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**A study of chromatin and rDNA
transcription in
*Saccharomyces cerevisiae***

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Abstract

Within the cell, DNA is compacted through its organisation into chromatin. A great deal is known about the chromatin structure of RNA Polymerase II-transcribed loci and its impact on transcription by this polymerase. In contrast, far less is known about the role of chromatin in RNA Polymerase I transcription. RNA Polymerase I transcribes rDNA genes within the nucleolus, producing 60% of all cellular transcripts. Studies into the chromatin structure of the rDNA locus have resulted in an unclear picture as to the nature of chromatin on actively transcribed rDNA genes. Furthermore, chromatin remodeling factors have so far only been shown to function on inactive rDNA repeats. This study used a combination of techniques to investigate the chromatin structure of actively transcribed rDNA repeats. In addition, the presence and function of chromatin remodeling factors at this locus was addressed. Chromatin immunoprecipitation analysis revealed that actively transcribed rDNA genes possess a form of chromatin structure composed of, at least, histones H2B and H3 and that histone H3 is dimethylated at lysine 4 and 36. Furthermore, chromatin remodeling factors Chd1p, Isw1p and Isw2p are present across the rDNA locus and are likely recruited by both histone tail modifications and rRNA. *In vivo* chromatin analysis was used to analyse the chromatin structure over the termination region of rDNA repeats and showed that Chd1p, Isw1p and Isw2p function redundantly to maintain a specific chromatin structure at inactive repeats. Finally, transcription run-on analysis demonstrated that Chd1p, Isw1p and Isw2p function redundantly to ensure efficient transcription.

These data therefore lead to an updated model of *S. cerevisiae* rDNA transcription, whereby RNA Polymerase I transcription occurs on a chromatin template. Chromatin modifications function to recruit chromatin remodeling factors that are required to ensure efficient transcription.

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Abbreviations

In addition to standard SI units and chemical symbols the following abbreviations were used:

A	adenine
ADP	adenosine 5'-diphosphate
ARS	autonomously replicating sequence
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cytidine
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CE	core element
CF	core factor
Ci	Curie
CKII	casein kinase II
CTD	C-terminal domain
CTP	cytidine 5'-triphosphate
d	2'-deoxy
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EM	electron microscopy
EtOH	ethanol
ETS	external transcribed spacer
G	guanine
GTP	guanosine 5'-triphosphate
H3 K4diMe	histone H3 dimethylated at lysine 4
H3 K36diMe	histone H3 dimethylated at lysine 36

HDAC	histone deacetylase
IP	immunoprecipitated sample
ITS	internal transcribed spacer
K4	lysine 4
K36	lysine 36
kb	kilobase
kDa	kilodaltons
mRNA	messenger RNA
nt	nucleotides
NTP	nucleoside 5'-triphosphate
NTS	non-transcribed spacer
NO	no antibody sample
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIC	pre-initiation complex
Pol I	RNA Polymerase I
Pol II	RNA Polymerase II
Pol III	RNA Polymerase III
PTRF	Pol I and transcript release factor
rDNA	ribosomal DNA
rInr	ribosomal initiator
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT-PCR	reverse transcription PCR
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SDS	sodium dodecyl sulphate
SL1	selectivity factor 1
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
ssDNA	single stranded DNA
T	thymine
TOT	total sample

tRNA	transfer RNA
TRO	transcription run-on
TTF-I	Pol I transcription termination factor
U	uracil
UAF	upstream activating factor
UBF	upstream binding factor
UPE	upstream promoter element
UTP	uridine 5'-triphosphate
UV	ultraviolet
YP	yeast peptone

Role of author

The research presented here is my own, original work and has not been submitted for any other degree.

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Introduction

Preface

RNA Polymerase I (Pol I) is responsible for the transcription of ribosomal DNA (rDNA) genes, resulting in the production of ribosomal RNA (rRNA), a vital catalytic and structural component of the ribosome. While rRNA genes account for 10% of the *Saccharomyces cerevisiae* (*S. cerevisiae*) genome, their transcription generates 60% of cellular transcripts, with ~2000 ribosomes produced per minute (Warner, 1989). Please note that hereafter, the term “yeast” refers specifically to *S. cerevisiae*.

The process of Pol I transcription in *S. cerevisiae* will be summarised below. This thesis has focussed on the question of chromatin and rDNA transcription. Thus, chromatin and chromatin remodeling factors are also discussed below.

1.1 The rDNA locus

Biogenesis of both rRNA and ribosomes takes place in the nucleolus. This organelle is a crescent-shaped structure lying alongside the nuclear envelope and occupying up to one-third of the nucleus. The nucleolus is divided into three sub-compartments: the fibrillar centre; the dense fibrillar component and the granular component. It is believed that the site of rRNA transcription lies in the transition zone between the fibrillar centre and the dense fibrillar component (Scheer and Hock, 1999). The nucleolus is a dynamic organelle and both its structure and location within the nucleus depends on normal Pol I transcription (Oakes et al., 1998).

Within the yeast nucleolus, the rRNA genes are located on Chromosome XII on a tandem array (Johnston et al., 1997). Each transcription unit is ~9.1kb in length (Goffeau et al., 1996) and produces a 35S polycistronic transcript. The transcription unit comprises the 18S gene, the 5.8S gene and the 25S gene. In addition, there are two external transcribed

sequences (5' ETS and 3' ETS) and two internal transcribed sequences (ITS) (Figure 1.1). ~150 copies of the rDNA transcription unit are found in budding yeast, arranged head-to-tail with individual transcription units separated by a non-transcribed spacer (NTS1 and NTS2). Located within the transcription unit are also an enhancer (see below), an origin of replication (ARS) and the 5S gene. This gene is transcribed from the opposite strand to the rDNA genes by RNA Polymerase III (Pol III).

In higher eukaryotes, rRNA genes are found on several chromosomes, forming arrays of tandem repeats known as nucleolus organiser regions. In humans for example, ~400 rRNA genes are divided into nucleolus organiser regions on chromosomes 13, 14, 15, 21 and 22. Furthermore, rRNA genes can be present as extrachromosomal copies. In maturing amphibian oocytes for example, rRNA genes are amplified, producing thousands of extrachromosomal copies (Raska, 2003).

In all organisms, approximately half of rRNA genes are transcriptionally silent and in yeast, the distribution of active and inactive copies is known to be random (Dammann et al., 1995). Silencing at the rDNA locus is discussed below (Section 1.8.2). Active transcription of rRNA genes visualised by electron microscopy produces a “Christmas tree” structure. The path of the gene is the trunk of the tree and the pre-rRNAs radiate away from the trunk, forming the branches of the tree. This structure was first seen in nuclear spreads from amphibian oocytes. Such structures were later shown to also exist in yeast (reviewed in Raska, 2003).

In yeast cells, Pol I transcription does not fluctuate during the cell-cycle. This is in stark contrast to mammalian cells where large oscillations in transcription are seen. rDNA transcription shuts down in mitosis, gradually increases during G1 phase and is maximal in S

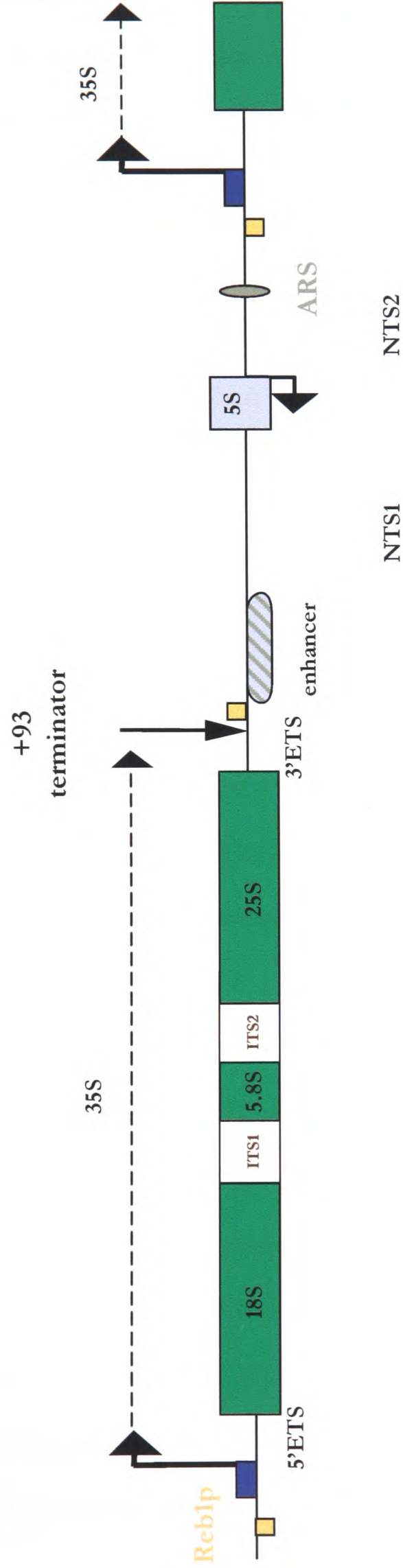


Figure 1.1: Diagram of the rDNA repeat. The direction of the 35S primary transcript is indicated by a dashed line. The 18S, 5.8S and 25S genic regions are shown in green. The external (ETS) and internal (ITS) transcribed sequences are shown. The non-transcribed spacer sequence is indicated (NTS). The Reb1p binding sites are shown in yellow. The promoter region is shown in blue. The Pol III-transcribed 5S gene is shown in grey. The +93 primary termination site is denoted by vertical arrow to the left of the Reb1p binding site. The autonomously replicating sequence (ARS) is denoted by a dark grey oval. The enhancer is shown as a grey dashed oblong. Note that diagram is representative and not drawn to scale. Diagram adapted from E. M. Prescott, D. Phil thesis, University of Oxford, 2002.

and G2 phases. The repression of transcription during mitosis is achieved through inhibitory phosphorylation of SL1 and UBF. In addition, essential UBF phosphorylation required for its activity is lost. During G1 phase, the inhibitory phosphorylation is lost and UBF is phosphorylated at two activatory sites, allowing for full activity during S and G2 phases (reviewed in Grummt, 2003 and Russell and Zomerdijk, 2005).

1.2 Eukaryotic RNA Polymerases

Transcription is the process by which RNA is synthesised from its template DNA. Almost all eukaryotic genes are transcribed by one of three RNA Polymerases. Pol I is dedicated solely to the production of rRNA. Transcription of protein-encoding genes by RNA Polymerase II (Pol II) produces messenger RNAs (mRNAs). In addition, Pol II is responsible for the production of a number of small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNAs). Pol III transcribes the remainder of the snRNAs, tRNA and 5S rRNA. The ratio of RNA to DNA in a rapidly growing *S. cerevisiae* cell is 50:1, with rRNA contributing 80% of this RNA (Warner, 1999). Finally, in *Arabidopsis thaliana*, a fourth polymerase has been identified as being required for amplification of small interfering RNAs (Onodera et al., 2005).

Pol I consists of 14 subunits, Pol II of 12 and Pol III of 13 (Ishihama et al., 1998). Those subunits belonging to Pol I are denoted “A”, those belonging to Pol II as “B” and those belonging to Pol III as “C”. All enzymes share 5 common subunits (ABC10 α , ABC10 β , ABC14.5, ABC23 and ABC27), whilst Pol I and Pol III share a further two subunits that are not found in Pol II (AC19 and AC40). The shared subunits are all essential for cell viability but there is no evidence that these five subunits are sufficient to form a functional enzyme (references in Bischler et al., 2002).

The structure of Pol I has been determined by electron microscopy (Klinger et al., 1996; Schultz et al., 1993b). These studies indicated that yeast Pol I is characterised by a 3nm wide, 10nm long groove which forms a putative DNA binding site. The structure is similar to *E. coli* holo-enzyme and Pol II lacking the non-essential subunits B4 and B7 (Schultz et al., 1993b). A more recent cryo-electron microscopy study elucidated the location of the 4 Pol I-specific subunits on the core enzyme (Bischler et al., 2002). Of great interest is the location of A43, the subunit known to interact with the transcription factor Rrn3p (see below) and the only essential Pol I-specific subunit. A43, together with A14, forms a stalk which is located at the site where the CTD of the large Pol II subunit exits the enzyme, suggesting this structure may fulfil a similar role to that of the CTD in Pol II (Bischler et al., 2002).

1.3 Transcription by RNA Polymerase I

1.3.1 Promoter structure

In contrast to the Pol II and Pol III promoters, the primary sequences of Pol I promoters are not highly conserved, resulting in species-specific transcription (Heix and Grummt, 1995). However, the general promoter architecture is conserved from yeast to man (Paule, M. R. 1998). This architecture consists of two *cis*-acting promoter elements, the core element (CE) and the upstream promoter element (UPE). The CE overlaps the start site of transcription, denoted as +1 (Klemenzen and Geiduschek, 1980), by 4-6bp and extends ~50bp upstream. The sequence surrounding +1 is known as the ribosomal initiator (rInr) and is AT-rich, allowing easy melting of the DNA duplex. The second promoter element, the UPE, is located upstream of the CE and extends to approximately -150. The CE is absolutely required for transcription and can direct low levels of transcription *in vivo* when the binding of factors to the UPE is blocked. However, the UPE is required for full transcription and formation of a stable pre-initiation complex (PIC) at the promoter (Keys et al., 1996).

1.3.2 Additional *cis*-acting elements within the rDNA locus

In addition to the CE and UPE, a number of other *cis*-acting elements are found within the rDNA transcription unit.

Upstream of the UPE lies a proximal terminator (PT) which binds the termination factor Reb1p. This element is unable to mediate termination as it is in the wrong orientation (Lang et al., 1994). It has been proposed to stimulate transcription in a number of ways, including protecting the promoter from read-through polymerases (Bateman and Paule, 1988; Henderson et al., 1989), remodeling chromatin over the promoter (Langst et al., 1998) and altering the structural organisation of the promoter region (Sander and Grummt, 1997).

The final *cis*-acting element to be discussed here is the enhancer, a 190bp element found approximately 100bp downstream of the rDNA transcription unit and overlapping the termination region (Elion and Warner, 1984; Elion and Warner, 1986). This is the sole enhancer element in *S. cerevisiae*, in contrast to higher eukaryotes which possess multiple such elements. The yeast enhancer is able to act bi-directionally to stimulate transcription at a promoter located over 2kb away (Johnson and Warner, 1989; Kulkens et al., 1992). It is unclear how the enhancer functions and a number of models have been proposed. Firstly, it has been suggested that the enhancer acts to assist stable PIC formation (McStay et al., 1997; Schultz et al., 1993a). A second model proposes that the enhancer acts to stimulate that rate of transcription from already active genes (Banditt et al., 1999; Morrow et al., 1993). The enhancer is known to bind the termination factor Reb1p and it is thought that Reb1p bound at the enhancer and PT causes the rDNA transcription unit to loop, bringing the termination region of one repeat close to the promoter of another repeat (Paule and White, 2000).

1.3.3 Initiation

Initiation of Pol I transcription involves the coordinated binding of a number of *trans*-acting factors to the *cis*-acting promoter elements described above (Figure 1.2). Basal transcription *in vitro* requires the core factor (CF), Rrn3p and Pol I. To achieve full transcription however, upstream activation factor (UAF) and TATA binding protein (TBP) are required, with UAF and TBP stimulating transcription 10-50 fold (Keener et al., 1998).

CF binds to the CE of the promoter (Bordi et al., 2001) and is composed of 3 TBP-associated factors (TAFs): Rrn6p, Rrn7p and Rrn11p (Keys et al., 1994; Lalo et al., 1996; Lin et al., 1996). CF interacts weakly with TBP via Rrn6p (Steffan et al., 1996), stimulating recruitment of CF to the promoter (Aprikian et al., 2000).

The UPE is bound by UAF, which contains 6 subunits: Rrn5p; Rrn9p; Rrn10p; histone H3 and histone H4 and Uaf30p (Keener et al., 1997; Keys et al., 1996; Siddiqi et al., 2001). Interaction of UAF with the UPE occurs in the absence of other factors and commits the rDNA to transcription (Bordi et al., 2001; Keys et al., 1996). In contrast to CF subunit mutants, mutants in UAF subunits Rrn5, Rrn9p and Rrn10p are viable, indicating that this complex is not essential but serves to stimulate transcription (Keys et al., 1996). The presence of histones within UAF may suggest that this complex is able to mark a promoter for transcription by wrapping the promoter DNA around these histones, relieving a repressive chromatin structure. UAF also plays a role in silencing rDNA transcription by Pol II. There is a cryptic Pol II promoter that overlaps the Pol I promoter and produces an equivalent 35S primary transcript (Conrad-Webb and Butow, 1995). This polymerase switch occurs in the absence of UAF, indicating a role for this factor in silencing Pol II transcription (Oakes et al., 1999; Vu et al., 1999). More recently, the Uaf30p subunit of UAF

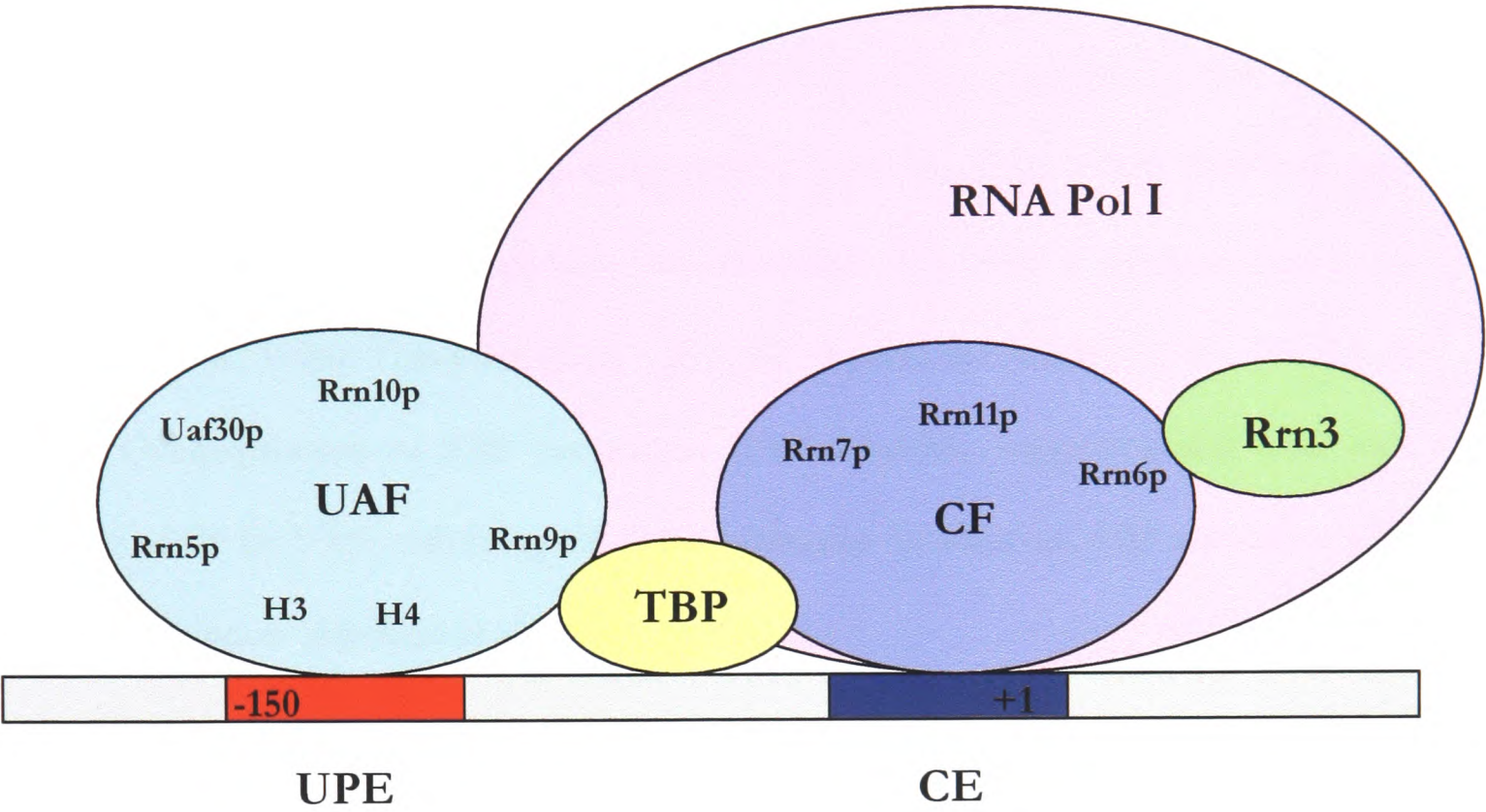


Figure 1.2: Diagram showing the *cis*-acting elements of the promoter and the *trans*-acting factors these elements bind. The rDNA promoter region is shown as a grey bar. The core element (CE) is shown in blue and the upstream promoter element (UPE) is shown in red. The CE overlaps the start site of transcription (“+1”). The UPE is found upstream of the CE and extends to -150 (“-150”). The *trans*-acting factors are shown as coloured circles. The upstream activating factor (UAF) is shown in light blue with its subunits (Rrn5p, Rrn9p, Rrn10p, Uaf30p, histone H3 and histone H4) shown. The proximity of Rrn9p to the TATA-binding protein (TBP, yellow) indicates their interaction. The core factor (CF) is shown in purple with its subunits (Rrn6p, Rrn7p, Rrn11p) shown. The proximity of Rrn6p to transcription factor Rrn3p (green) indicates their interaction. RNA Polymerase I is shown in pink and makes contact with Rrn3p via the A43 subunit. See text for more details. Note that diagram is representative and not drawn to scale.

was characterised and found to be required for silencing of Pol II transcription within the rDNA locus, with little effect on the activatory function of UAF (Siddiqi et al., 2001).

