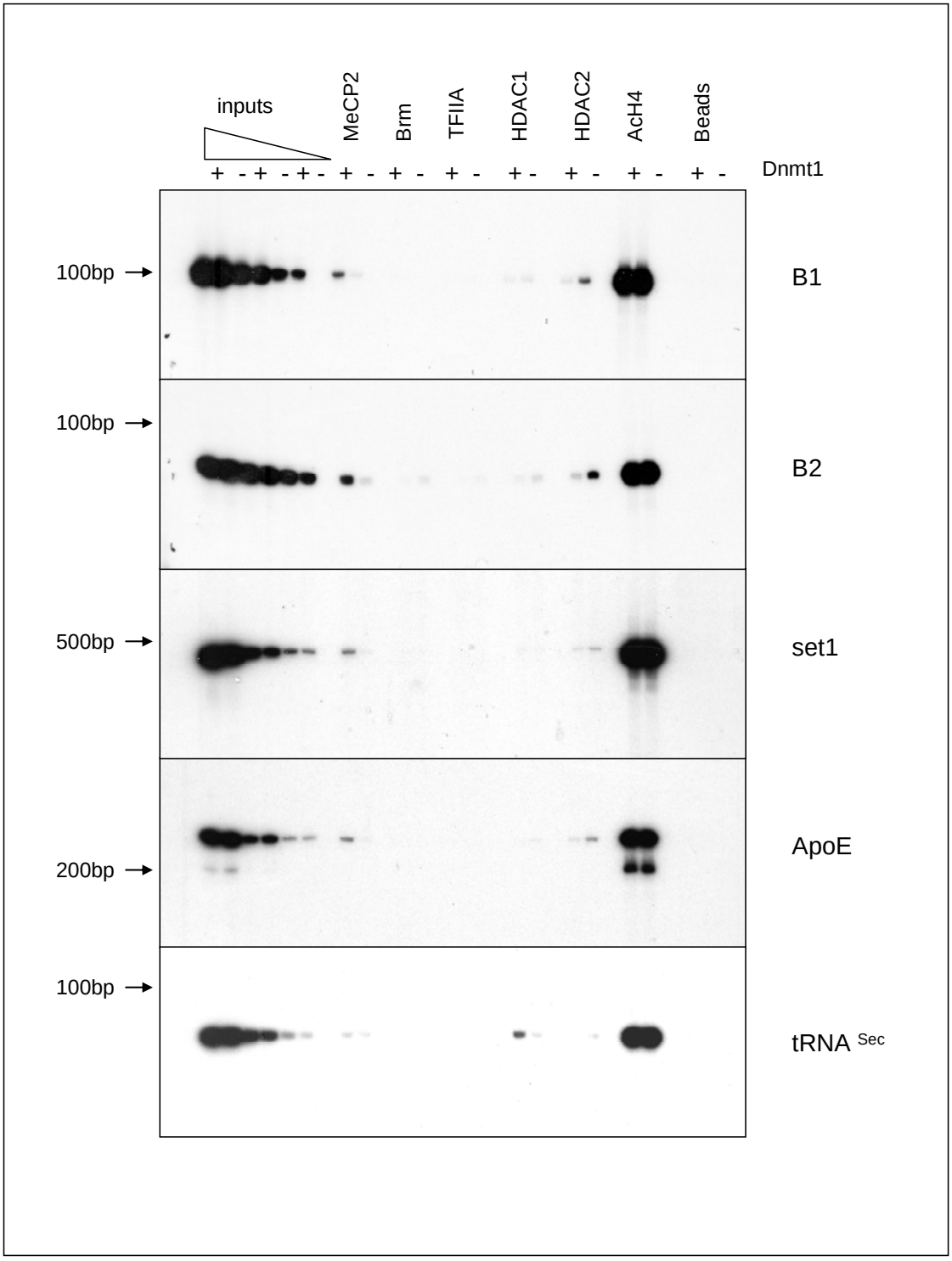


Figure 5-12 ChIP analysis of the occupancy of the components of corepressor complexes, namely Brm, HDAC1, HDAC2 on B1 and B2 in Dnmt1$^{+/+}$ p53$^{-/-}$ cells and Dnmt1$^{n/n}$ p53$^{-/-}$ cells. MeCP2 showed reduction in occupancy in the *Dnmt1$^{n/n}$ p53$^{-/-}$* fibroblasts, as before (figure 5.9). Surprisingly, no other proteins were detected in the *Dnmt1$^{+/+}$ p53$^{-/-}$* fibroblasts, apart from HDAC1 at tRNA^{Sec} . Increased occupancy of HDAC2 on B1, B2 and ApoE was detected in *Dnmt1$^{n/n}$ p53$^{-/-}$* cells. Input DNA was first normalized and then diluted to 10%, 2% and 0.4% to ensure quantitative PCR reactions. TFIIA antibody was used as a negative control antibody. Samples with beads-only were used as a general negative control for ChIP analysis. Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter C (ChIP) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-^{32}P] dCTP. Autoradiograph. Marker sizes are indicated.

The lack of proteins bound to the promoters could also simply be due to altered expression of these proteins in the cells. To confirm that the proteins are expressed in both *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells, total cell extracts were made and Western blot analysis performed (figure 5.13). Actin served as a loading control. It was confirmed that Brm, HDAC1 and HDAC2 are all expressed in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ cells. They are also expressed in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells, and their levels seem to increase in these cells. This may be due to higher solubility of these proteins in the total cell extract, as their presence on chromatin may be reduced.