TBP is a DNA-binding protein that interacts with the minor groove of the TATA element at Pol II and requires severe distortion of the DNA. TBP is also required for high levels of Pol I transcription, but not for basal transcription (Keener et al., 1998). TBP interacts directly with both UAF and CF, although the interaction with UAF is stronger (Lin et al., 1996; Steffan et al., 1996). This interaction with UAF requires the Rrn9p subunit (Steffan et al., 1998). Overexpression of TBP can rescue UAF deletions, suggesting that TBP aids recruitment of CF by UAF, stabilising CF at the promoter. In addition, TBP stimulates high levels of transcription (Aprikian et al., 2000).

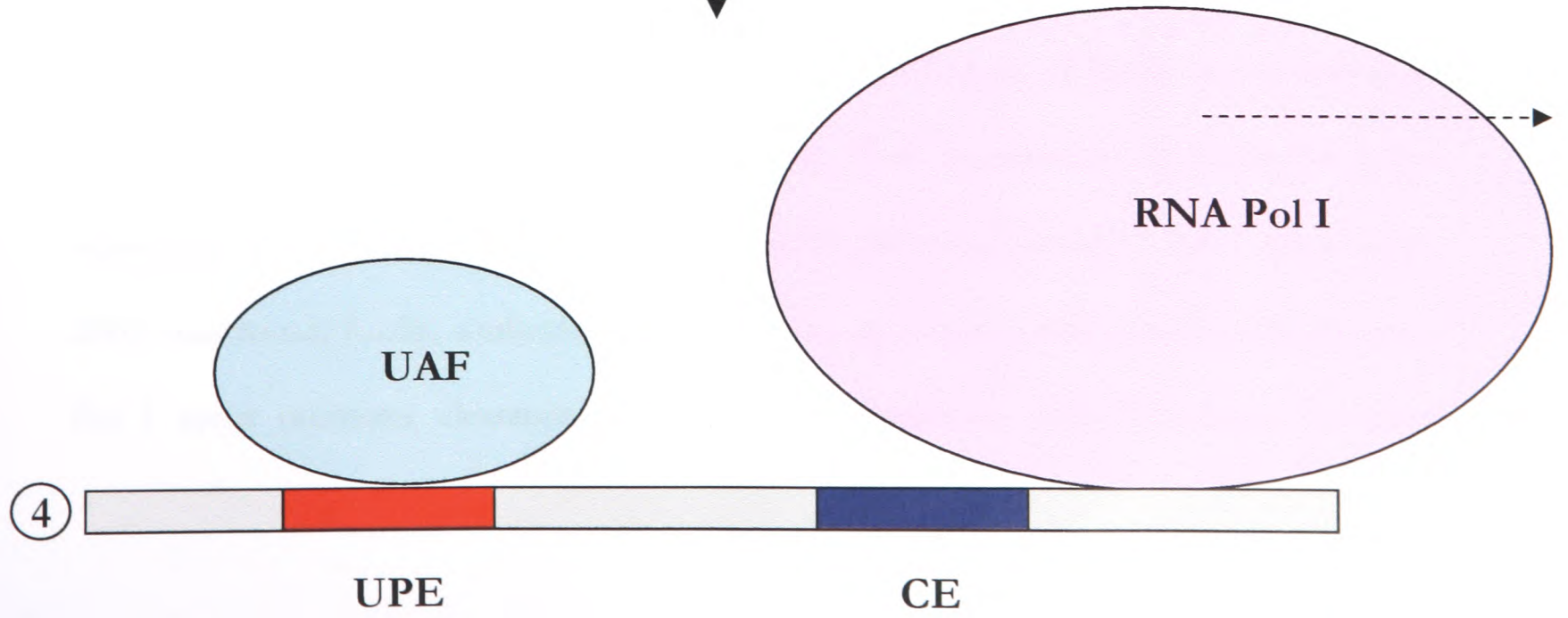
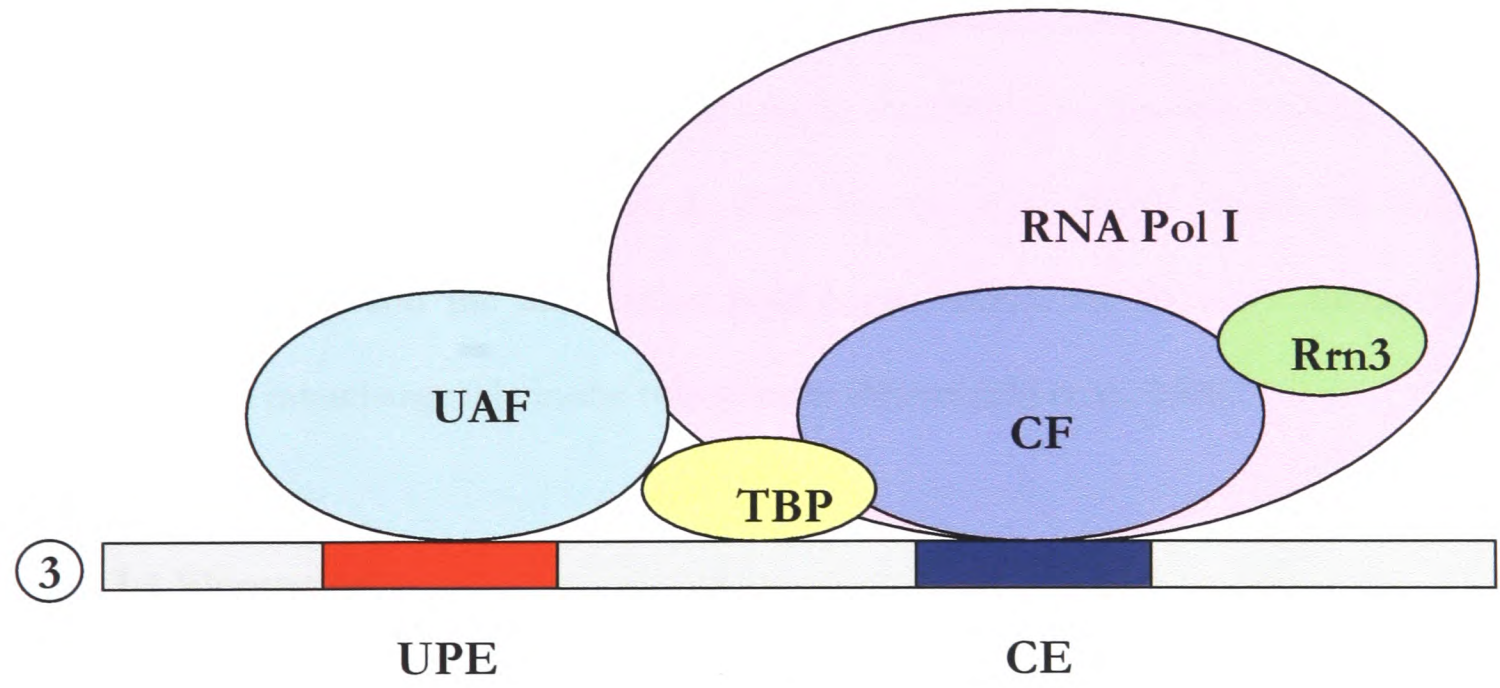
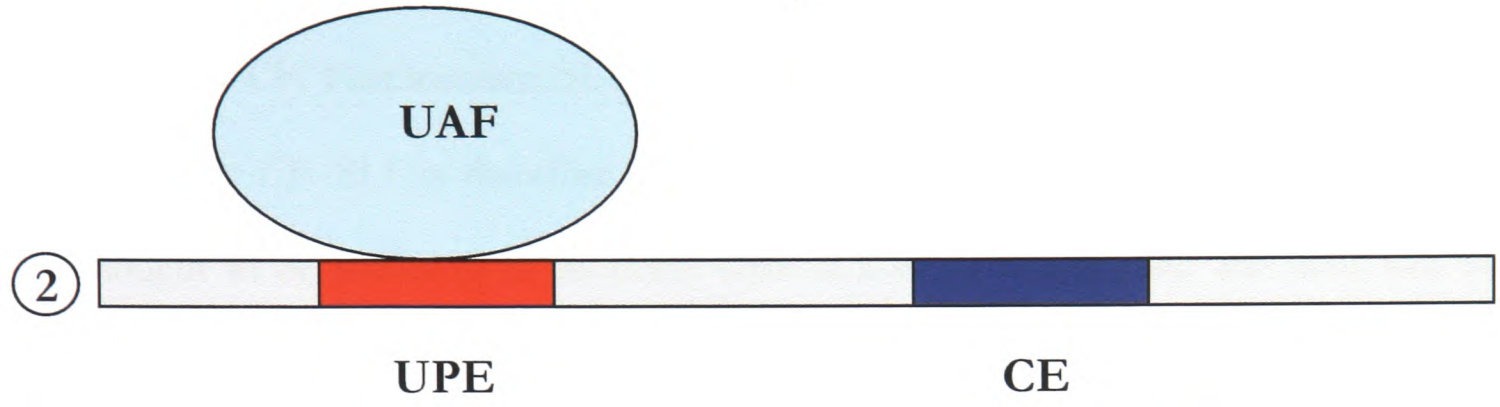
An early screen identified genes involved in rRNA synthesis, *RRN1-9*, which have been, in part, described above as components of UAF or CF (Nogi et al., 1991a). *RRN3* encodes the transcription factor, Rrn3p that directly interacts with Pol I, stimulating its recruitment to the promoter (Yamamoto et al., 1996). This interaction occurs via the Pol I subunit A43. In addition, Rrn3p makes contact with CF via Rrn6p, suggesting that Rrn3p acts as a bridge between Pol I and CF (Peyroche et al., 2000). This interaction is crucial for CF recruitment, as CF cannot be recruited to the promoter in the absence of Pol I (Aprikian et al., 2001). Whilst Pol I can be recruited to the promoter in the absence of Rrn3p, efficient transcription only occurs in the presence of Pol I complexed with Rrn3p (Aprikian et al., 2001; Milkereit and Tschochner, 1998). It has therefore been suggested that Rrn3p, when complexed with Pol I, may cause the polymerase to adopt an active conformation. Formation of the Pol I-Rrn3p complex is accompanied by an increase in the phosphorylation state of Pol I, suggesting a mechanism whereby transcription could be regulated (Fath et al., 2001). In contrast, studies of the mammalian homologue of Rrn3p, TIF-IA, indicate that it is the

phosphorylation of TIF-IA that regulates its interaction with Pol I (Cavanaugh et al., 2002). Phosphorylation of Rrn3p is known but it is not required in yeast to effect Rrn3p-Pol I complex formation. Both studies however agree with previous data indicating that Rrn3p/TIF-IA dissociates from Pol I upon transcription (Aprikian et al., 2001; Milkereit and Tschochner, 1998). Phosphorylation/dephosphorylation of Rrn3p and/or Pol I may therefore act as a switch to regulate formation of a stable PIC and possibly the shift to elongation (see below).

The order of PIC assembly is shown in Figure 1.3. Firstly, UAF binds to the UPE. This occurs in the absence of any other factors and designates the rDNA repeat for transcription (Keys et al., 1996). Next, TBP recruits CF, acting as a bridge between the UAF and CF. Stable recruitment of CF cannot occur in the absence of Pol I, itself complexed with Rrn3p (Aprikian et al., 2001). Thus, the recruitment of Pol I-Rrn3p is concomitant with the recruitment of CF. The association of UAF, CF, TBP and Pol I-Rrn3p forms a stable PIC, competent for initiation of transcription. Finally, dissociation of the PIC occurs upon addition of nucleotides (Aprikian et al., 2001) and Pol I enters productive elongation (see below).

The system of *trans*-acting factors and *cis*-acting elements described above in yeast shows both similarities and differences to the system in higher eukaryotes and at present appears to be more complex (Grummt and Pikaard, 2003; Moss and Stefanovsky, 2002). Mammalian cells possess the same architecture of *cis*-acting elements as yeast, the UPE and the CE. However, the factors that bind to these elements are not all homologous to the yeast factors. PIC formation is initiated by the binding of selectivity complex (SL1/TIF-IB). This is followed by the binding of the HMG1 box upstream binding factor (UBF), an interaction that is stabilised by SL1. Binding of UBF results in DNA bending and the formation of an

Figure 1.3: Diagram showing the order of pre-initiation complex assembly. (1) The rDNA promoter region is shown as a grey box, with the UPE and CE shown in red and blue respectively. (2) UAF binds to the UPE in the absence of any other factors, designating the rDNA repeat for transcription. (3) TBP recruits CF, bridging CF and UAF. Stable recruitment of CF requires the Pol I-Rrn3p complex. Thus CF recruitment brings Pol I-Rrn3p to the promoter. The association of UAF, CF, TBP and Pol I-Rrn3p forms a stable PIC, competent for initiation of transcription. (4) Finally, dissociation of the PIC occurs upon addition of nucleotides and Pol I enters productive elongation.



“enhanceosome”, followed by the recruitment of Pol I-RRN3 by SL1 (Friedrich et al., 2005). In contrast to the yeast system, both UBF and SL1 contact the UPE and CE, and both factors are thought to remain at the promoter, forming a reinitiation scaffold (Friedrich et al., 2005). It is also thought that two dimers of UBF bind whilst the stoichiometry of SL1 is unknown. SL1 contains TBP and 3 TAFs, two of which show homology to Rrn6p and Rrn7p of CF. Furthermore, SL1 recruits Pol I-RRN3, mirroring the recruitment of Pol I-Rrn3p by CF. SL1 is therefore thought to be homologous to CF. UBF however is not thought to be homologous to UAF. UBF is a single polypeptide and does not bear any structural resemblance to UAF. Whilst UAF is thought to be restricted to the promoter, UBF binds throughout the rDNA transcription unit (O'Sullivan et al., 2002). In addition, UBF is found at high levels in the cell at $\sim 5 \times 10^5$ copies per cell (O'Sullivan et al., 2002) whilst UAF is found at low levels at $\sim 1 \times 10^3$ copies per cell (Bier et al., 2004). The functional homologue of UBF in the yeast system may be the HMG-box protein Hmo1p which is able to bind and bend DNA (Gadal et al., 2002; Kamau et al., 2004). Finally, the conservation between Rrn3p and the mammalian homologue RRN3/TIF-IA is so strong that these factors are interchangeable in the two systems (Moorefield et al., 2000).

1.3.4 Elongation

As yeast Pol I clears the promoter and enters elongation phase, it is no longer associated with any of the factors found in the PIC. UAF remains bound at the UPE, acting as a scaffold for reinitiation whilst CF, TBP and Rrn3p all leave the promoter as shown in Figure 1.3 (Aprikian et al., 2001; Milkereit and Tschochner, 1998). Loss of Rrn3p occurs during or just after promoter clearance (Bier et al., 2004). This requirement of Rrn3p-Pol I for transcription offers a mode of regulation. Specific phosphorylation of Pol I (Fath et al., 2001) may recruit Rrn3p, whilst dephosphorylation may facilitate dissociation of Rrn3p from Pol I upon promoter clearance and entry into elongation. This hypothesis has been

strengthened by the recent discovery that the CTD phosphatase Fcp1p is required for efficient Pol I transcription (Fath et al., 2004). This phosphatase is not required for formation of the Rrn3p-Pol I complex, suggesting it may facilitate elongation.

In contrast to Pol II transcription, only a small number of elongation factors have been identified for Pol I transcription. It has been shown that the basal Pol II transcription factor, TFIIF, acts during Pol I transcription (Iben et al., 2002). TFIIF is localised to sites of active transcription within the mouse nucleolus and is specifically associated with SL1 (homologous to CF) and Pol I. Furthermore, neither the phosphorylation nor ATP hydrolysis activities of TFIIF are required, indicating that TFIIF stimulates Pol I transcription at a post-initiation stage and most likely during elongation. In addition, elongation requires either topoisomerase I or II. Loss of these enzymes leads to the appearance of extrachromosomal rDNA rings, suggesting that topoisomerases are required to overcome torsional strain during elongation (Kim and Wang, 1989).

As is seen above, far fewer factors have been identified as being required for transcription by Pol I than by Pol II. Pol I elongation complexes are densely packed along the active rRNA genes with approximately 50 complexes per gene, transcribing at a rate of approximately 60 nucleotides per second (French et al., 2003). This transcription rate is almost 2-fold faster than the rate estimated for Pol II, of ~23nt per second (Shermolen and O'Farrell, 1991). Such a rapid transcription rate, coupled with the dense packing of transcription complexes along an active rDNA repeat, has been used as an argument against a chromatin structure on active rRNA genes. This will be discussed in greater detail in Section 1.8.1.

1.3.5 Termination

Termination of Pol I transcription has been studied in a range of eukaryotes from yeast to *Xenopus* to mouse. A common architecture of two DNA sequence elements has been found, comprising a binding site for a sequence-specific DNA binding protein and an upstream element that encodes the final ~10nt of the primary transcript (Reeder and Lang, 1998).

Termination of transcription was first thought to occur at +210 with respect to the end of the 35S primary transcript (Kempers-Veenstra et al., 1986; van der Sande et al., 1989). However, further studies indicated that the primary site of termination is located at +93 with a failsafe terminator located at +250. ~90% of transcripts are terminated by the +93 terminator, with ~10% reading through to the +250 failsafe terminator (Reeder et al., 1999). The +93 termination site is directly downstream of the first element of the terminator, the 10-12bp T-rich release element. Downstream of the T-rich element is the 11bp Reb1p binding site. These two elements function in a two-step termination process, with transcripts first being paused and then released (Lang et al., 1994; Reeder and Lang, 1997). The elements found within the yeast termination region are shown in Figure 1.4. In addition, the sites of Rnt1p cleavage are shown (see below).

The pause element is composed of Reb1p, bound to its site on rDNA. Reb1p is homologous to mammalian TTF-I and both factors possess Myb homology DNA binding domains (Reeder et al., 1999). The function of Reb1p is orientation-dependent (Lang and Reeder, 1993) and termination efficiency is increased if the affinity of Reb1p for its binding site is increased (Reeder et al., 1999). The release element is T-rich (on the non-template strand) and located 25nt upstream of the Reb1p binding site. Mutation of nucleotides within this element has been shown to diminish termination (Lang and Reeder, 1995). Furthermore, this element can function when the Reb1p binding site is replaced by the lac

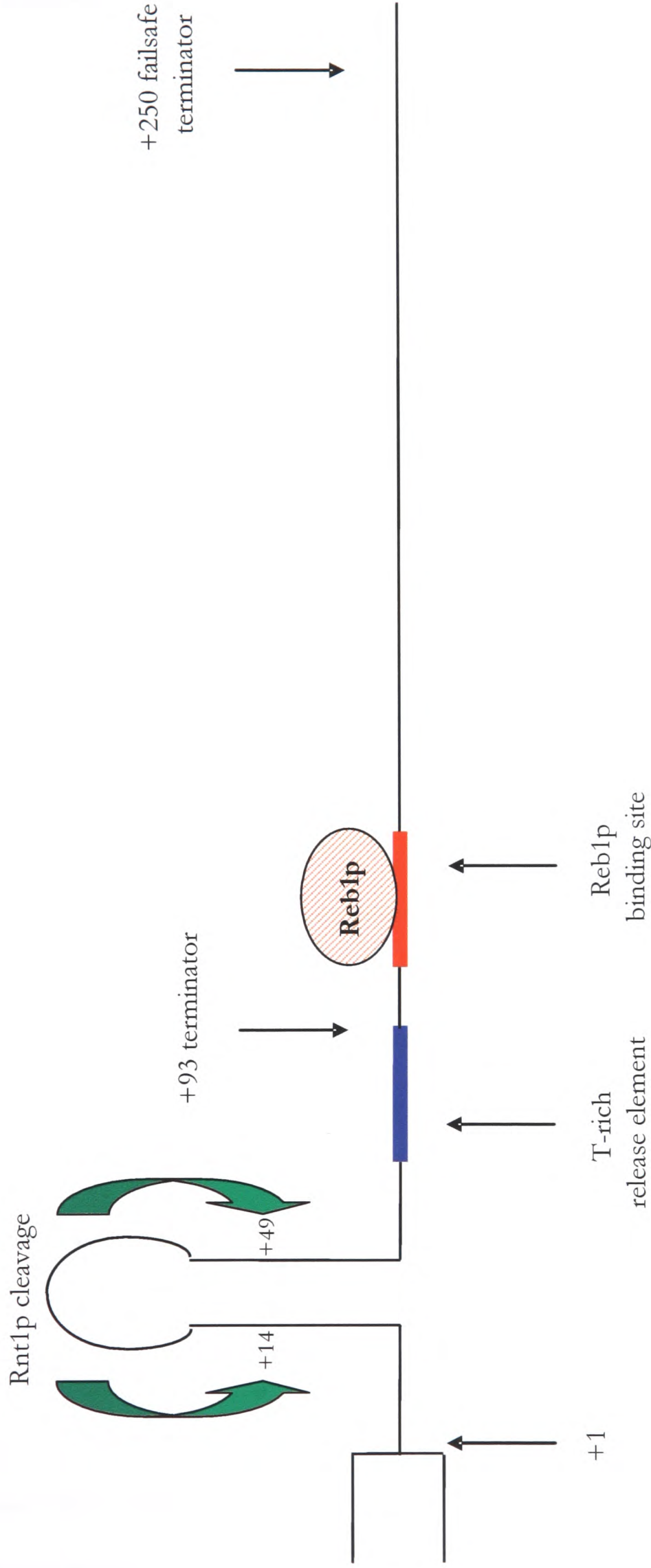


Figure 1.4: Diagram of the yeast rDNA termination region. The open rectangle denotes the 3' end of the 25S genic region with the first nucleotide of the 3' ETS denoted as "+1". The black line represents the termination region. The 11bp Reb1p binding site is shown in red and the 10-12bp T-rich release element is shown in blue. Reb1p is shown as a dashed red circle. The +93 primary and the +250 failsafe termination sites are shown by vertical arrows. The hairpin structure that forms within the 3'ETS is shown. Rnt1p cleaves at two sites within this structure, denoted as "+14" and "+49". Cleavage is shown by the two green arrows. Note that diagram is representative and not drawn to scale.

repressor binding site (Jeong et al., 1995). However, release of paused transcripts is much slower than when the Reb1p binding site is present, indicating that Reb1p functions not only to pause the polymerase, but also plays a direct role in transcript release (Lang and Reeder, 1995).

The two-step model of termination indicates that elongating polymerases are stalled by Reb1p bound to its binding site. The location of the Reb1p site causes the polymerase to pause over the T-rich release element. The polymerase is then proposed to backtrack away from the pause site, resulting in mismatch between the transcript and the template. A stable rRNA-rDNA hybrid cannot form and the polymerase therefore cannot elongate further. The ternary complex dissociates, achieving termination and transcript release (Reeder and Lang, 1997). It is predicted that the presence of a non-homopolymeric release element ensures that the polymerase does not enter an iterative cycle of slippage and elongation but rather effects termination (Jeong et al., 1996).

In higher eukaryotes, the same terminator architecture is seen (Reeder and Lang, 1998). In contrast to a single Reb1p binding site at the site of termination, eight Sal boxes are found in mouse. The Reb1p homologue, TTF-I, binds to these Sal boxes. In humans, downstream sequence elements with homology to the Sal box are found and the human homologue of mTTF-I, hTTF-I, has been identified. Furthermore, both mouse and humans possess an upstream release element (Mason et al., 1998). In contrast to yeast, a release factor is required in mouse. This factor was identified and named Pol I and Transcript Release Factor (PTRF) (Jansa et al., 1998; Mason et al., 1997). PTRF associates with the largest Pol I subunit A194 and requires the upstream T-rich element to effect release (Jansa and Grummt, 1999; Mason et al., 1997). PTRF is also known to bind TTF-I, suggesting that it may form a bridge between the polymerase and TTF-I. Furthermore, PTRF binds the 3' end

of the rRNA primary transcript although the consequences of this binding are not clear (Jansa et al., 1998). Finally, it is thought that PTRF also functions to enhance transcription through Pol I recycling and can be phosphorylated at multiple sites, suggesting a means of regulating its activity (Jansa et al., 2001).

The requirement of a release factors appears to be conserved from mouse to yeast (Tschochne and Milkereit, 1997). It was found that Reb1p-paused transcripts could be released upon addition of the murine release factor (Mason et al., 1997). This is in contrast to studies indicating that Reb1p could effect full termination of Pol I (Lang et al., 1994; Lang and Reeder, 1995). It is thought that yeast Pol I used in these experiments was already complexed with the yeast release factor. It has since been shown that PTRF is able to release murine Pol I transcripts paused by Reb1p or TTF-I, or yeast Pol I transcripts paused by Reb1p. However, PTRF is unable to release yeast Pol I transcripts paused by TTF-I. Thus it appears that the yeast system requires an as-yet-unidentified release factor that is homologous to PTRF. Furthermore, some of the interactions occurring at the terminator are conserved from yeast to mouse, whilst others are species specific (Jansa and Grummt, 1999).

More recently, a Pol I subunit, A12, and the endoribonuclease Rnt1p have been implicated in termination (Prescott et al., 2004). Nascent transcription profiles in the strains lacking Rpa12p and Rnt1p indicated that these factors are required for efficient transcription termination. Loss of Rpa12p led to high levels of read-through into the downstream intergenic spacer and into the downstream promoter whilst loss of Rnt1p caused termination to occur further downstream than normal. It is suggested that Rpa12p may interface between Reb1p and Rnt1p whilst Rnt1p may be required to ensure the location and efficiency of termination.

1.4 Processing of the primary transcript

Once the 35S primary transcript has been transcribed by Pol I, it must be processed into the mature 18S, 5.8S and 25S transcripts. An early, co-transcriptional cleavage event is performed by the endoribonuclease Rnt1p (Figure 1.4). Rnt1p is homologous to bacterial RNaseIII (Elela et al., 1996) and is also required for snRNA and snoRNA cleavage (Chanfreau et al., 1997; Chanfreau et al., 1998). Rnt1p cuts at a tetraloop containing a conserved AGNN sequence 13-16bp from the cleavage sites within the 3' ETS (Chanfreau et al., 2000; Chanfreau et al., 1998). These cleavage sites are found at +14 and +49 relative to the 3' end of the primary transcript (Kufel et al., 1999).

Once Rnt1p has cleaved the primary transcript, a rapid series of further cleavages occur. In addition, the transcripts are modified. In total, ~170 proteins and 70 snoRNAs are involved in the post-transcriptional stages of ribosome assembly (Dez and Tollervey, 2004). Processing and modification of rRNA transcripts is reviewed in Granneman and Baserga, 2005 and Dez and Tollervey, 2004.

1.5 An RNA Polymerase I holoenzyme?

Evidence from plants has suggested that a Pol I holoenzyme may exist that contains Pol I and all necessary transcription factors pre-assembled, prior to PIC formation on the promoter (Kenzior and Folk, 2001). The requirement of an Rrn3p-Pol I complex for efficient transcription initiation, and the need for Rrn3p-Pol I to be present to effect CF binding to the CE would suggest the possibility of a Pol I holoenzyme (Aprikian et al., 2001; Milkereit and Tschochner, 1998). The discovery of a complex containing Pol I, transcription factors such as Rrn3p and TBP, Reb1p and rRNA processing factors adds weight to this idea (Fath et al., 2000). However the components of this complex do not associate in stoichiometric amounts and the question of a Pol I holoenzyme remains open.

A related question regards the fate of Pol I after each round of transcription. A study in mammalian cells showed that Pol I subunits dissociate and rapidly exchange between the nucleoplasm and the nucleolus (Dundr et al., 2002). However, a more recent study in yeast indicated that Pol I remains intact when not engaged in active transcription with no detectable exchange of Pol I subunits (Schneider and Nomura, 2004). These contrasting results could suggest that yeast Pol I transcription is very different to mammalian transcription. However it is also suggested that technical differences can explain the opposing results (Schneider and Nomura, 2004). Thus it is thought most likely that Pol I does not dissociate after each round of transcription and can stably perform multiple rounds of transcription.

1.6 Chromatin

The length of a eukaryotic genome (~ 2 metres in humans) compared to the diameter of the cell nucleus ($\sim 10^{-5}$ metres) necessitates the compaction of DNA. This is achieved through the packaging of DNA into a nucleoprotein complex, chromatin, via a series of hierarchical coiling steps. ~ 146 bp DNA is wrapped around a histone octamer to form a nucleosome. The histone octamer is composed of 8 core histones: two H2A/H2B dimers and one H3/H4 tetramer (Luger et al., 1997). Coiling around the octamer causes the DNA to become severely distorted. This high energy cost is compensated for by the histone:DNA interactions which occur every 10bp, resulting in 7 histone:DNA contacts per DNA coil (Langst and Becker, 2004). Further stabilisation occurs through contacts between the H4 tail and the exposed surface of H2A/H2B dimer on an adjacent nucleosome, although this stabilisation appears to be absent in *S. cerevisiae* (Luger et al., 1997; White et al., 2001). It is now recognised that chromatin not only serves to condense the DNA but also plays a crucial role in gene regulation (Krebs et al., 2000; Lee and Young, 2000). In general, the packaging of DNA into chromatin is not compatible with the access required by DNA

binding factors that regulate replication and transcription and repair damaged DNA. However it should be noted that pioneer transcription factors exist that are capable of binding to and opening compacted chromatin (Cirillo et al., 2002). To overcome a repressive chromatin structure, three types of mechanism exist (Figure 1.5). The first involves covalent modification of histone protein tails (Berger, 2002; Fischle et al., 2003). The second mechanism involves the incorporation of histone variants into the nucleosome (Korber and Horz, 2004). Finally, chromatin remodeling factors use the energy liberated by ATP hydrolysis to disrupt DNA-histone interactions (Flaus and Owen-Hughes, 2004; Langst and Becker, 2004).

Histone tail modifications and histone variants are discussed in this section. Chromatin remodeling factors are discussed below in Section 1.7.

1.6.1 Histone tail modifications

All four histones possess N-terminal domains known as “tails” that protrude from the nucleosome. In yeast, these tails range in size from 14 to 30 amino acids. Histone tails are rich in basic amino acids and are subject to a wide range of modifications, including acetylation, methylation, phosphorylation and ubiquitylation. Within the tails, lysines (K) are acetylated, methylated and ubiquitylated, arginines are methylated and ADP-ribosylated, and serines and threonines are phosphorylated. The complexity of modifications is enhanced by the knowledge that lysine residues can be mono-, di- or trimethylated and arginine residues can be mono- or dimethylated (Martin and Zhang, 2005; Peterson and Laniel, 2004; Vaquero et al., 2003). Furthermore within the globular domains, K56 of histone H3 and K91 of histone H4 are also acetylated (Xu et al., 2005; Ye et al., 2005). The combination of histone tail modifications has been proposed to form a code that is read out by effector proteins, resulting in activation/repression of transcription (Strahl and Allis, 2000). More recently, it has been suggested that tail modifications may influence gene expression through

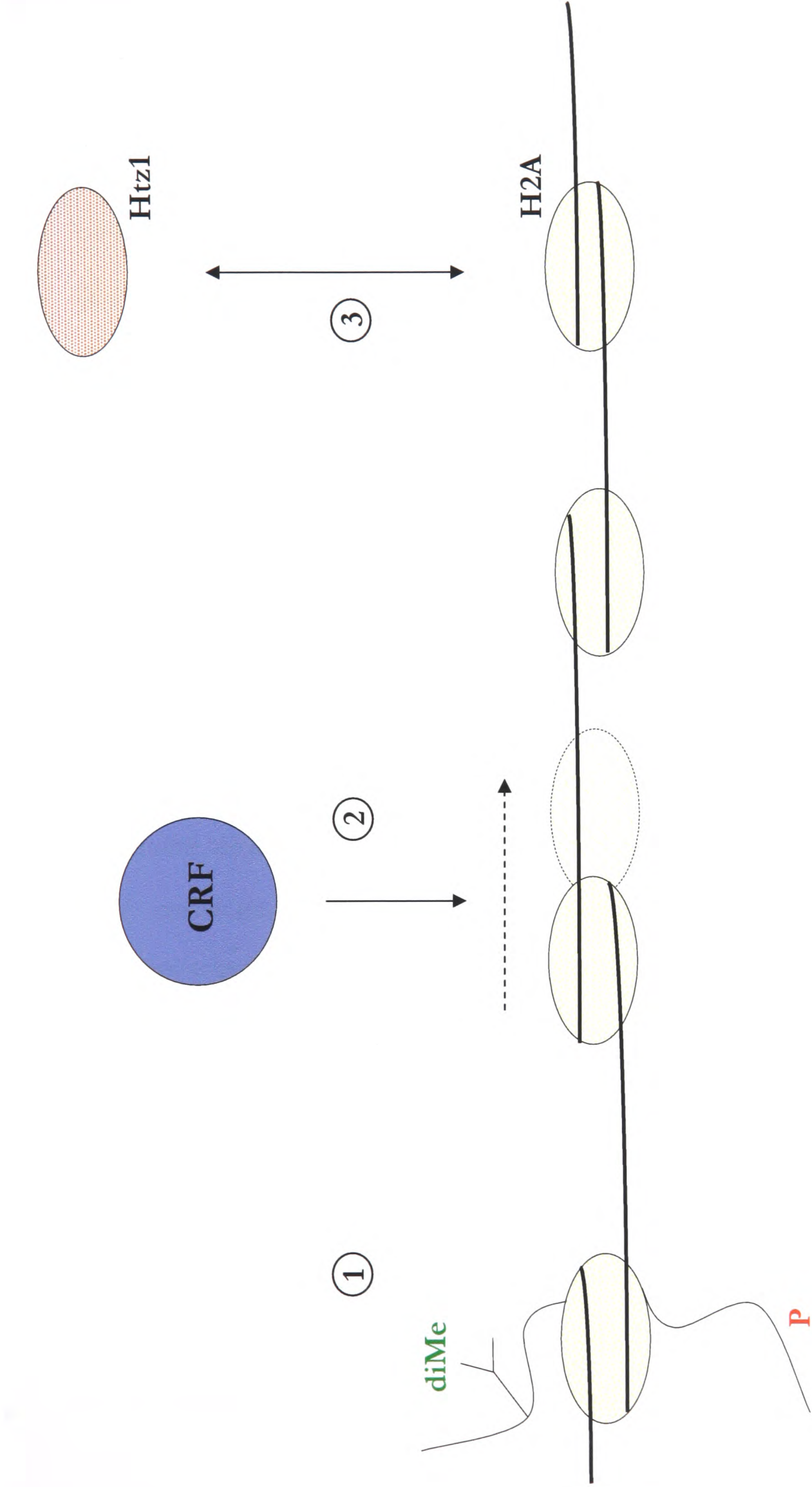


Figure 1.5: Diagram summarising the three mechanisms by which chromatin structure can be altered. (1) N-terminal histone tails undergo modifications at key residues. For example, histone H3 serine 10 undergoes phosphorylation (red "P"). A number of residues can undergo mono-, di- or tri-methylation; for example histone H3 lysine 4 and lysine 36 can be dimethylated (green "diMe"). (2) Chromatin remodeling factors (CRF) such as Chd1p and Isw1p can catalyse the movement of nucleosomes along DNA templates. (3) Histone variants can be incorporated into chromatin e.g. the SWR1 complex catalyses the incorporation of the histone H2A variant Htz1.

a simpler, cumulative charge neutralisation model, and not through a complex, combinatorial code (Dion et al., 2005; Henikoff, 2005). What is clear, however, is that histone modifications facilitate the recruitment of effector proteins that influence gene expression e.g. Isw1p binds to the tails of histone H3 methylated at K4 (Santos-Rosa et al., 2003). Histone tail modifications are also self-propagating e.g. phosphorylation of serine 10 on histone H3 recruits the histone acetylase Gcn5 that then catalyses acetylation of K14 (Zhang and Reinberg, 2001). A further example of cross-talk amongst modifications is the dimethylation of histone H3 K4 by Set1p. This modification requires the prior ubiquitylation of histone H2B K123 by Rad6p (Dover et al., 2002; Sun and Allis, 2002).

In addition to the numerous modifications and the cross-talk amongst them, different modifications have different effects on the chromatin in which they are found. Acetylation, phosphorylation, ubiquitylation and arginine methylation are almost exclusively activatory for transcription, whereas lysine methylation can be either activatory or repressive. *S. cerevisiae* possesses three histone methyltransferases, Set1p, Set2p and Dot1p that are responsible for methylation of histone H3 K4, 36 and 79 respectively (Sims et al., 2003). All three modifications serve to activate transcription. For the purposes of this thesis, methylation by Set1p and Set2p will be discussed.

Set1p is a methyltransferase that specifically methylates histone H3 at K4 (Boa et al., 2003; Briggs et al., 2001; Nagy et al., 2002; Roguev et al., 2001). Set1p is a component of the COMPASS complex (Krogan et al., 2002a; Miller et al., 2001) and is involved in both activation and repression of transcription (Krogan et al., 2002a; Santos-Rosa et al., 2003). Of particular interest to this thesis, Set1p is required for silencing at the rDNA locus (Briggs et al., 2001; Bryk et al., 2002; Fingerman et al., 2005).

Set1p is responsible for both di- and trimethylation of histone H3 and both these marks are associated with actively transcribed Pol II genes (Bernstein et al., 2002; Santos-Rosa et al., 2002). Dimethylation can be associated with silenced regions e.g. rDNA (Briggs et al., 2001), as well as active regions; trimethylation however is only found at active loci (Santos-Rosa et al., 2002). The patterns of di- and trimethylation differ, with dimethylation seen throughout coding regions, whilst trimethylation is usually restricted to the 5' end of the coding region (Ng et al., 2002; Santos-Rosa et al., 2002). Under stress conditions though, trimethylation is seen at the 3' end of genes, possibly signalling impaired transcription (Zhang et al., 2005). The restricted pattern of trimethylation is thought to occur due to the interaction of Set1p with the serine 5 phosphorylated CTD of Pol II (Ng et al., 2002). Multiple domains of Set1p itself also function to regulate trimethylation (Schlichter and Cairns, 2005). Set1p activity further requires the Rtf1 and Paf1 components of the Paf1 complex, linking histone H3 K4 methylation and transcription elongation (Krogan et al., 2003a; Ng et al., 2002).