In conclusion, this study provides the first evidence that DNA methylation and DNA methylation-mediated repression alone may not be silencing these SINE families. MBD proteins were found to be bound on methylated B1s and B2s, but when removed, no increase in transcriptional activity was observed. Their sole involvement in B1 and B2 repression is therefore unlikely. HDACs and Brm were detected in A31 cells, but when their dependence on DNA methylation and MBD proteins was tested in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ fibroblasts, ChIP analysis failed to detect them, possibly due to the absence of p53. Their involvement stays therefore unclear. If these complexes are involved in B1 and B2 repression, it is likely to be in a DNA methylation-independent manner.

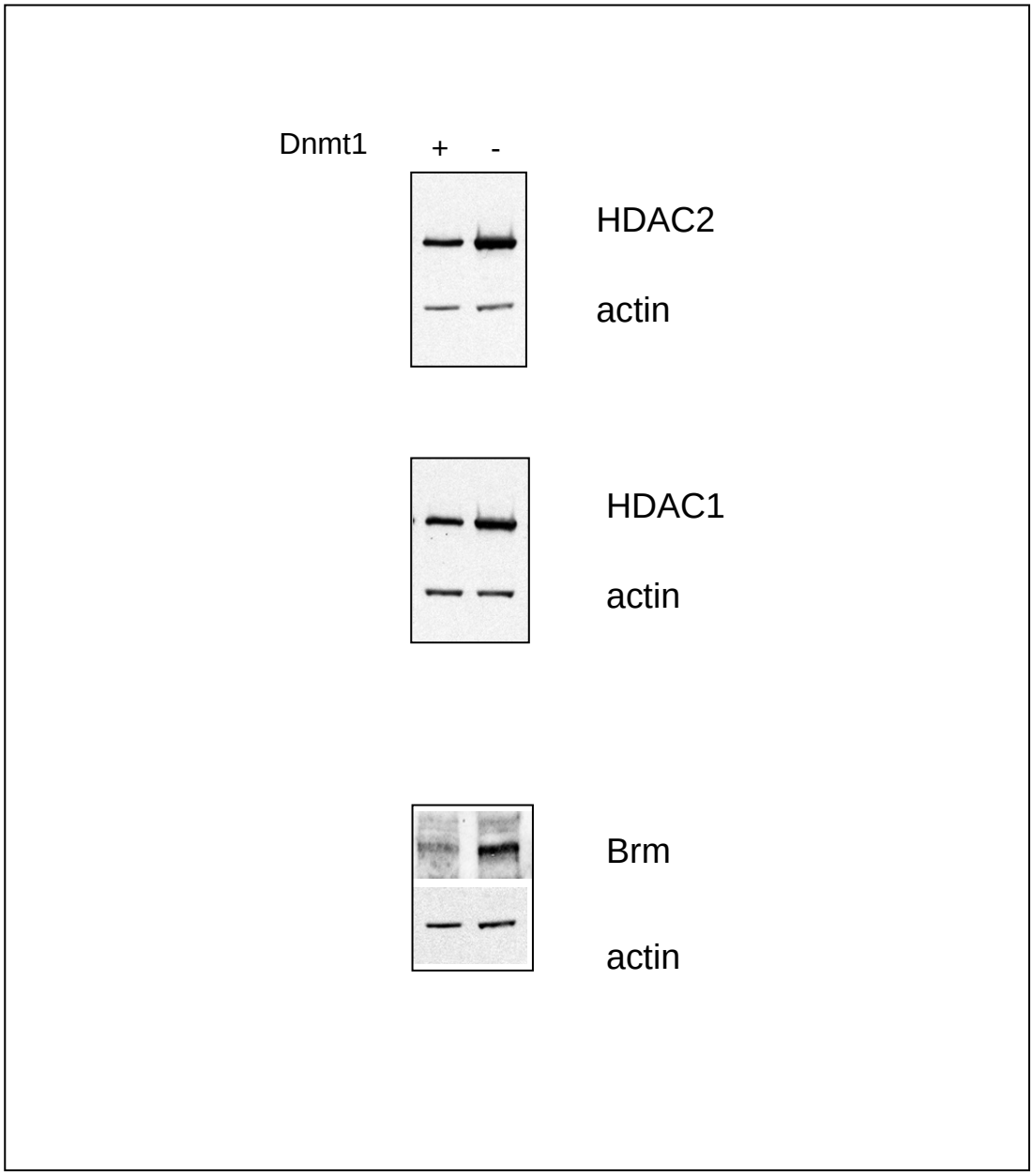


Figure 5-13 Western analysis of protein levels of HDAC2, HDAC1 and Brm in Dnmt1$^{+/+}$ p53-/- and Dnmt1$^{n/n}$ p53$^{-/-}$ cells.
Western analysis shows that levels of both proteins were not reduced in the *Dnmt1$^{n/n}$ p53$^{-/-}$* fibroblasts. Total cell extracts were used. Actin was used as a loading control.

5.2.5 Histone H1 is not responsible for silencing B1 and B2 transcription

Functional assays suggested that viral infections stimulate SINE transcription in transformed cells by unmasking their chromatin structure and making them more accessible for *in vitro* transcription (Carey and Singh, 1988; Russanova et al., 1995). Adenovirus also unmasks a subset of Alus in HeLa chromatin (Li et al., 2000). Accessibility of Alus for restriction cleavage increases after heat shock treatment or cycloheximide treatment of HeLa and 293 cells (Li et al., 2000). So these studies indicate that there is a tight correlation between Alu transcription and chromatin accessibility. Adenoviral infection in HeLa cells also results in a slight increase of nucleosome spacing, suggesting that linker histone H1, which influences the length of linker DNA between nucleosomes, may be involved in SINE repression. However, when Bio-Rex 70 ion-exchange resin was used to deplete H1 from HeLa cells, it resulted in a very mild 2-to 2.5-fold increase of Alu expression (Russanova et al., 1995). Alus were further shown to possess a nucleosome positioning signal *in vitro* and *in vivo* (Englander and Howard, 1995; Englander et al., 1993). Assembling a methylated Alu template with histone tetramers leads to complete transcriptional repression compared to only 2- to 3-fold reduction of naked Alu DNA by methylation alone; however, assembling the Alu template with histone octamers was methylation insensitive (Englander et al., 1993). In contrast to Alu, ion-exchange resin-mediated depletion of linker histone H1 had a major effect on alleviating B2 repression (Carey and Singh, 1988; Russanova et al., 1995). The effect of linker histone H1 on B1 repression has not yet been established.