Set2p is a histone methyltransferase that specifically dimethylates histone H3 at K36 (Strahl et al., 2002). K36 dimethylation (H3 K36diMe) is found on actively transcribed genes, with build-up seen towards the 3' end of the transcribed region (Morillon et al., 2003a). Set2p is found throughout coding regions (Krogan et al., 2003c) and has been shown to interact with the serine 2 phosphorylated CTD of RNA Pol II (Krogan et al., 2003c; Li et al., 2003; Li et al., 2002; Schaft et al., 2003; Xiao et al., 2003) via a Set2p domain known as Set2p-Rpb1p interaction domain (Kizer et al., 2005). The interaction of Set2p with the serine 2 phosphorylated CTD is necessary for its methyltransferase activity (Krogan et al., 2003c; Xiao et al., 2003). Furthermore, Set2p recruitment is also dependent on the presence of Paf1 complex components Rtf1 and Cdc73 (Krogan et al., 2003c). These requirements are very similar to those for Set1p methylation of H3 K4 and suggest that Set2p and H3 K36 methylation is linked to transcription elongation.

The modification of histone tails provides a mechanism of factor recruitment. Many proteins involved in the regulation of gene expression possess domains that are able to interact with, and be recruited to, modified histones. The bromodomain, found in the acetyltransferase Gcn5 and the chromatin remodeling complex NoRC, is able to bind to acetylated lysine residues (Mamorstein and Roth, 2001; Zeng and Zhou, 2002). The chromo (chromatin organisation modifier) domain was first identified in two *Drosophila* proteins HP1 and Polycomb (Paro and Hogness, 1991) and has since been found in a large number of proteins including the *S. cerevisiae* chromatin remodeling factor Chd1p (Woodage et al., 1997). Chromodomains interact with methylated lysine residues, as well as DNA and RNA (Akhtar et al., 2000; Brehm et al., 2004). Histone tail modifications thus facilitate the recruitment of a wide range of factors to chromatin, facilitating further modifications and effecting chromatin remodeling.

The lifetime of a given histone tail modification will directly affect its ability to influence the chromatin environment in which it is found, and to recruit protein factors. Acetylation marks are removed by histone deacetylases (HDACs), whilst phosphorylation can be removed by phosphatases. Methylation however may be a more permanent mark as the half-life of histones and methyl-lysine residues is the same and identification of demethylases has proved problematic (Bannister et al., 2002). This would suggest therefore that in contrast to the dynamic marks of acetylation and phosphorylation, methylation is a long-term mark. Such a view however appears incompatible with the known dynamic role of histone tail methylation in transcription (Morillon et al., 2005; Zhang et al., 2005). Recent studies however identified enzymes that can remove methyl groups from both lysine and arginine residues. LSD1 is able to demethylate histone H3 K4 and is conserved from *Schizosaccharomyces pombe* to man (Shi et al., 2004). However the enzyme is absent from *S. cerevisiae*. The enzyme can demethylate di- and monomethylated lysine residues, but not

trimethylated residues. Arginine methylation can be overcome by the action of PADI4, an arginine deiminase that converts methylated arginine into citrulline (Cuthbert et al., 2004; Wang et al., 2004). However the enzyme is non-specific and can convert unmethylated arginine to citrulline. It is therefore still not clear how *S. cerevisiae* brings about specific and efficient removal of lysine and arginine methylation during the dynamic transcription reaction.

1.6.2 Histone variants

The incorporation of histone variants into chromatin allows further modification. Histone variants can differ from the major histones by as little as one amino acid or can show large number of changes. Whilst histones H2B and H4 do not possess histone variants, histones H2A and H3 have large numbers of variants, especially histone H2A. Not all variants are expressed in all organisms however. In *S. cerevisiae*, there are two variants of H2A, known as H2A and Htz1. Htz1 is the homologue of H2A.Z whilst H2A most resembles the variant H2A.X. The major form of H3 in *S. cerevisiae* also most resembles a variant histone found in higher eukaryotes, namely H3.3 (Kamakaka and Biggins, 2005).

Htz1 is incorporated into chromatin by the 13 subunit complex SWR1. This complex is able to bind chromatin through its interaction with Bdf1, a bromodomain-containing factor that binds the acetylated tails of histones H3 and H4. Genetic interactions were found between SWR1 and elongation factors such as components of the Paf1 complex, and the methyltransferases Set1p and Set2p, suggesting that incorporation of the variant histone Htz1 is linked in some way to transcription (Korber and Horz, 2004; Krogan et al., 2003b).

In *Drosophila*, the histone H3 variant H3.3 is expressed throughout the cell cycle and is deposited at transcriptionally active rDNA in a replication-independent pathway. It is thought that incorporation of this histone variant may mark the rDNA repeat as active and aid future rounds of transcription (Ahmad and Henikoff, 2002).

1.7 Chromatin remodeling factors

As described above, chromatin remodeling factors use the energy from ATP hydrolysis to disrupt chromatin. These enzymes contain an ATPase subunit that belongs to the SNF2 superfamily and are grouped into four classes according to their domain structure: SWI/SNF2, ISWI, CHD/Mi-2 and INO80 (Sif, 2004). SWI/SNF ATPases contain a bromodomain and INO80 class members have a split ATPase domain. ISWI ATPases possess a SANT domain and CHD class members have chromodomains and PHD fingers, although the latter are absent in *S. cerevisiae* (Langst and Becker, 2004; Woodage et al., 1997). Whilst members of all classes show ATPase activity, the targets of this activity differ between the different enzymes. Swi2 is maximally stimulated by free DNA whilst Mi-2 activity is only induced by nucleosomal DNA. Further, specific examples are given below.

1.7.1 Chd1p

The CHD/Mi-2 class was first discovered in mouse (Delmas et al. 1993) and is conserved throughout eukaryotes, with Chd1p being the sole member in *S. cerevisiae* (Woodage et al. 1997). Chd1p contains three different domains – two N-terminal chromodomains, a centrally-located helicase/ATPase domain and a C-terminal DNA-binding domain (Figure 1.6A). The DNA-binding domain interacts with the minor groove of DNA at AT-rich sequences (Stokes and Perry 1995). The chromodomain was first identified in the *Drosophila* Polycomb protein due to its similarity to the protein HP1 (Paro and Hogness 1991). Both are associated with heterochromatin, suggesting that Chd1p might act to repress transcription. In agreement with this, a study on murine CHD1 showed that it associates with an HDAC activity and with a co-repressor known as NCoR that functions to repress nuclear hormone receptor transcription (Tai et al., 2003). However, CHD1 from a number of species has been shown to function in active transcription, suggesting multiple functions for this remodeling factor. In *Drosophila*, CHD1 localises to areas of high transcriptional

Figure 1.6: Chromatin remodeling factors. (A) Domain structure of *S. cerevisiae* Chd1p. The two chromodomains are shown as dark blue circles. The ATPase domain is shown as a black dashed rectangle and the DNA binding domain is shown as a light blue oval. (B) The domain structure of *S. cerevisiae* Isw1p and Isw2p. The ATPase domain is shown as a black dashed rectangle and the SANT domain is shown as a red rectangle. (C) Diagrams representing the Isw1 complexes found in the cell. The Isw1a complex is composed of Isw1p and Ioc3p. The Isw1b complex is composed of Isw1p, Ioc2p and Ioc4p. (D) Diagram representing the Isw2 complex found in the cell. The Isw2 complex is composed of Isw2p, Itc1p, Dpb4p and Dls1p. Note that all diagrams are representative and not drawn to scale. The diagrams in (C) and (D) show the components of the complexes and the placement of protein factors does not necessarily represent interactions between these factors.

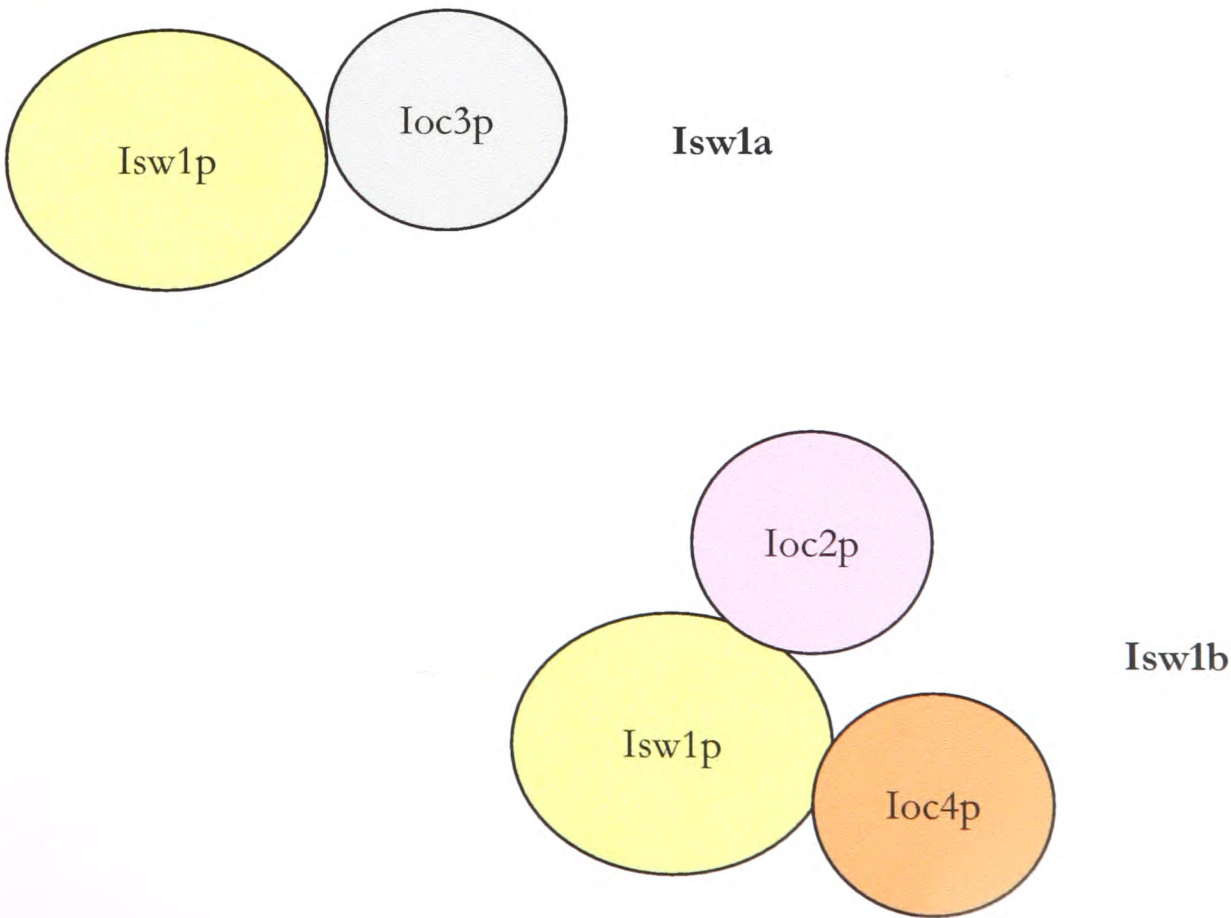
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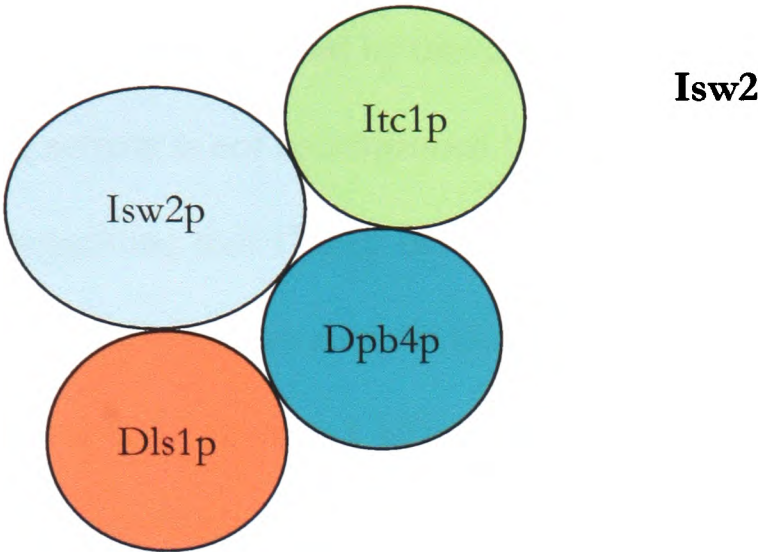
B



C



D



activity known as puffs (Stokes et al. 1996) and this interaction is dependent on the chromo- and ATPase domains. In addition, CHD1 co-localises with SSRP1, an HMG box-containing protein. This result is also seen for mammalian CHD1 (Kelley et al., 1999). In *S. cerevisiae*, Chd1p has also been shown to function in active transcription. The *in vitro* ATP hydrolysis activity of Chd1p is stimulated by free and nucleosomal DNA but not by core histones. This remodeling activity is not accompanied by changes in the amount or mobility of nucleosome particles, suggesting that Chd1p does not remove histones from DNA (Tran et al., 2000). The activity of Chd1p has recently been extended to include the assembly of nucleosome arrays. Loss of *S. cerevisiae* Chd1p was shown to deleteriously affect the replication-independent assembly of chromatin arrays (Robinson and Schultz, 2003). Furthermore, *Drosophila* CHD1 catalyses the transfer of histones from a chaperone onto DNA and can convert randomly distributed nucleosomes into a periodic array (Lusser et al., 2005). This chromatin assembly function of Chd1p suggests it may act to reassemble nucleosomes in the wake of an elongating polymerase. Consistent with this theory, Chd1p has emerged as a factor involved in Pol II elongation. Chd1p interacts with Rtf1, a component of the transcription elongation complex Paf1; and Spt4-5 and Spt16-Pob3, both essential elongation factors (Krogan et al., 2002b; Simic et al., 2003). The Paf1 complex itself interacts with Spt4-Spt5 and Spt16-Pob3, the yeast homologues of DSIF and FACT respectively. Furthermore, Rtf1, Spt5 and Chd1p co-localise to chromatin being actively transcribed by Pol II and all Chd1p domains are required for its role in elongation (Simic et al., 2003). The interaction of Chd1p with casein kinase II (CKII), which is known to phosphorylate Spt5, suggests Chd1p may function in elongation by regulating the activity of CKII (Krogan et al., 2002b). As discussed above, actively transcribed genes are associated with histone tail modifications including lysine methylation. The role of Chd1p in active transcription necessitates a mechanism for the localisation of Chd1p on active genes. A recent study has suggested a means of Chd1p recruitment, via the tails of histone H3. Chd1p was shown to

interact with H3 K4 dimethylated (H3 K4diMe) tails via its second chromodomain (Pray-Grant et al., 2005) and it has been suggested that Chd1p can also bind to H3 K36diMe tails (Hampsey and Reinberg, 2003). Finally, Alen et al. have shown that Chd1p is required for efficient transcription termination of *CYC1*. Furthermore, Chd1p functions redundantly with Isw1p and Isw2p to effect termination at the *GAL10-7* locus (Alen et al., 2002).

1.7.2 Isw1p

The ISWI class of chromatin remodeling factors is highly conserved throughout eukaryotes and is represented in *S. cerevisiae* by Isw1p and Isw2p (Tsukiyama et al. 1999). All ISWI members possess a SANT domain, in addition to the ATPase domain (Figure 1.6B). In the cell, Isw1p is found in two distinct complexes, Isw1a and Isw1b (Figure 1.6C). Isw1a contains Ioc3p whilst Isw1b contains Ioc2p and Ioc4p (Vary et al. 2003). Isw1p is able to produce regularly spaced nucleosomal arrays (Tsukiyama et al., 1999) and the Isw1a and Isw1b complexes show equivalent nucleosome-stimulated ATPase activity, presumably due to the common Isw1p subunit. However, Isw1a shows stronger nucleosome sliding and spacing activity than Isw1b (Vary et al., 2003). *Drosophila* ISWI requires the N-terminal tail of histone H4 for its activity (Clapier et al., 2001). This functions at a stage subsequent to the interaction of dISWI with the nucleosome and requires the dISWI C-terminal domain, which includes the SLIDE and SANT domains. The SLIDE domain is inserted into the major groove of DNA whilst the negatively charged SANT domain points away from the DNA and may contact positively charged histone tails (Grune et al., 2003). Isw1p in yeast has both positive and negative effects on gene expression, with repression seen at a number of loci including *PHO8* (Moreau et al. 2003). Distinct complexes of Isw1p also function at a single locus. The *MET16* locus is first repressed by Isw1a. Upon activation, Isw1a is displaced and Isw1b is recruited, ensuring regulation of early elongation and termination (Kent et al. 2001, Morillon et al. 2003a). Isw1p may also act as a monomer, independent of

its Ioc partners (Vary et al., 2003). Finally, Isw1p has been shown to function redundantly with Isw2p at the *FIG1* and *MET17* loci (Kent et al. 2001).

1.7.3 Isw2p

Isw2p in yeast is a positive regulator of early sporulation (Trachtulcova et al. 2000) but also acts to repress a number of early meiotic genes by creating an inaccessible chromatin structure over the promoter (Goldmark et al. 2000; Fazzio et al. 2001). In the cell, Isw2p is associated with Itc1p, Dpb4p and Dls1p (Figure 1.6D; Gelbart et al., 2001; McConnell et al., 2004). The Isw2 complex interacts with both naked DNA and nucleosomal arrays in the absence of ATP (Gelbart et al., 2001). A model thus suggests that Isw2 binds linker DNA in the absence of ATP. ATP binding compacts the Isw2 structure. Hydrolysis of ATP to ADP then promotes release of Isw2 from DNA. Finally, loss of ADP facilitates rebinding of Isw2 to DNA, resulting in nucleosome translocation (Fitzgerald et al., 2004). These data have more recently been confirmed and extended to show that Isw2 function requires a basic patch on the histone H4 tail, paralleling the requirement of this tail for Isw1 binding (Fazzio et al, 2005). The Dls1p subunit of Isw2 is required for its remodeling activity at certain loci *in vivo* but is not required for the interaction of Isw2 with chromatin (McConnell et al., 2004). The function of this Isw2 subunit is therefore not clear. The interaction of Isw2 with chromatin has recently been shown to be transient, suggesting that Isw2 scans the genome, searching for its targets (Gelbart et al., 2005). Isw2 is known to repress transcription of early meiotic genes including *INO1* (Sugiyama and Nikawa, 2001). This repression of *INO1* is dependent on the DNA-binding protein Ume6p (Kent et al., 2001) and is independent of the HDAC Rpd3p-Sin3p (Goldmark et al., 2000). There are, however, targets of Isw2 that are independent of Ume6p but dependent on the Rpd3p-Sin3p complex (Fazzio et al., 2001). An example of a Ume6p-independent target is *PHO3* (Kent et al., 2001), suggesting the existence of an unidentified factor able to recruit Isw2 to this locus.

A number of interactions are known to occur between Chd1p, Isw1p and Isw2p. These three remodeling factors have been shown by co-immunoprecipitation to physically interact (Gavin et al., 2002). Furthermore, genetic interactions have been shown, as a $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ triple mutant is lethal at 37°C (Tsukiyama et al., 1999). Finally, Chd1p, Isw1p and Isw2p function redundantly to effect efficient transcription termination at the *GAL10-7* locus (Alen et al., 2002).

1.8 Chromatin and the rDNA locus

The current view of chromatin and the rDNA locus is that inactive repeats are bound up in silent heterochromatin, whilst active repeats are devoid of nucleosomes. However, a number of studies over the years have suggested a more complicated picture than at first thought, with active rDNA repeats perhaps possessing a non-canonical chromatin structure. In support of the first view though, the currently published functions of histone tail modifications and chromatin remodeling factors on rDNA repeats in both yeast and higher eukaryotes are limited to gene silencing. The evidence for and against active rDNA repeats possessing chromatin structure is discussed, as well as the role of histone modifications and chromatin remodeling complexes in rDNA silencing.

1.8.1 Chromatin structure of rDNA repeats

Studies on the chromatin structure of rDNA repeats have been carried out for a number of years by numerous groups using a range of different techniques. The picture that has emerged from these studies is unclear, as studies appear, at first glance, to contradict each other. Psoralen cross-linking studies have suggested that inactive repeats are nucleosomal, whilst active repeats are devoid of nucleosomes. However, a number of studies have indicated that active rDNA repeats possess chromatin structure, although it differs from that seen on inactive repeats. The evidence for these theories is discussed below.