H1 was long considered as a global repressor of gene activity through its compaction of chromatin, because it is often depleted on active chromatin and can cause inhibition of transcription *in vitro* (Bresnick et al., 1992; Shimamura et al., 1989; Smith and Hager, 1997). However, new evidence showed that cells and tissues can tolerate very low levels of H1 and that only a small percentage of genes are effected in their expression (Fan et al., 2003; Fan et al., 2005).

Fan et al. (2003) prepared mouse embryonic stem cells with three out of six H1 subtypes (H1c, H1d, H1e) deleted. That led to about 50% reduction in linker H1 content in these cells. This resulted in dramatic global changes in chromatin structure, involving decreased global nucleosome spacing and reduced chromatin compaction. Surprisingly, expression of only few genes was affected as shown by microarray analysis, some with positive and some with negative effects. The largest group of effected genes were imprinted or repressed by methylation. B1 and B2 expression was not studied.

RNA from two H1cde triple knock-out ES cell lines together with wild type ES cell line (derived from littermate embryos) was obtained (Y.Fan, gift) and analysed here for B1 and B2 expression using RT-PCR. ARPPPO mRNA was used as a loading control. Imprinted H19 gene, whose RNA levels were shown to be upregulated in KO ES cells served as a positive control. RT-PCR confirmed that H19 mRNA increases in both triple KO ES cell line when compared to the wild type ES cell line, but surprisingly, neither B2 nor B1 expression increased in the triple KO ES cells (figure 5.14). This is in striking contrast with previous studies which suggested that B2 was derepressed by H1 depletion. Several explanations are possible. Firstly, because the reduction in H1 content is only 50%, the remaining H1 may still be maintaining repression of SINEs in the ES cells. However, considering the substantial overall relaxation of the chromatin structure, if linker H1 was the major repressor some changes would be expected. The results are consistent with those of Fan et al (2005) that despite large changes in chromatin structure, only small numbers of genes were affected, some of them even with lower expression. Secondly, the previous data which showed derepression of B2 when H1 was removed from chromatin using ion-exchange columns could have depleted other important components which resulted in the observed derepression. The lack of increase in activity is in agreement with evidence that increased nucleosome spacing has a positive effect on Alus accessibility for RNA polymerase III (Li et al., 2000). Here, the nucleosome spacing is actually reduced upon H1 removal which together with unchanged methylation of B1 and B2 may still keep B1 and B2 repressed. In conclusion, Alus were not affected by H1 depletion and perhaps B1 and B2 may not be either.

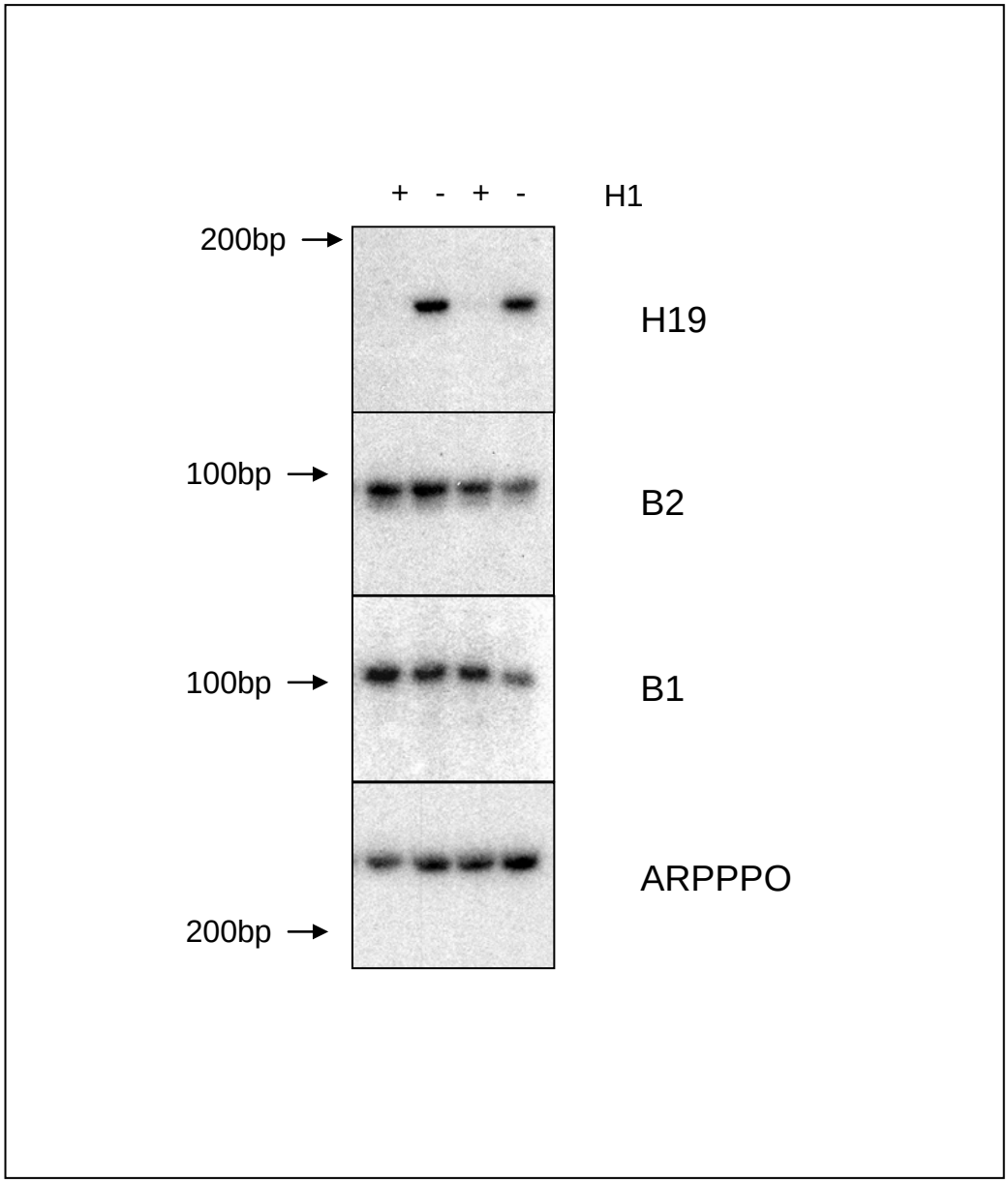


Figure 5-14 RT-PCR shows that H1 depletion does not increase B1 and B2 expression in ES cells.
Consensus B1 and B2 primers were used to detect B1 and B2 RNAs. ARPPPO was used as a loading control. H19 was used as a positive control. Samples are shown in duplicates. Samples with no Superscript added during cDNA synthesis were used as a negative control for DNA contamination (data not shown). Primers used in each PCR reaction are listed in Table 2-3 and indicated with letter R (RT-PCR) in the second column and M (mouse) in the third column of the table. PCR reactions contained [α-32P] dCTP. Autoradiograph. Marker sizes are indicated.

5.3 Discussion

B1 and B2 are rodent SINEs which are present each in about half a million copies in the mouse genome. Despite their abundance, relatively few B2 transcripts and even fewer B1 transcripts (Carey et al., 1986; Maraia, 1991) can be detected in cultured cells or mouse tissues, indicating that like human Alus, expression of these repetitive elements is repressed. Compared to Alus, mechanisms of repression of B1s and B2s have not yet been studied much. Numerous studies, however, showed that both B1 and B2 expression increases as a response to various stimuli and that led to hypotheses about how they may be repressed.

In cells infected with various viruses, the increase is thought to be mediated through activity of TFIIIC and/or TFIIIB (Larminie et al., 1999; White et al., 1990; Yoshinaga et al., 1986). Strikingly, it was revealed in this study that RNA polymerase III machinery is present on many B1 and B2 sequences. Data presented here showed that both TFIIIB and TFIIIC are present on tested B1s and B2s at comparable levels with active genes. Increased availability of additional TFIIIB and TFIIIC might therefore activate those B1s and B2s which were previously unoccupied.

More global changes in chromatin not dedicated to RNA polymerase III components were observed previously (Li et al., 2000; Russanova et al., 1995). Cell stress conditions increase transcription of both B1 and B2 (Liu et al., 1995; Schmid, 1998). It is not known how this is mediated, but in the case of Alus, it is believed to be via chromatin and template availability.

Observations here suggest that at least some B1 and B2 genes avoid being sequestered by chromatin proteins and maintain their promoters accessible to RNA polymerase III machinery, arguing against the general template masking that has been suggested before (Li et al., 2000; Russanova et al., 1995). Chromatin remodelling following stimuli such as viral infection and cell stress may, however, unmask other B1 and B2 genes which are normally inaccessible and unoccupied by RNA polymerase III components. It is reasonable to consider that to mount a quick response resulting in the increase of B1 and B2 expression

which is observed within half an hour of stimulation, preassembled complexes would be a key. *In vitro*, RNA polymerase III transcription complex can assemble in less than 15 minutes (R.White, personal communication), the rate of the *in vivo* assembly is not known.

However, as the very low levels of transcripts detected in uninduced cells and tissues imply, these preassembled complexes are somehow inactive. The observed low occupancy of detected RNA polymerase III may be a sign of a repression. RNA polymerase III access and function was thought to be inhibited by chromatin proteins or by mediators of DNA methylation-derived repression, the MBD proteins. ChIP analysis in A31 cells and in mouse embryonic fibroblasts showed that MBD1, MeCP2 and MBD2 are present on both B1 and B2 sequences, potentially mediating repression of the RNA polymerase III complex. HDAC1, HDAC2 and Brm were detected on B1 and B2 sequences in A31 cells. It is not clear whether these are recruited via MBD proteins or independently and whether they can establish repression of B1 and B2 transcription. RT-PCR analysis of B1 and B2 RNA in MeCP2 null mice, however, showed no increase, suggesting that MeCP2 may not play a non-redundant role in their repression. This would agree with a previous study showing that human Alus are bound by MeCP2, but MeCP2 does not seem to repress them (Yu et al., 2001). More MeCP2 null individuals and wild type mice and tissues should however be examined to avoid individual differences. Tissue-specific cofactors might allow regulation in other cell types.