The largest group of studies suggesting active rDNA repeats are devoid of regular chromatin have been carried out by the Sogo lab. These studies used the ringed compound psoralen from the plant genus *Psoralea*. Psoralen can intercalate between the two strands of duplex DNA and, upon exposure to ultraviolet (UV) light, react with thymidines forming covalent cross-links. The cross-linked DNA is purified, digested with restriction enzymes and separated by gel electrophoresis. This cross-linked DNA will migrate more slowly than unmodified DNA. Early studies applying this technique in both *Dictyostelium* and mouse cells found that rDNA separates into two bands. The heavily cross-linked, slow migrating band was found to correspond to active rDNA repeats, whilst the fast migrating band corresponded to inactive repeats (Conconi et al., 1989; Sogo et al., 1984). From the heavy psoralen cross-linking of the active repeats, it was concluded that they were devoid of nucleosomes as regular nucleosomes would inhibit strong psoralen cross-linking. The psoralen cross-linking technique was then applied to *S. cerevisiae* with the same results (Dammann et al., 1993). In addition, early electron microscopy analysis indicated that actively transcribed rDNA is in an unbeaded conformation, suggesting a lack of nucleosomal structure (Labhart and Koller, 1982).

Whilst the psoralen cross-linking results suggest that active rDNA repeats are devoid of nucleosomes, a number of studies have indicated that chromatin structure is present during Pol I transcription. *Staphylococcal* nuclease and DNase I digestion of *S. cerevisiae* 35S rDNA suggested the presence of regular nucleosome structure across coding sequences (Lohr, 1983). Furthermore, cross-linking studies in *Physarum*, *Tetrahymena* and *Xenopus* showed the presence of histones on coding regions (Colavito-Shepanski and Gorovsky, 1983; Dimitrov et al., 1990; Dimitrov et al., 1992; Prior et al., 1983). Histone acetylation has also been identified on actively transcribed rRNA genes from rat tumour cells (Mutskov et al., 1996).

These studies were carried out using UV or formaldehyde cross-linking, followed by immunoprecipitation and DNA analysis by Southern blot. In order to reconcile these studies with the psoralen cross-linking studies, it has been suggested that histone signals are derived from contaminating inactive repeats (Lucchini and Sogo, 1998). However, limitations of psoralen are also known, as it has been shown that histone:DNA interactions, different from those seen in regular nucleosomes, are possible and such interactions allow extensive psoralen cross-linking (Conconi et al., 1984). Thus it has been suggested that these two sets of studies could be reconciled by the presence of a chromatin structure on rDNA that consists of non-canonical nucleosomes, or simple histone:DNA interactions (Toussaint et al., 2005). The case for chromatin structure playing a role in Pol I transcription has been furthered recently by a study showing that depletion of histones H3 and H4 inhibits Pol I transcription (Tongaonkar et al., 2005).

There is therefore still no consensus with regard to the chromatin structure of actively transcribed Pol I repeats. It does seem clear though that some form of structure involving nucleosome components is likely to play a role in Pol I transcription.

1.8.2 Silencing at the rDNA locus

Silencing at the rDNA locus is thought to occur by a number of different mechanisms. In addition to the roles described above for the methyltransferase Set1p in active Pol II transcription, Set1p is also required for the silencing of Pol II transcription at the rDNA locus. Pol II-transcribed genes inserted into rDNA are silenced by Set1p-mediated histone H3 K4 methylation (Briggs et al., 2001). This mechanism is independent of the known silencing factor Sir2p and involves the RNA recognition motif of Set1p (Bryk et al., 2002; Fingerman et al., 2005).

A second mechanism for silencing at the rDNA locus involves the HDAC Sir2p (Bryk et al., 1997; Smith and Boeke, 1997) and active rDNA transcription (Buck et al., 2002; Cioci et al., 2003). It is thought that Sir2p interacts with rDNA and causes deacetylation of histone H3 and H4 tails. This loss of acetylation may alter the rDNA chromatin structure, forming a binding site for a further factor such as Sir3p that is able to propagate the silenced state (Hoppe et al., 2002).

Finally, it has recently been shown that the chromatin remodeling complex Swi/Snf is required for rDNA silencing of RNA Pol II-transcribed genes. It is not clear whether Swi/Snf plays a direct or indirect role in silencing; however its function is independent of both Set1p and Sir2p (Dror and Winston, 2004).

In higher eukaryotes, the chromatin remodeling complex NoRC establishes rDNA silencing. NoRC is composed of two factors, TIP5 and the ATPase SNF2h. TIP5 interacts with histone H4 acetylated at K16 via its bromodomain, and with TTF-I which is bound to the promoter-proximal terminator. NoRC induces chromatin remodeling via its SNF2h subunit, the mammalian homologue of ISWI. NoRC also facilitates histone deacetylation and CpG methylation by recruiting the HDAC-containing complex SIN3 and DNA methyltransferases respectively. Methylation and deacetylation then propagate the silenced state. The chromatin remodeling complex NoRC thus establishes and maintains a silent chromatin state (Zhou and Grummt, 2005 and references therein).

1.9 Aims of this thesis

Much is known about the chromatin structure of Pol II-transcribed mRNA genes. In addition, numerous studies have investigated the roles of chromatin remodeling factors at Pol II loci. Chromatin and chromatin remodeling factors have been shown to have both positive and negative effects on Pol II transcription and detailed analyses have been

undertaken to begin to understand how Pol II transcribes through its chromatin template. In contrast, studies into the chromatin structure of rRNA genes have given mixed results. At first, it would appear that active genes are devoid of chromatin structure whilst inactive genes are silenced by heterochromatin. There have however been a number of studies indicating that active rRNA genes possess core histones and chromatin structure. These latter studies were criticised as they did not differentiate inactive repeat signals from active repeat signals. In addition, nucleosome components were localised to active rDNA repeats by Southern blot. This gives an all-or-nothing signal and does not allow the determination of histone profiles across the repeats. The overall aim of this thesis was therefore to study the chromatin structure and histone modifications of (active) rRNA genes using strains that allow the differentiation of signals arising from active and inactive repeats. The use of ChIP analysis, coupled to real-time PCR allows not only the association of histones with active repeats to be ascertained, but produces profiles of association over the chosen region. In addition to the study of nucleosome components, this study also aimed to investigate the role of chromatin remodeling factors at the rDNA locus.

In Chapter 2, ChIP analysis is used to study chromatin structure at actively transcribed repeats. It is found that nucleosome components histones H2B and H3 are present on active repeats. Furthermore, the N-terminal tails of histone H3 are modified by both K4 and K36 dimethylation, with K4 methylation dependent on Set1p. In Chapter 3, it is shown that chromatin remodeling factors Chd1p, Isw1p and Isw2p are present across the rDNA locus. Chd1p recruitment may be facilitated by both K4 dimethylation and rRNA. In Chapter 4, *in vivo* chromatin analysis is used to analyse chromatin structure over the termination region in active rDNA repeats. In addition, the function of chromatin remodeling factors is assessed. It is found that a strain possessing only actively transcribed rDNA repeats shows a number of differences in chromatin structure over the termination region, as compared to the wild-type. Furthermore, Chd1p, Isw1p and Isw2p function redundantly to maintain a specific

chromatin structure at inactive repeats. Finally, in Chapter 5, transcription run-on analysis is used to analyse the Pol I nascent transcription profile. It is found that Chd1p, Isw1p and Isw2p function redundantly to ensure efficient transcription termination.

These results allow an updated model of the chromatin environment during rDNA transcription and the factors that function at rDNA repeats. This model is presented in Chapter 6.

Chapter 2: Analysis of chromatin at the rDNA locus

Preface

The potential role of chromatin in the transcription of actively transcribed rRNA genes has been investigated for a number of decades, using a variety of techniques and carried out in a wide range of organisms, from yeast to slime mould to humans. Electron microscopy (EM), psoralen cross-linking and nuclease digestion techniques have all been used but have led to differing views on the chromatin structure of active rDNA repeats. The dense packing of Pol I elongation complexes along active rRNA genes (~50 complexes per gene) and the rate at which these elongation complexes transcribe (~60 nt per second; French et al., 2003) suggests that actively transcribed rDNA repeats are devoid of nucleosomes. This theory is supported by evidence from both EM and psoralen cross-linking studies (Conconi et al. 1989, 1993). However, Pol II is known to transcribe through nucleosomal templates (Hartzog, 2002; Studitsky, 2004), giving a clear precedent for polymerase transcription through a nucleosome template. Evidence from nuclease digestion studies suggests that active rDNA repeats possess a regular chromatin organisation (Lohr, 1983). Cross-linking studies in *Xenopus*, *Tetrahymena* and *Physarum* have shown that coding sequences are associated with core histones (Colavito-Shepanski and Gorovsky, 1983; Dimitrov et al., 1990; Dimitrov et al., 1992; Prior et al., 1983). Finally, it has recently been shown that partial histone depletion inhibits Pol I transcription (Tongaonkar et al., 2005). Thus the common view of actively transcribed rDNA repeats being devoid of nucleosomes does not appear as clear cut as may be first imagined.

The aim of this chapter was to apply the newer technique of chromatin immunoprecipitation (ChIP) coupled to real-time PCR analysis to investigate the profiles of nucleosome components across the rDNA locus in *S. cerevisiae*. This technique was first employed to probe for the presence of core histones across active rDNA repeats. The

analysis was then extended by probing for modified histones. Finally, the effect of deleting histone modifying enzymes was investigated.

2.1 Chromatin immunoprecipitation analysis

ChIP analysis is a powerful technique, allowing the identification of chromatin-associated factors (Figure 2.1A). The technique uses formaldehyde to cross-link DNA and its associated factors. Formaldehyde is a chemical that cross-links primary amino and thiol groups. Cross-links are further propagated to amino acids, for example tyrosine and arginine (Metz et al., 2004), facilitating the rapid cross-linking of DNA:protein complexes for analysis.

After cross-linking, the cells are ruptured and the DNA is sonicated to produce small fragments of a predetermined size. Addition of an antibody against a chosen factor e.g. a core histone or transcription factor, followed by incubation with beads that bind the antibody allows isolation of specific cross-linked complexes. Treatment with proteinase K digests the protein factors in the isolated complexes and the cross-links are reversed by high temperature. DNA bound to the chosen factor is then purified and its sequence analysed using real-time PCR. Compared to normal PCR analysis, real-time analysis allows relative quantitation of the association of the chosen factor with the amplified DNA sequence. The full analysis thus produces a profile of association of the chosen factor across a chosen locus.

In this study, primer pairs spanning the rDNA locus were employed (Figure 2.1B). The primers were designed to give coverage of the entire rDNA locus, from upstream of the promoter (primer pair “3' of 5S”), across all genic regions and downstream over and past the sites of transcription termination (primer pairs “2+3”, “5” and “7”). Primer pairs amplifying Pol II loci were chosen as controls for a number of ChIP experiments (Figure 2.1C and D).

Figure 2.1A: Schematic of the chromatin immunoprecipitation analysis protocol. 50ml cultures were grown overnight and used as described in the schematic. For a more detailed description of the protocol, see Chapter 8: Methods.

A

Treat *S. cerevisiae* culture with formaldehyde



Break cells and sonicate DNA



Treat with antibody overnight at 4°C



Incubate with beads, wash and elute chromatin:DNA complexes



Treat with proteinase K then incubate at 65°C overnight to reverse cross-links



Purify DNA by Qiagen column and analyse by real-time PCR

B

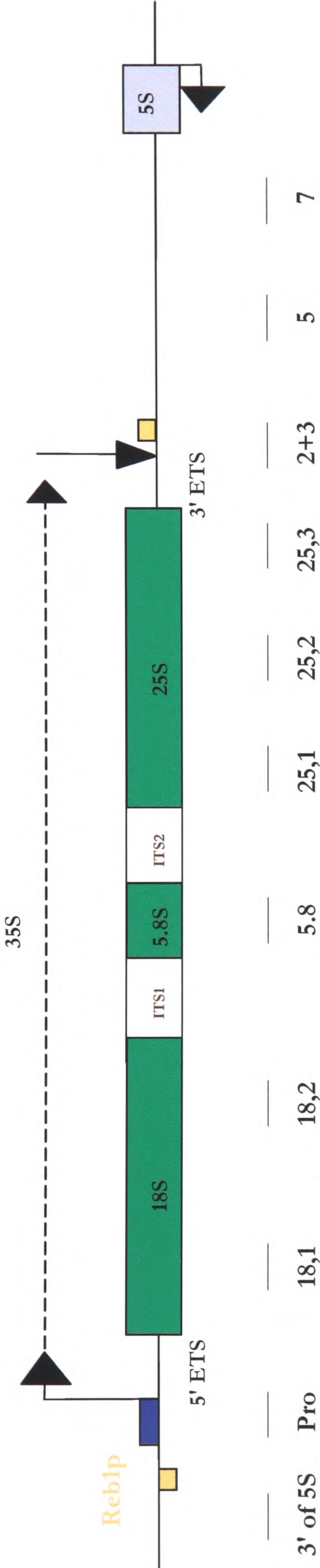
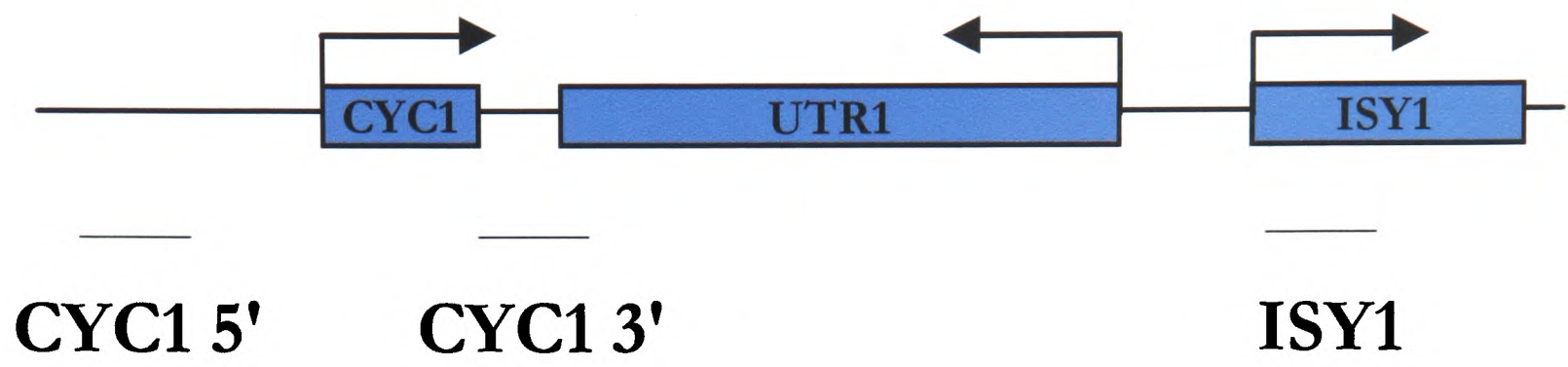


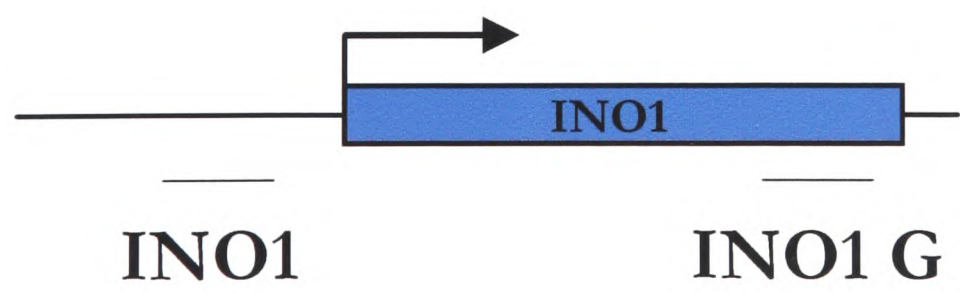
Figure 2.1B: Location of regions probed within the rDNA repeat during chromatin immunoprecipitation analysis. Black lines denote regions amplified during real-time PCR analysis. Regions amplified are named according to their location within the rDNA repeat. The sequences of primer pairs used to amplify these regions during real-time PCR analysis are listed in Chapter 8: Methods. For a full description of the elements found within the rDNA repeat, see Figure 1.1 of Chapter 1: Introduction. The diagram is representative and is not drawn to scale.

Figure 2.1C and D: Location of regions probed within Pol II loci during chromatin immunoprecipitation analysis. Genes are shown as blue boxes. Black lines denote regions of the *CYC1/UTR1/ISY1* locus (**C**) and *INO1* locus (**D**) amplified during real-time PCR analysis. Regions amplified are named according to their location within the respective Pol II locus. The sequences of primer pairs used to amplify these regions during the real-time PCR analysis are listed in Chapter 8: Methods. All diagrams are representative and are not drawn to scale.

C



D



2.2 Profile of RNA Polymerase I across the rDNA repeat

To ensure the specificity of the ChIP assay, the analysis was first carried out on a strain carrying the Pol I subunit *RP414* tagged with HA, γ AS14 (Bischler et al., 2002; Peyroche et al., 2000). As can be seen in Figure 2.2, very low signals are seen upstream of the promoter (3' of 5S) for Pol I, with the first high signal found over the promoter region (Pro). High signals then continue across the genic region (18,1 – 25,3) and over the +93 primary termination site (2+3). Signals finally drop 5- and 10-fold respectively over regions 5 and 7 (compared to region 2+3), indicating that the polymerase has terminated and is disengaging from the template. Whilst acting as a control for the ChIP analysis, the data presented here also indicate the presence of Pol I at certain regions of the repeat when it is not actively engaged in transcription. High signals seen over the promoter region indicate that Pol I is present although it is known to not be engaged in transcription (see transcription run-on analysis in Chapter 5). As Pol I should be absent from Pol II-transcribed loci, ChIP analysis was also carried out using primer pairs located upstream of *CYC1* (*CYC1* 5'), downstream of *CYC1* (*CYC1* 3') and within *ISY1* (*ISY1*). As seen in Figure 2.2, Pol I is absent from all three regions analysed. The very low signals seen for Pol I upstream of the promoter and downstream of the termination site (regions 3' of 5S, 5 and 7) and the absence of signal from Pol II-transcribed loci confirm that the protocol is working efficiently and with high specificity.

2.3 Profile of histone H2B across the rDNA repeat in wild-type and all-active strains

Previous studies supporting the theory that actively transcribed rDNA repeats are nucleosomal have been criticised as they did not differentiate signals derived from inactive repeats from those derived from active repeats. As inactive repeats are packaged into heterochromatin (Santoro et al., 2002), it could be argued that core histones seen in these

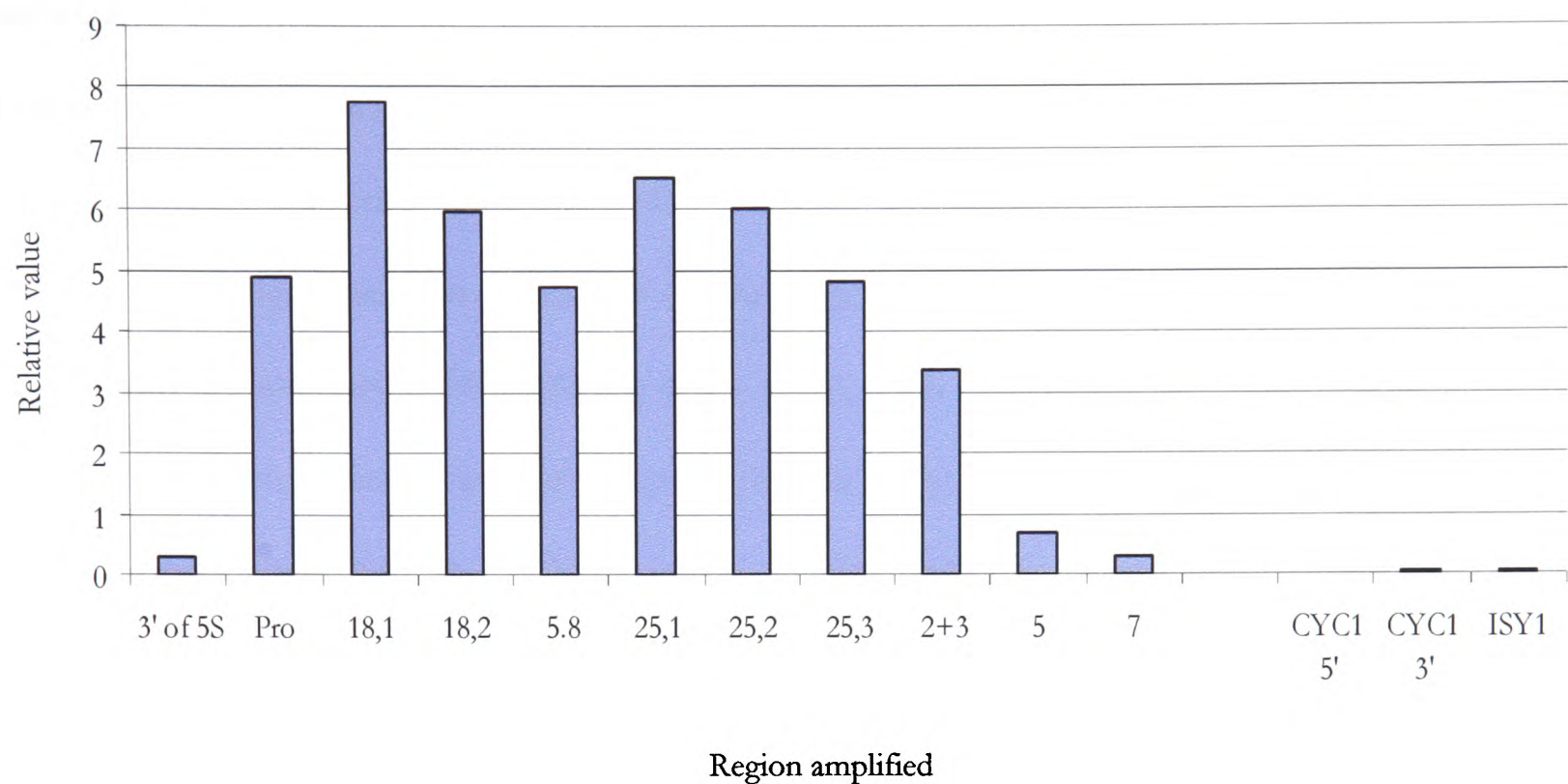


Figure 2.2: Profile of RNA Polymerase I at Pol I- and Pol II-transcribed loci. Chromatin immunoprecipitation analysis in a yAS14 strain using an HA antibody against the HA-tagged Pol I subunit A14. RNA Polymerase I is found across the rDNA repeat. High signals are seen over the promoter (Pro), throughout the genic region (18,1 – 25,3) and over the +93 termination region (2+3). Low signals upstream of the promoter (3' of 5S) and downstream of the terminator (5, 7) indicate that little polymerase is engaged upstream of the promoter and that most of the polymerase has dissociated prior to reaching regions 5 and 7. Analysis of a Pol II-transcribed locus (CYC1 5', CYC1 3', ISY1) produces background signals, consistent with the absence of Pol I from the locus and confirming the sensitivity of the ChIP analysis. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and an average data set is presented.

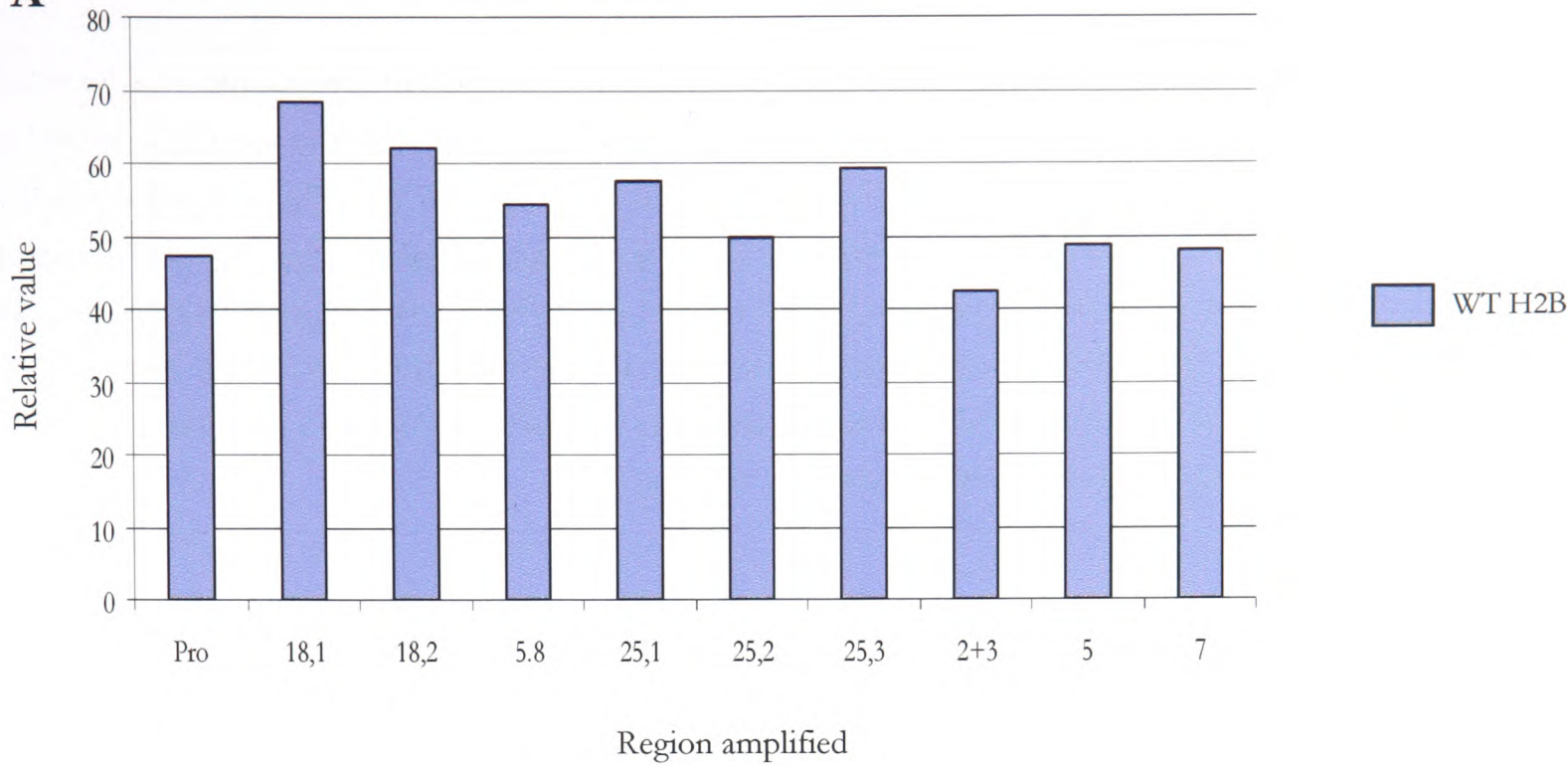
studies derive from inactive repeats. To overcome this problem, ChIP analysis was carried out in a strain possessing only 42 rDNA repeats (NOY886). This low repeat number is maintained stably by the strain, as determined by Southern blotting (Jones et al., 2005). Furthermore, all repeats are actively transcribed at all times, allowing distinction between signals derived from active and inactive rDNA repeats (French et al., 2003; Kobayashi et al., 1998). ChIP analysis was first performed on the isogenic wild-type strain (NOY1051) using an antibody against histone H2B (Zhang et al., 2005). High signals, varying by at most two-fold, are seen across the entire rDNA repeat (Figure 2.3A). This profile is not unexpected since approximately half the rDNA repeats in this wild-type strain are inactive and heterochromatic. Next, the ChIP analysis was repeated on the all-active strain. In contrast to the theory that actively transcribed repeats are devoid of chromatin, histone H2B signals are still seen across the entire repeat (Figure 2.3B). By comparing histone H2B signals from wild-type and all-active strains (Figure 2.3C), it can be calculated that active repeats contribute, on average, 40% of the H2B signal seen in the wild-type strain (see Chapter 8: Methods).

2.4 Profile of histone H3 across the rDNA repeat in wild-type and all-active strains

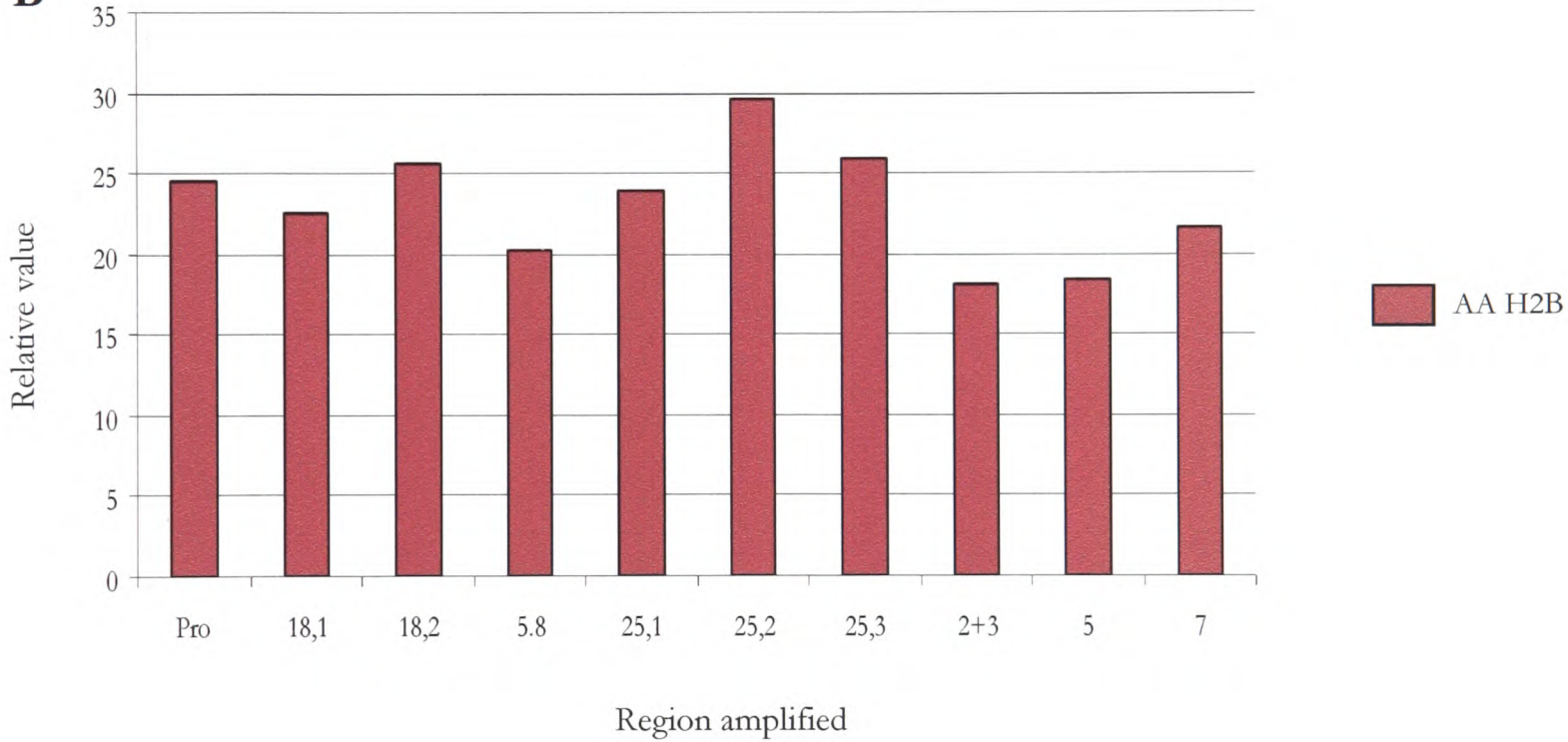
The presence of high levels of H2B across actively transcribed rDNA repeats suggests that chromatin structure may be compatible with transcription by Pol I. To investigate this further, ChIP analysis was performed using an antibody against core histone H3. The analysis was first carried out in the wild-type strain and shows that H3 is associated with rDNA repeats at high levels (Figure 2.4A). Repeating the analysis in the all-active strain, we find that, like histone H2B, histone H3 signals are seen across active rDNA repeats (Figure 2.4B). Comparing wild-type and all-active histone H3 signals, it can be calculated that all-active repeats contribute, on average, 25% of the total histone H3 seen in the wild-type

Figure 2.3: Histone H2B is associated with actively transcribed rDNA repeats. (A) In an NOY1051 wild-type strain (WT), histone H2B is found at high levels across the entire rDNA repeat. (B) In an NOY886 all-active strain (AA), histone H2B signals are still present across the entire repeat. (C) Comparison of WT and AA signals reveals that histone H2B remains associated with rDNA repeats during transcription. By calculating the ratio of H2B in the all-active strain to H2B in the wild-type strain across all amplified regions (see Chapter 8: Methods), it is found that the active repeats contribute, on average, 40% of histone H2B seen in the wild-type strain. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed two times and average data sets are presented.