B1 and B2 were then studied in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ embryonic fibroblasts, which have less than 5% of the normal DNA methylation level. ChIP analysis showed a significant reduction in tested MBD proteins MBD2 and MeCP2 binding to methylated genes. RT-PCR comparison of levels of B1 and B2 RNA in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells, however, failed to detect any increase in B1 or B2 expression. This suggested that DNA methylation-dependent MBD proteins do not mediate the repression of B1 and B2 genes. Occupancy of RNA polymerase III factors in the two cell lines was also studied, but no increase in occupancy of any of the RNA polymerase III components was seen when DNA methylation was reduced. Occupancy of RNA polymerase III remained reduced compared to active genes, suggesting a MeCP2 and MBD2 independent effect. MBD1 that was not included in the study due to technical difficulties may still mediate repression as

it also binds unmethylated as well as methylated sequences (Jorgensen et al., 2004). Its effect could be DNA methylation-independent. MBD1 was shown to interact with H3K9 HMTs and the persistent level of methylated H3K9 in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells is in support of this possibility.

The binding of HDAC1, HDAC2 and Brm was studied in this system and was not detected in either of the cell lines. This was unexpected, as these proteins were detected on B1 and B2 in A31 cells. Careful interpretation about the significance of lack of these proteins on genes is needed here, as the most obvious difference between A31s and these cells is the lack of p53 protein. P53 is a global repressor of RNA polymerase III transcription and is known to interact with various chromatin repressor and chromatin remodelling complexes such as HDACs and mSin3a (Murphy et al., 1999) and SWI/SNF (Lee et al., 2002). It is likely to cause changes in the binding of these proteins to their target sequences.

Chromatin was suggested as a possible major repressor of template availability and activity. B2 activity was reported to increase upon removal of linker histone H1 (Carey and Singh, 1988; Russanova et al., 1995). Triple null mutant cells were created, resulting in a 50% decrease in H1 content (Fan et al., 2003). This led to significant changes in chromatin structure. However, a surprisingly small number of genes were affected, resulting in increased expression of some and decreased expression of others (Fan et al., 2005). RNA from these cells was used to re-investigate the effect of H1 on activity of B2s and establish the effect of H1 on B1 activity. The RNA level of the control H19 gene increased in both triple KO ES cells when compared to the wild type ES cells, but surprisingly neither B2 nor B1 expression increased in triple KO ES cells. H1 was also shown to have little effect on Alus in human cells (Russanova et al., 1995). It is possible that during the ion-exchange resin removal of H1, another important component is removed, resulting in the B2 increase. Deletion of the H1 genes provides a better control of specificity.

Taken together, the data from MeCP2 null mice and *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells suggest that DNA methylation and DNA methylation-dependent MBD proteins are not responsible for B1 and B2 repression, although further studies are necessary. A better system for assessing the role of DNA methylation in B1 and B2 silencing would be to use the Dnmt3^{-} cells (Okano et

al., 1999), which, through prolonged passage in culture established virtually no detectable CpG methylation (0.6%) and p53 levels were not deliberately altered (Gilbert et al., 2007).

DNA methylation-mediated silencing may also act in concert with histone deacetylation. It was shown recently that there are two distinct classes of methylated genes (Lande-Diner et al., 2007). One category is automatically induced following the removal of DNA methylation. In the other group, methylated genes could not be activated by demethylation. However, they underwent strong induction when cells were subjected to combination of demethylation and TSA treatment (Lande-Diner et al., 2007). TSA is a histone deacetylase inhibitor and its treatment prevents deacetylation of histones. Future study should therefore determine whether this is not the case of SINE repression by treating *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells with TSA.

Future studies should also include another chromatin remodelling protein, Lsh. Lsh is a member of SNF2 of chromatin remodelling complexes (Jarvis et al., 1996) that was previously mainly associated with pericentromeric chromatin (Yan et al., 2003). However, its deletion was found to result in global loss of DNA methylation in mouse cells, comparable to that of Dnmt1$^{n/n}$ (Dennis et al., 2001). Mainly repetitive sequences were shown to be demethylated (Dennis et al., 2001). SINEs were amongst sequences found to be deregulated in Lsh$^{-/-}$ cells (Huang et al., 2004), however, this deregulation was not determined further as the study focused mostly on LTR repeats. Slight enrichment of Lsh at SINEs was observed by ChIP analysis (Huang et al., 2004). mRNA and protein levels of Dnmt1 and Dnmt 3a/3b were unchanged in the Lsh$^{-/-}$ cells, as was their methyltransferase activity. It was therefore suggested that based on its chromatin remodelling activity, Lsh may regulate chromatin accessibility for DNA methyltransferases. Lsh's homologue DDM1 (Dennis et al., 2001) also cooperates with DNA methyltransferases and contributes to transcriptional silencing of transposons in plants (Lippman et al., 2004). Lsh cooperation with Dnmts may be similar in mammalian cells. Despite the fact that Dnmt1 deletion did not result in increased expression of SINEs in this study, Lsh may be involved in additional, independent way. As its plant homologue DDM1, it could involve SINE-derived double stranded RNA to maintain their silenced status (Lippman et al., 2004).

6 Chapter 6 - Final discussion

6.1 Introduction

Despite the abundance of the templates, both human and rodent SINEs are normally expressed at a very low level. There are several reasons why it may be important to suppress their transcription. Firstly, they could present a burden for limited cell resources. Secondly, their activity could result in undesired amplification, which can be potentially very harmful (Deininger and Batzer, 1999). Thirdly, taken from a view that they may be merely DNA parasites, in order for them to amplify, they have to get to the next generation. Such amplification is only possible for retroelements in germ lines, where they are indeed more active (Li et al., 1999). In somatic cells, it is therefore not to their advantage. Taken together, there is no incentive for the host cell to keep SINEs constitutively active or for SINEs themselves to be constitutively active. Exposure to various cell stress stimuli increase this activity many folds. One view is that this increase simply disrupts normal SINE regulation, inadvertently causing a transient, non-specific increase in dormant SINE activity. However, as more and more functions are being described, whether original or acquired, it is clear that using the vast potential they represent might be beneficial to a host cell in certain situations (see Chapter 1).