A



B



C

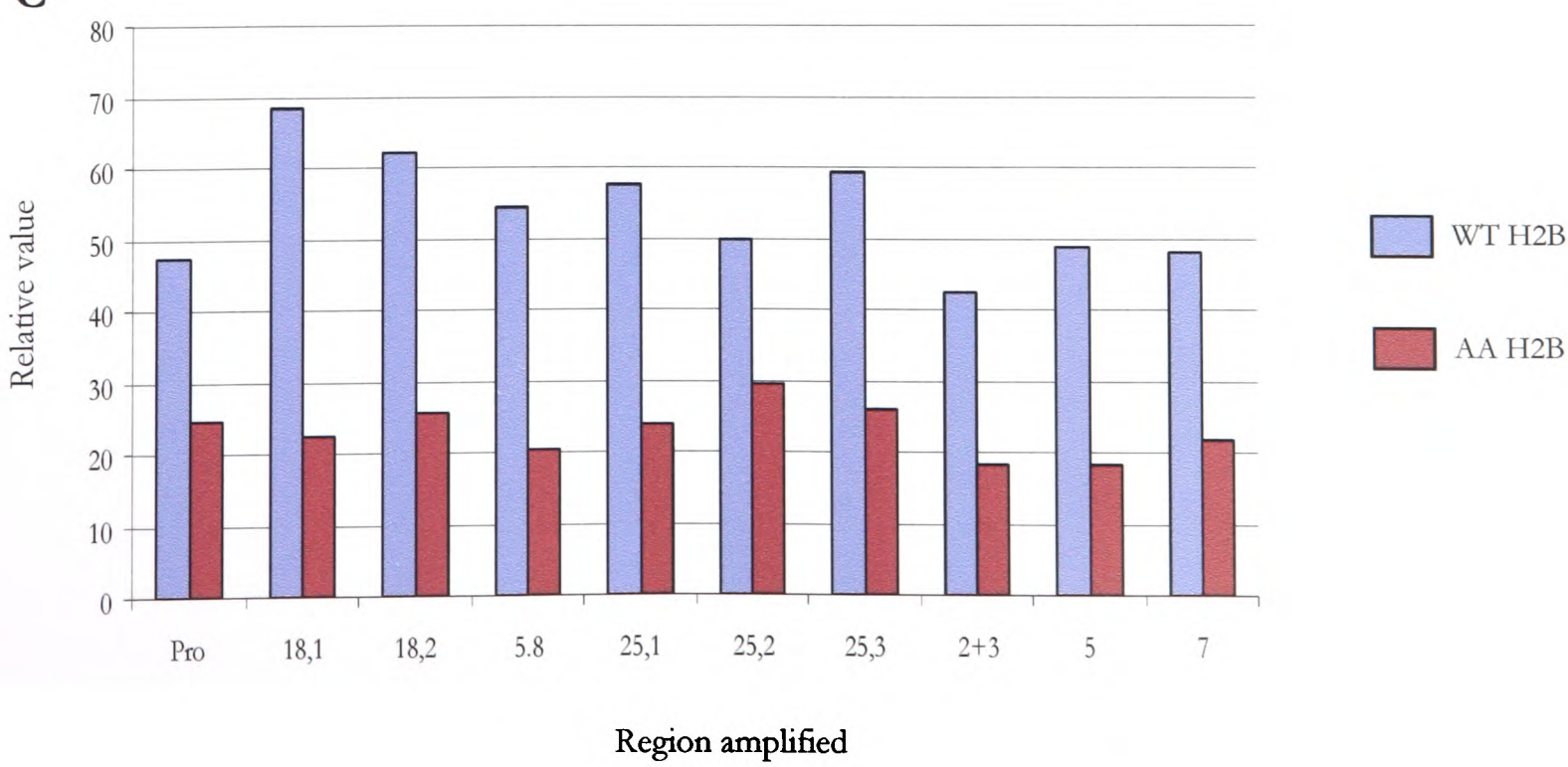
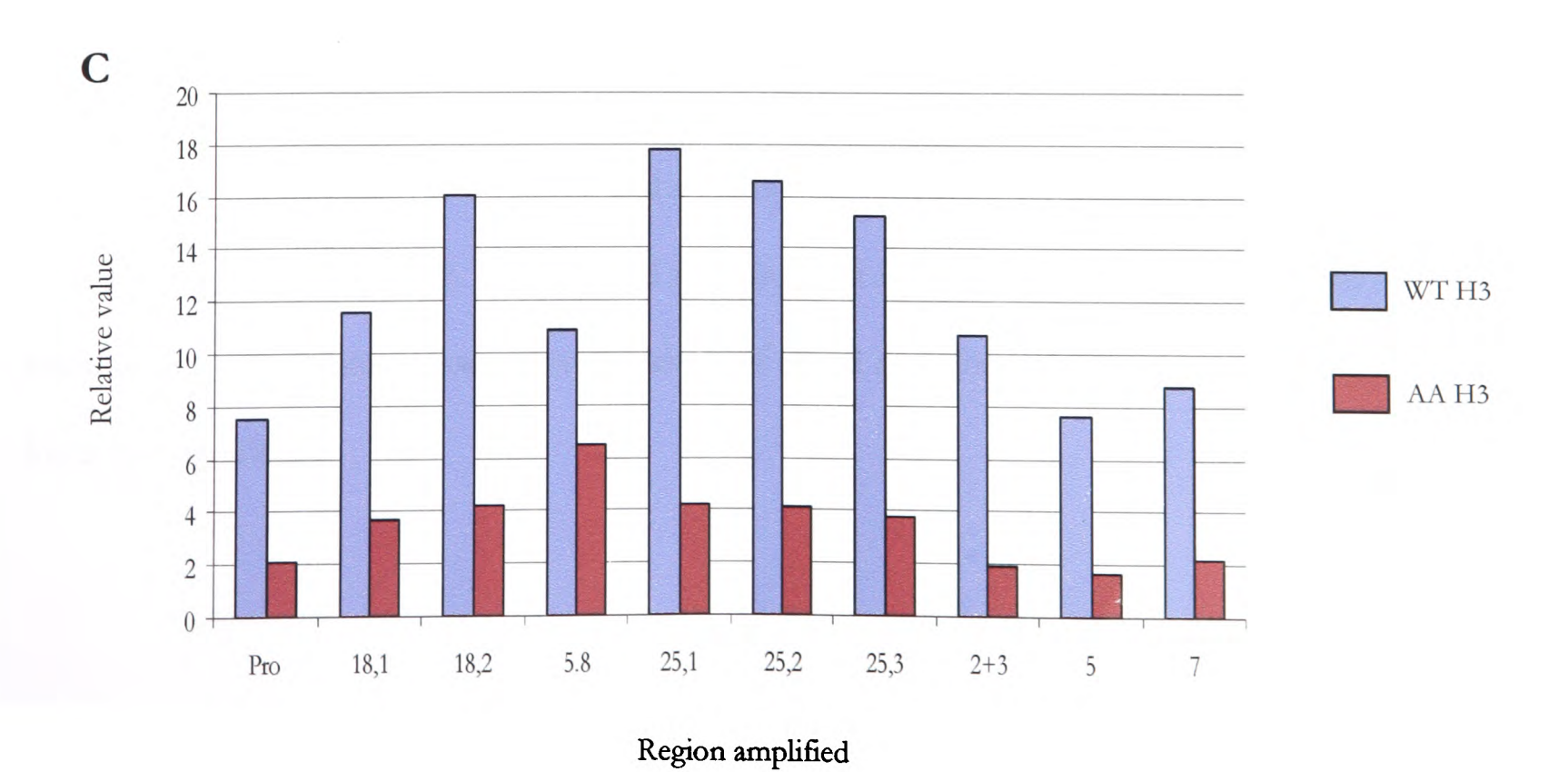
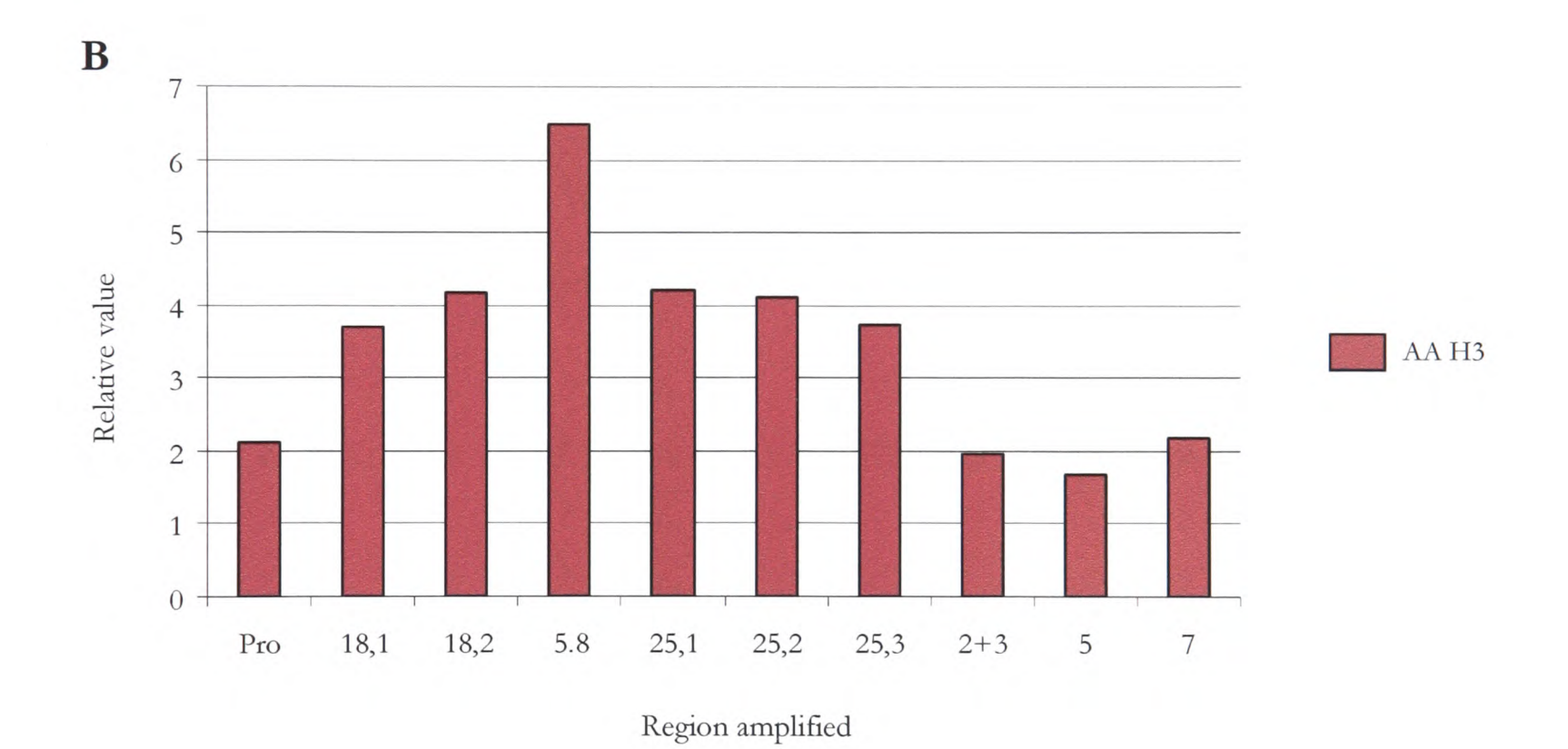
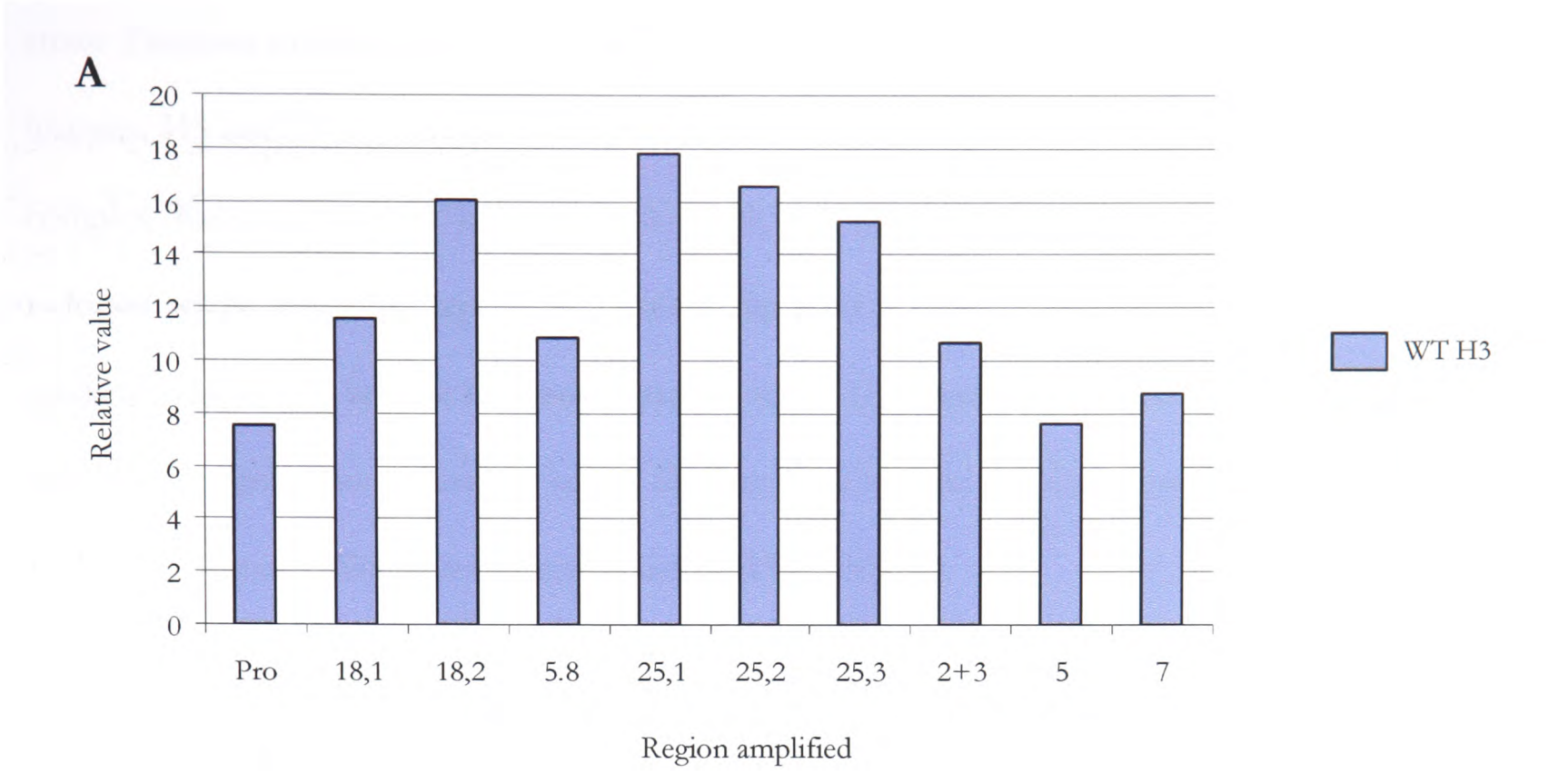


Figure 2.4: Histone H3 is associated with actively transcribed rDNA repeats. (A) In an NOY1051 wild-type strain (WT), histone H3 is found at high levels across the entire rDNA repeat. (B) In an NOY886 all-active strain (AA), histone H3 signals are still present across the entire repeat. (C) Comparison of WT and AA signals reveals that histone H3 also remains associated with rDNA repeats during transcription. By calculating the ratio of H3 in the all-active strain to H2B in the wild-type strain across all amplified regions (see Chapter 8: Methods), it is found that the active repeats contribute, on average, 25% of histone H3 seen in the wild-type strain. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and average data sets are presented.



strain. Previous studies predict that histone H3 would be found over the rDNA promoter as histones H3 and H4 are known components of UAF, a component of the Pol I initiation complex (Keener et al., 1997). However, for both wild-type and all-active strains, histone H3 is found across the entire repeat. In addition, the level of signal seen over the promoter is unlikely to be due to histone H3 derived from a single UAF molecule bound there. Thus the histone H3 signal seen across the repeat likely reflects histone H3 that forms, with histone H2B, a nucleosome-like structure across active rDNA repeats.

Note that neither Figure 2.3 nor Figure 2.4 includes data from the “3' of 5S” primer pairs. For histones H2B and H3, very high signals were obtained over this region (WT, AA = 114, 98 for H2B and 27, 13 for H3) that obscured the profiles seen over the rest of the repeat. The very high signals seen may be related to the proximity of the replication origin, as the primer pairs are located with the B box of the ARS.

2.5 Profiles of histones H2B and H3 at *INO1* in wild-type and all active strains

To confirm that the results seen for histone H2B and H3 across actively transcribed rDNA repeats are not due to indirect effects of the use of the NOY886 strain possessing 42 rDNA repeats, the analyses described above were performed with real-time PCR analysis of the *INO1* genic region (INO1 G, Figure 2.1D). As can be seen in Figure 2.5, levels of H3 and H2B at *INO1* are similar in wild-type and all-active strains, with no large drop-off in signal seen for the all-active strain. As the level of histone at a Pol II locus should be similar in both strains, these data indicate that the levels of signal seen for H2B and H3 at the rDNA locus are significant.

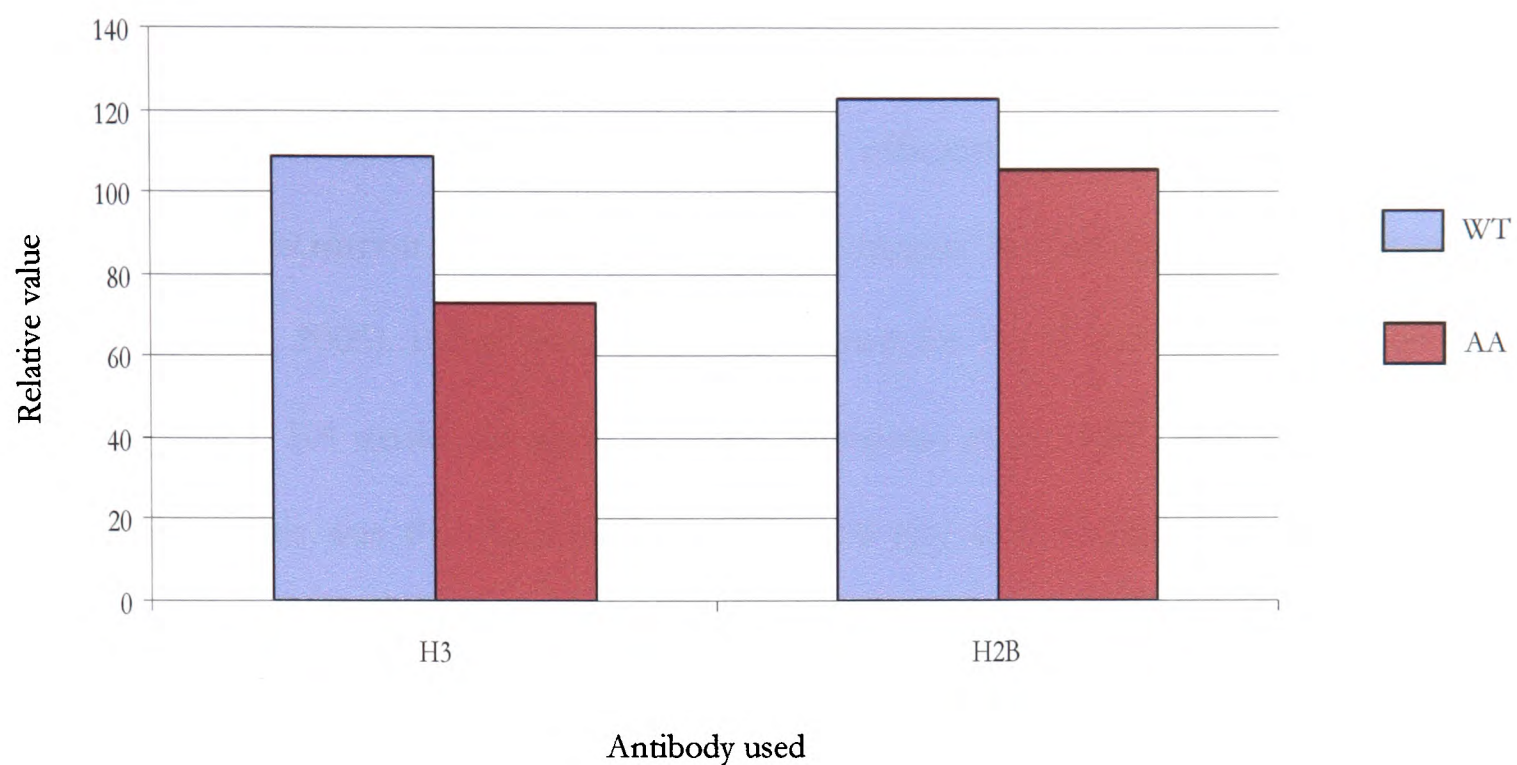


Figure 2.5: Association of nucleosome components within the *INO1* genic region. Both histone H3 (left-hand data set) and histone H2B (right-hand data set) are present at similar levels at *INO1* in a NOY1051 wild-type (WT) strain and a NOY886 all-active (AA) strain. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed two times and an average data set is presented.

2.6 Profile of H3 K4 dimethylation across the rDNA locus

The presence of both histones H3 and H2B across active rDNA repeats suggests that a chromatin structure is compatible with active transcription. At Pol II loci, it has been shown that histone tail modifications are associated with efficient transcription by facilitating recruitment of transcription factors and serving to propagate further modifications (Berger, 2002; Fischle et al., 2003). It has previously been shown for Pol II loci that methylation of histone H3 tails at K4 marks the gene as active (Bernstein et al., 2002; Santos-Rosa et al., 2002). ChIP analysis was therefore performed in wild-type and all-active strains using an antibody against H3 K4diMe. It can be seen that in a wild-type strain, H3 K4diMe is present across the entire rDNA repeat, with a pronounced peak over the 25S genic region (Figure 2.6A). Furthermore, all-active repeats also possess histone H3 K4 dimethylation (Figure 2.6B). However the profile shows a more diffuse peak than is seen in a wild-type strain (Figure 2.6C). Comparing the dimethylation at inactive (WT-AA) and active repeats, it can be seen that active rRNA genes are dimethylated across the entire repeat, whilst inactive repeats show a peak of methylation over the 25,1 and 25,2 genic regions. It has previously been shown that histone H3 K4 dimethylation is required for silencing of Pol II-transcribed genes inserted at the rDNA locus (Briggs et al., 2001; Bryk et al., 2002). Therefore the more pronounced peak seen in the wild-type strain may reflect high levels of dimethylation specific to, and required for, silenced inactive repeats.

2.7 Profile of H3 K36 dimethylation across the rDNA locus

A second modification to histone H3 tails, dimethylation at K36, has also been correlated with active transcription (Xiao et al., 2003). ChIP analysis was therefore carried out using an antibody against histone H3 K36diMe to determine whether a second histone tail modification is present at active rDNA repeats. In a wild-type strain (Figure 2.7A), it can be seen that H3 K36diMe is present across the rDNA repeat, with highest levels seen within

Figure 2.6: Histone H3 lysine 4 dimethylation is present across the rDNA repeat. (A) In an NOY1051 wild-type strain (WT), histone H3 K4 dimethylation is found across the entire rDNA repeat, with a pronounced peak over the 25S genic region. **(B)** In an NOY886 all-active strain (AA), histone H3 K4 dimethylation is present across the entire repeat. The strong peak of dimethylation is absent; instead a more diffuse profile is seen. **(C)** Comparison of WT and AA signals reveals that histone H3 K4 dimethylation is present on both inactive and active rDNA repeats. As actively transcribed repeats lack a pronounced peak of dimethylation, the peak seen in the wild-type strain likely derives from inactive repeats. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and average data sets are presented.