6.2 The effect of DNA methylation and chromatin on the activity of SINEs

Expression of both human and rodent SINEs was studied here in physiological conditions with respect to its regulation by DNA methylation and other chromatin factors, including the mechanisms by which such regulation may be achieved. Both human and rodent SINEs were shown here to be bound by the MBD proteins MeCP2, MBD1 and MBD2, which suggested that the effect of DNA methylation may be mediated via these proteins. Whether MBD proteins mediate repression of SINEs was then investigated. Their involvement in recruitment of chromatin remodeling and corepressor complexes was studied. In addition, the

importance of their presence was studied using systems with low or near absent DNA methylation or systems where the MBD was removed.

HDAC1/2 and components of chromatin remodeling and corepressor complexes were detected on SINEs in this study. Brahma was specifically enriched only on both human and rodent SINEs and a control methylated gene. It is a component of a SWI/SNF2 remodeling complex. In this case, it may be recruited via MeCP2, which was shown before to interact with Brahma, resulting in repression (Harikrishnan et al., 2005). Other proteins detected in this study on human Alus were components of the NuRD and SIN3 complexes. HDAC1/2, NuRD and SIN3 were shown to associate with MBD proteins before and they may therefore be recruited by MBD proteins to the SINE's methylated DNA. Yet, they could also be recruited independently, for example via p53 (Murphy et al., 1999) or H3K9me3 (Stewart et al., 2005).

Apart from SINEs, some of the NuRD and SIN3 components were also detected on some actively transcribed genes. HDACs on the other hand, were detected on all of the tested genes. That was surprising, but given the complexity of their action, these complexes are no longer regarded as solely connected to transcriptionally silenced genes. It may be that there is an intricate 'dance' of associations, with these changing places over time. The positive-acting complexes may be recruited during initiation or elongation, followed by recruitment of negative-acting complexes (Sin3-HDAC) during attenuation of transcription. As an example could serve transcriptional regulation by oestrogen-receptor-α (ERα) at the pS2 gene. Once bound by oestradiol and recruited to target DNA containing oestrogen responsive elements, ERα induces an ordered and cyclical recruitment of coactivator and corepressor complexes containing HAT, HMT or ATP-dependent remodelling activities (Metivier et al., 2003).

The importance of the observed occupancy of MBD proteins and corepressor complexes on SINEs was then investigated by studying their effect in near methylation-free systems or, in the case of MeCP2, by its direct removal. The effect of the absence of MBD proteins and corepressor complexes was, however, only investigated with regards to rodent SINEs, because of the lack of a suitable 'methylation-free' system for Alu research. Human methylation-free cells (HCT116 DKO) were shown in this study to have decreased levels of p53, which is

a known RNA polymerase III repressor. The observed increase in SINE RNA levels and all other tested RNA polymerase III transcripts were probably affected by this. Support for a p53 effect came from the observed increase in levels of 5S rRNA. Expression of 5S rRNA was shown before not to be affected by DNA methylation (Besser et al., 1990). Because its RNA levels were upregulated in HCT116 DKO cells, it was likely an effect of decreased levels of p53, rather than of the lack of DNA methylation. *Saccharomyces cerevisiae*, a DNA methylation-free organism, was also used to study an integrated Alu element, but problems were encountered with the chosen Alu construct, as it appeared to affect a nearby RNA polymerase II promoter. Although this is very interesting, it was not ideal in this study.

Whether MBD proteins' absence will affect the activity of rodent SINEs was studied using two systems – MeCP2 null mice and *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. RT-PCR analysis of B1 and B2 expression in MeCP2 null mice kidneys showed no increase in B1 and B2 RNA levels. This suggested that MeCP2 may not play a non-redundant role in their repression, at least in kidney tissue. If it plays any, it is well compensated by other mechanism(s). B1 and B2 were also studied in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ embryonic fibroblasts, which have less than 5% of the normal DNA methylation level. ChIP analysis showed a significant reduction of MeCP2 and MBD2 binding to SINEs, showing that their presence is DNA methylation-sensitive. RT-PCR comparison of *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ and *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells, however, detected no increase in B1 or B2 RNA levels. This is consistent with results obtained from MeCP2 KO mice, where lack of MeCP2 did not result in increased B1 and B2 expression. This is also consistent with a previous study showing that human Alus are a major binding target of MeCP2, but that MeCP2 does not seem to repress them (Yu et al., 2001). MBD2 also does not seem to repress rodent SINE activity, as its removal did not result in increased SINE RNA levels. SINE expression remained at comparable level in DNA methylation-free cells (*Dnmt1*$^{n/n}$ *p53*$^{-/-}$) relative to levels in cells with normal DNA methylation (*Dnmt1*$^{+/+}$ *p53*$^{-/-}$).

How is SINE repression mediated in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells? Attempts were made to detect occupancy of components of corepressor and chromatin remodeling complexes, that were found to occupy SINEs in other mouse cells (A31); however, these failed to be detected even in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$, making

interpretation of their absence in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ difficult. A potential explanation for the absence of the complexes in both cell lines is the lack of p53. p53 is known to interact with HDACs, which were shown both in HeLa cells and in A31 cells to be present on SINEs (Murphy et al., 1999). The extent of the p53 effect is, however, not known. Because p53 is known to repress SINEs, levels of SINE RNA in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ may already be partially elevated compared to levels in other cells, disguising the potential effect of any DNA methylation-independent action of corepressor complexes.

SINE repression could also be mediated by MBD1. MBD1 can bind both methylated and unmethylated DNA through its different domains and it was detected here on Alus in HeLa cells and on both B1 and B2 in A31 cells. Due to lack of suitable antibody, it was not tested in the *Dnmt1*$^{+/+}$ *p53*$^{-/-}$/*Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. It is likely to occupy B1 and B2 in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ cells and it is possible that it remains bound to unmethylated SINEs in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. It is not known to interact with HDAC1/2 or SIN3 and NuRD, but it is known to establish repression via different partners. It is known to interact with HMTs Suv39h1 and SETDB1 (Fujita et al., 2003b; Sarraf and Stancheva, 2004), which specifically methylate H3K9. Levels of methylated H3K9 were comparable on B1 and B2 in *Dnmt1*$^{+/+}$ *p53*$^{-/-}$/*Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells, suggesting that MBD1 may still be present on SINEs. MBD1 further associates with MCAF/AM-related proteins (Ichimura et al., 2005; Sarraf and Stancheva, 2004) and together with SETDB1 establishes transcriptional repression. H3K9 methylation was also shown to be connected to an RNAi mechanism and Alus were shown to be sources of miRNAs and to match certain miRNAs as targets (Borchert et al., 2006; Smalheiser and Torvik, 2006). However, this repressive effect of MBD1 has only been shown for RNA polymerase II transcription and H3K9 methylation has never been connected to repression of RNA polymerase III transcription.

Overall, SINEs repression was not found to be mediated by DNA methylation or the DNA methylation-dependent MBD proteins, MeCP2 and MBD2. If MBD1 and/or corepressor and chromatin remodelling complexes bound to SINEs regulate SINE transcriptional activity, it is via DNA methylation-independent mechanism(s).

Future study should include TSA treatment to determine whether DNA methylation-mediated silencing does not act in concert with histone

deacetylation. Some methylated genes could not be activated by demethylation, but they undergo strong induction when cells are subjected to combination of demethylation and TSA treatment (Lande-Diner et al., 2007). SINEs may be similarly regulated.

As mentioned earlier, future studies should also include the chromatin remodelling protein Lsh. Its deletion was found to result in global loss of DNA methylation in mouse cells, comparable to that of $Dnmt1^{n/n}$ (Dennis et al., 2001). Mainly repetitive sequences were shown to be demethylated (Dennis et al., 2001) and SINEs were amongst deregulated sequences found to be in $Lsh^{-/-}$ cells (Huang et al., 2004), however, this deregulation was not determined further as the study focused mostly on LTR repeats. Slight enrichment of Lsh at SINEs was observed by ChIP analysis (Huang et al., 2004). Lsh was suggested to regulate chromatin accessibility for DNA methyltransferases. Despite the fact that Dnmt1 deletion did not result in increased expression of SINEs in this study, Lsh may be involved in additional, independent way. As its plant homologue DDM1, it could involve SINE-derived double stranded RNA to maintain their silenced status (Lippman et al., 2004).

NuRD complex binding to Alus should be also further examined with respect to its association with cohesin on Alus. ChIP analysis demonstrated specific association of hRAD21, SNF2 and mi2 with DNA elements containing Alu element (Hakimi et al., 2002). This binding depended on DNA methylation status of Alu elements. Binding of the cohesin complex to Alu containing DNA was stronger after 5-azacytidine treatment. Chromatin remodelling activity of SNF2h was shown to be important, as transfection of cells with SNF2h mutant containing mutation in the nucleotide-binding motif that abrogates ATP hydrolysis resulted in disruption of hRAD21 binding to DNA elements containing Alus (Hakimi et al., 2002). It may be that SNF2h/NuRD-mediated binding of hRAD21 to Alus plays a role in their transcriptional regulation.

Another aspect of chromatin that was also considered is the linker histone H1. H1 is connected to chromatin-mediated repression (Laybourn and Kadonaga, 1991; Shimamura et al., 1989). It is often more abundant on silent genes and regions of chromosomes (heterochromatin) which are less transcriptionally active. Its removal was shown to result in major changes in chromatin

compaction (Fan et al., 2005). It was reported previously that is has an effect on B2 transcription (Carey and Singh, 1988; Russanova et al., 1995), but not on Alu (Russanova et al., 1995). It was reexamined here after its removal was shown to have little effect on general RNA polymerase II activity (Fan et al., 2005), and a great proportion of the few genes upregulated were normally repressed by DNA methylation. RT-PCR from RNA extracted from triple H1 KO mouse embryonic fibroblasts was used here to study B1 and B2 activity. No increase in B1 or B2 RNA levels was detected, as compared to wild type cells, suggesting that H1 is not the main or single mediator of repression. This is in agreement with the work of Fan et al. (2005), showing that only small percentage of RNA polymerase II genes and their activity is affected by H1.

Histone H1 was not itself found to be repressing SINEs. However, it has been suggested that chromatin may act through positioning of nucleosomes over SINE promoters (Englander and Howard, 1995). This seems unlikely, because all SINEs studied here have preassembled transcriptional complexes, which suggests that H1 or nucleosomes do not cause an obstruction for their assembly. Furthermore, it was shown that yeast RNA polymerase III can transit during its transcriptional activity through nucleosomal DNA by mobilising histones along the templates (Studitsky et al., 1997).

A striking discovery in this work was the detection of preassembled RNA polymerase III transcription complexes on all tested human and rodent SINEs. These were present both in wild type cells (HeLa, A31) and *Dnmt1*$^{+/+}$ *p53*$^{-/-}$ cells and their occupancy did not increase in *Dnmt1*$^{n/n}$ *p53*$^{-/-}$ cells. This suggests that occupancy is not affected by methylated DNA or perhaps chromatin in general. The occupancy of TFIIIC and TFIIIB on SINEs was equivalent to their occupancy on active RNA polymerase III transcribed genes, while the occupancy of the RNA polymerase III was significantly lower on SINEs then on active genes, suggesting a deficiency in RNA polymerase III loading. Since levels of SINE RNAs in the cells are low and the occupancy of RNA polymerase III is lower on SINEs, these complexes may be regarded as inactive. The nature of the defect in RNA polymerase III loading onto SINEs is not known. A direct effect of DNA methylation or MBD proteins can, however, be excluded. Also, an unprecedented insight into the number of RNA polymerase III transcription factors present in the cell is presented here. If it is the case that all SINEs capable of recruiting

transcription complexes to their promoters (excluding those with inactive promoters) can be occupied by transcription complexes, there must be a high number of transcription complexes present in the cell. Recent preliminary data show that indeed there are far more TFIIIB molecules than originally thought (White, personal communication).

6.3 Conclusions

To conclude this work, MBD1, MeCP2 and MBD2 and components of chromatin remodelling and corepressor complexes were detected on both human and rodent SINEs, while SIN3 and NuRD were also detected on human SINEs. Removal of DNA methylation and DNA methylation-dependant MBD proteins did not result in increased B1 and B2 transcriptional activity, showing that repression of SINE activity is still maintained.

It was shown here that despite their low transcriptional activity, SINEs are occupied by RNA polymerase III transcription factors TFIIIB and TFIIIC. There is also a low level of RNA polymerase III present on SINEs, suggesting that SINEs have a defect in its loading. Occupancy of the RNA polymerase III does not increase at DNA methylation-'free' SINEs, suggesting that this defect is DNA methylation-independent. This contradicts the common belief, that SINEs do not bind the RNA polymerase III transcription machinery *in vivo* and that their promoters are masked by chromatin, or MBD proteins. It also brings a new insight into the number of RNA polymerase III transcription factors present in the cell.

How the repression of SINEs is mediated is not clear, but data in this study show that it is likely to be DNA methylation-independent. TSA studies will need to exclude that histone deacetylation is not required in addition to DNA demethylation. Repression may be mediated via chromatin remodelling complexes, which were shown to bind SINEs. However, these would be recruited and maintained via DNA methylation-independent mechanisms, such as p53 and H3K9 methylation. So far, involvement of chromatin remodelling complexes in SINEs repression has not been shown except for Lsh (Huang et al., 2004), which was not studied here and will need to be addressed in future experiment. Brahma, which was shown to be recruited by MeCP2 and result in repression (Harikrishnan et al., 2005) is otherwise a 'neutral' component of SWI/SNF2

remodelling complexes and it is used by both activating and repressing complexes to remodel chromatin. Brahma was also shown to be present together with MeCP2 on actively transcribed genes during natural cycles of methylation and demethylation after RNA polymerase II transit (Metivier et al., 2008). The DNA methylation-independent repression of SINEs is likely to be at multiple levels. Depending on the stimuli and the function they perform, a certain amount of SINE RNA may be needed. It is therefore desirable that a cell could control these at many levels, which could be used individually or in a compounded manner in order to achieve a certain magnitude of response. Many mechanisms of control would be expected and, when needed, some of these mechanisms are alleviated in order to obtain the required levels of SINE RNA.

The significance of the presence of DNA methylation on SINEs also remains unknown. It was speculated that DNA methylation only silences genes that are inactive and does not affect genes that may be active (Bird, 2002). Potentially active SINEs with preassembled RNA polymerase III complexes may therefore be 'immune' to repressive effect of DNA methylation. Evidence that DNA methylation does not repress actively transcribed genes was given recently. Completion of genome-wide microarray analysis of DNA methylation in *Arabidopsis* indicated that, in addition to its expected distribution in silenced heterochromatin, DNA methylation is also common across ORFs. Even more unanticipated is the presence of DNA methylation in the ORFs of many actively transcribed genes (Zhang et al., 2006; Zilberman et al., 2007). Further example of DNA methylation on active genes was given by study of ERα-controlled genes (Kangaspeska et al., 2008; Metivier et al., 2008). Actively transcribed genes were showed to be exposed to cycles of DNA methylation/demethylation, where DNA methylation occurs after the cyclical occupancy of ERα and RNA polymerase II (Kangaspeska et al., 2008). DNA methyltransferases were shown to be involved in the methylation and active demethylation of CpGs (Metivier et al., 2008).

DNA methylation on SINEs could be useful to cells in other ways, such as reduction of non-homologous recombination by introducing CpG to TpG mutations (Bird, 1980). These mutations can also silence retrotransposition, as shown with a B1 element where a single base mutation is responsible for a many-fold decrease in transpositional activity (Dewannieux and Heidmann, 2005). However, a primary function of DNA methylation as a mechanism of introduction

of these mutations seems unlikely, as most of the preserved CpGs (apart from CpG islands) are present in SINE DNA. It was also speculated that methylated SINEs contribute to genomic imprinting as they are highly methylated in somatic tissues and female germ cells, but methylation in male germ cells is restricted (Hellmann-Blumberg et al., 1993; Rubin et al., 1994). However the fact, that imprinted regions are usually poor in SINEs (Greally, 2002), does not support that.

For the future, one can confidently predict that research of SINEs will yield further surprises.

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