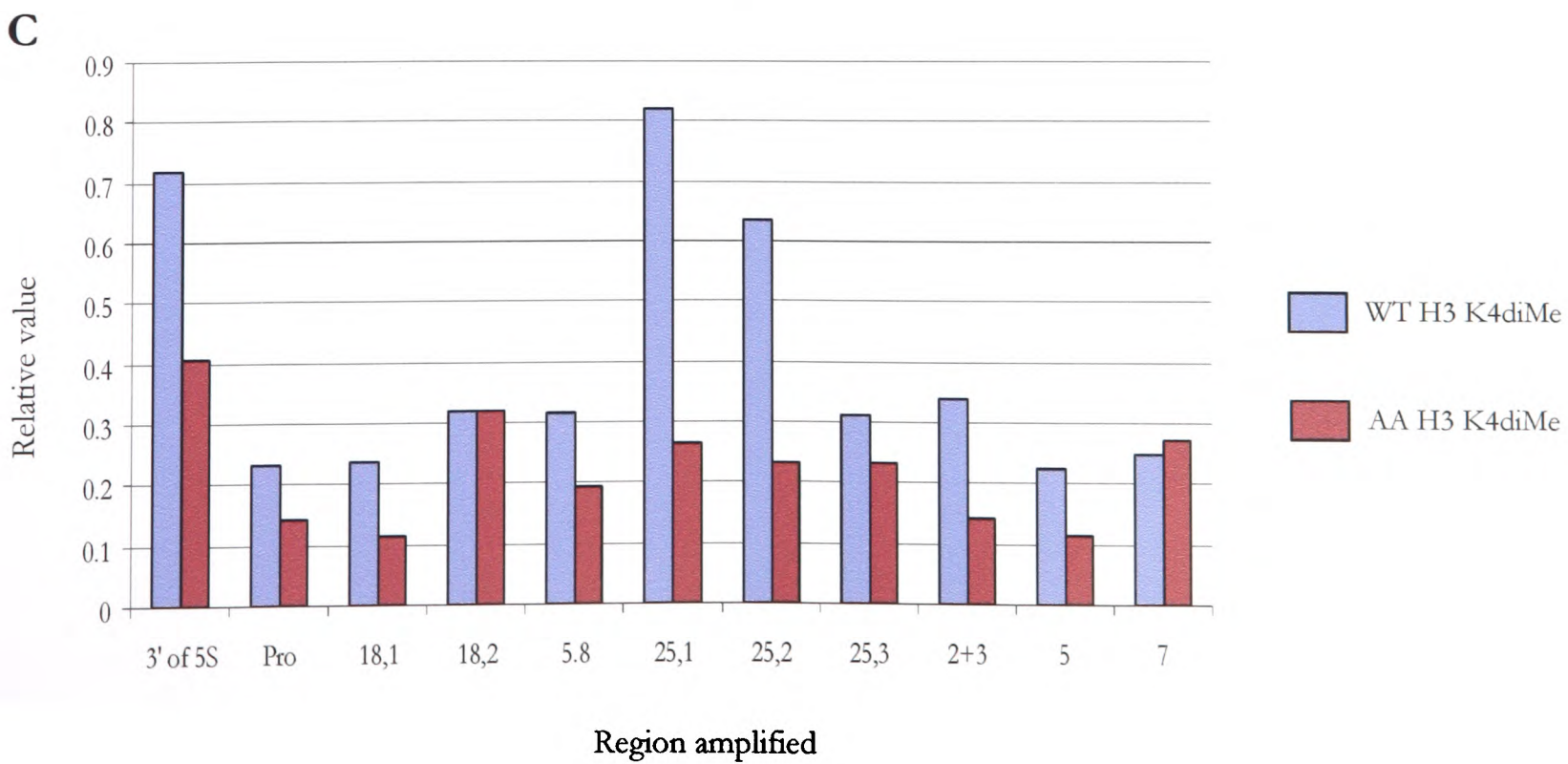
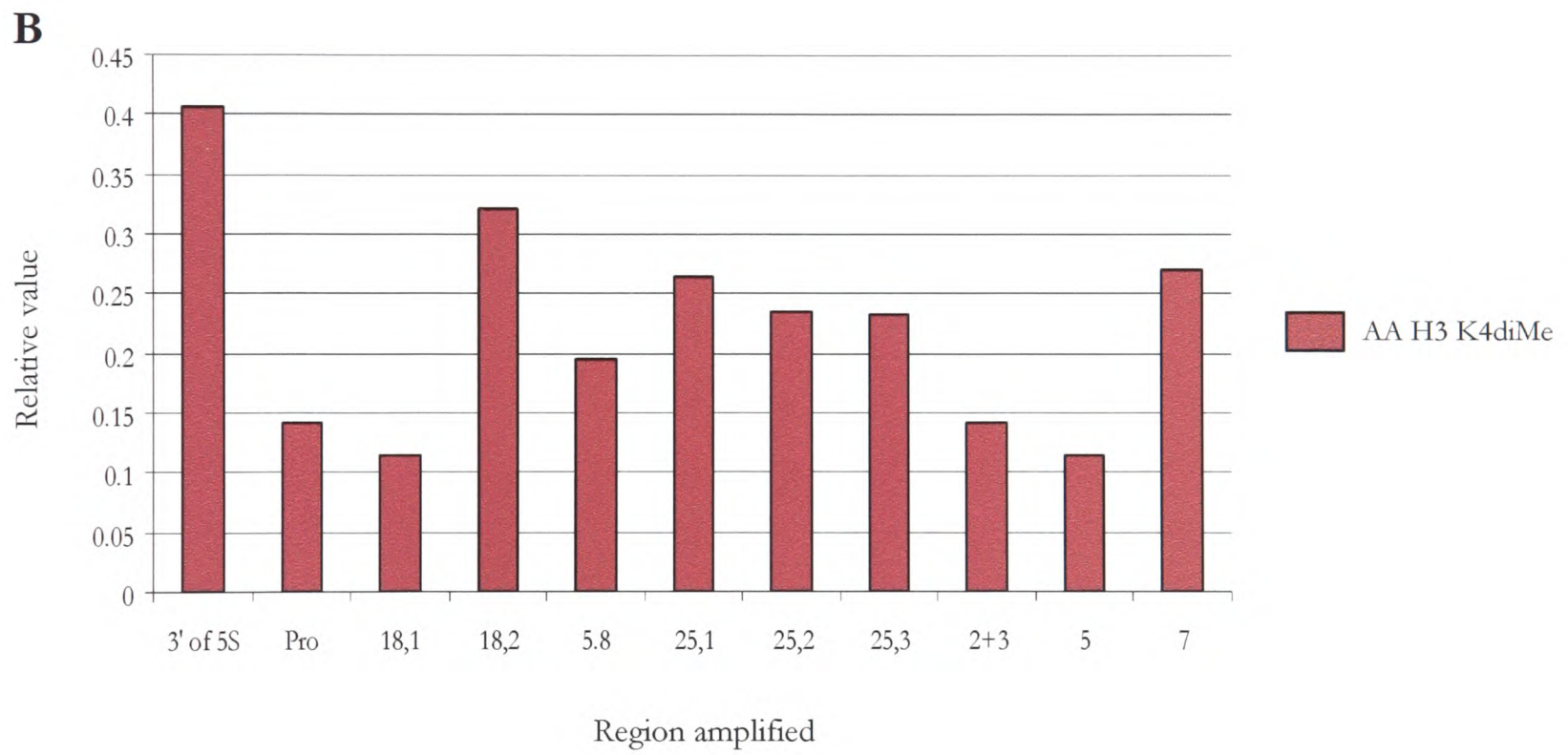
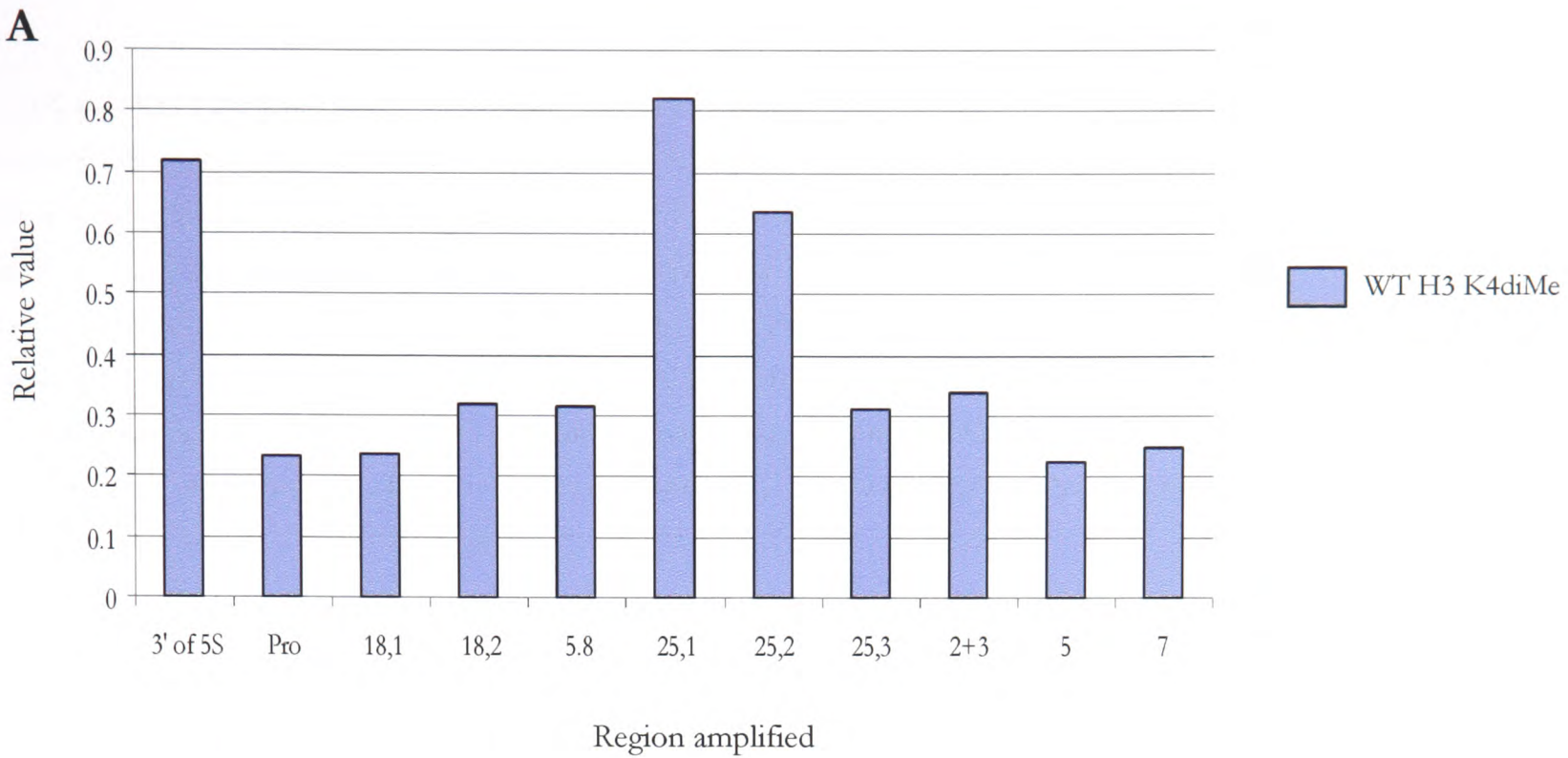
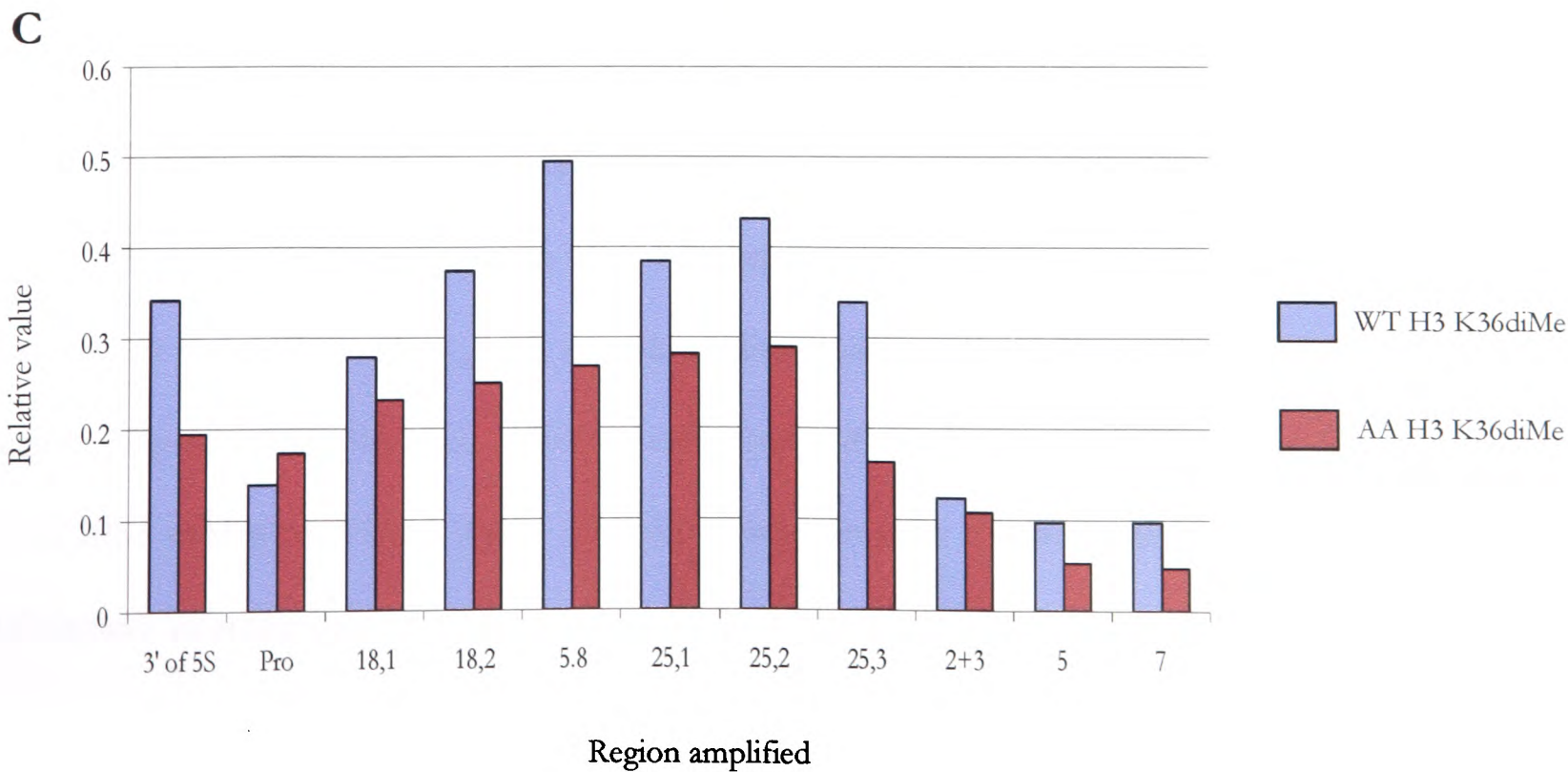
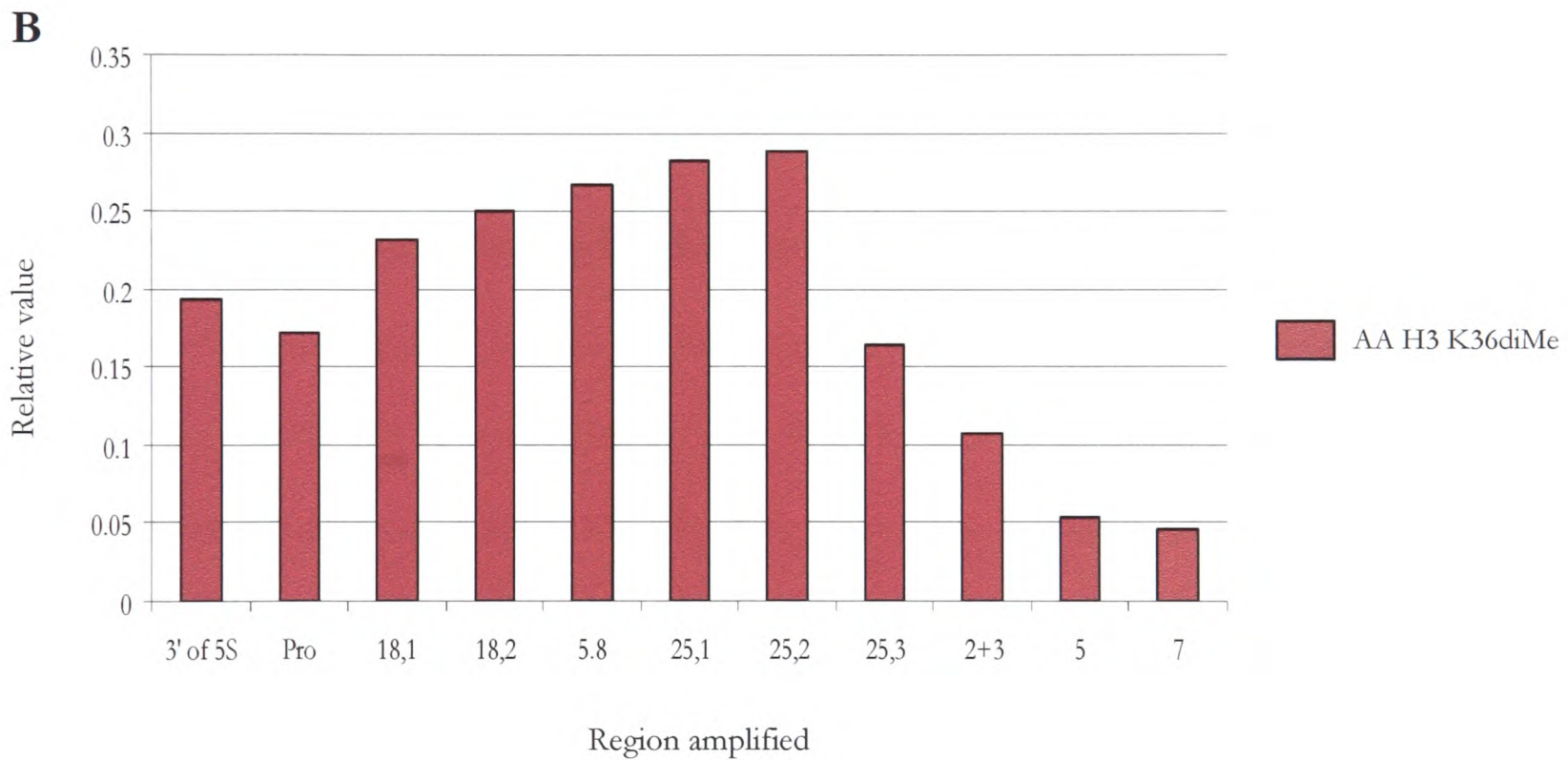
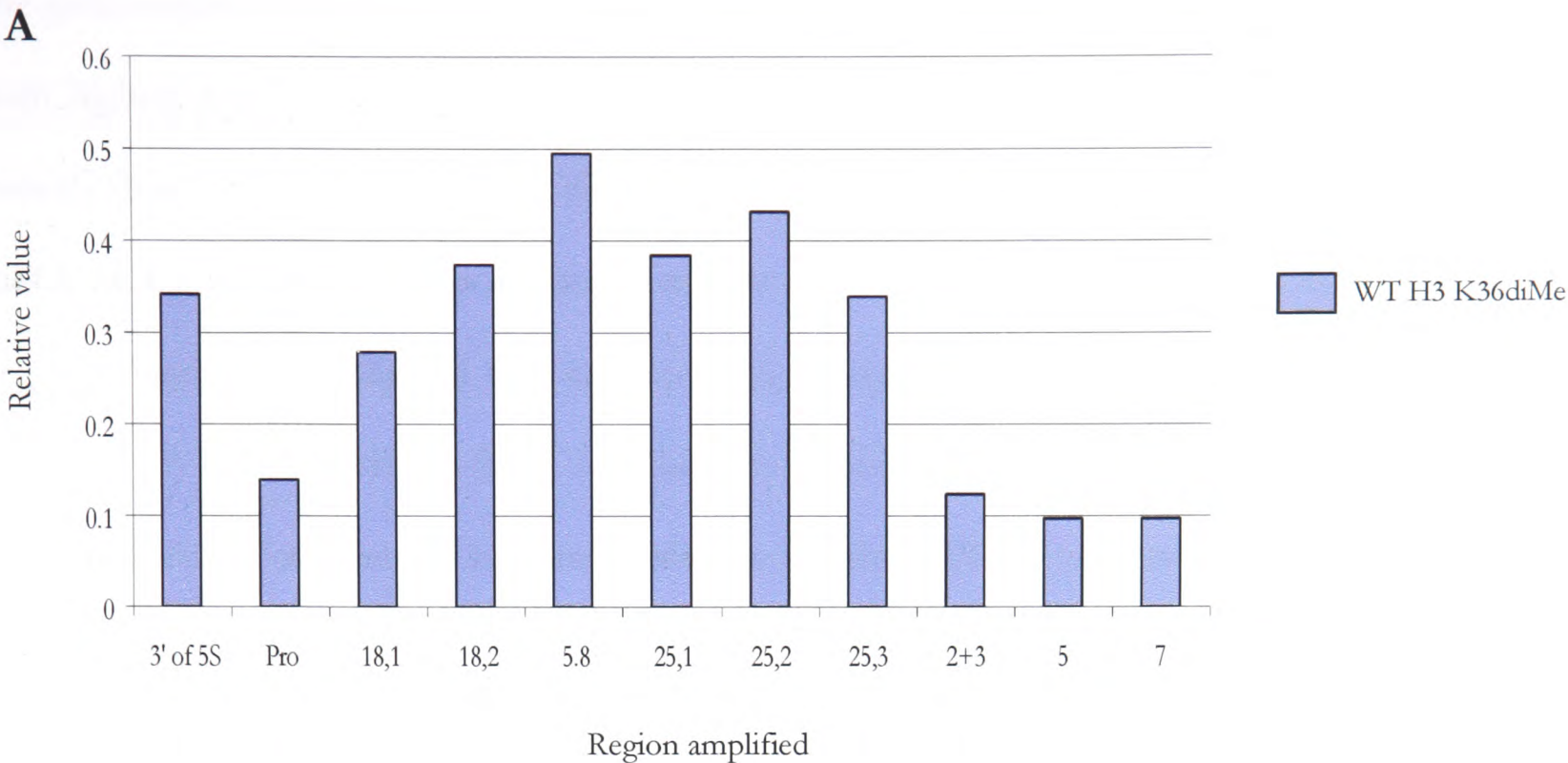


Figure 2.7: Histone H3 lysine 36 dimethylation is present across the rDNA repeat. (A) In an NOY1051 wild-type strain (WT), histone H3 K36 dimethylation is found across the entire rDNA repeat, with highest levels seen in the genic region. **(B)** In an NOY886 all-active strain (AA), histone H3 K36 dimethylation is present across the entire repeat, with highest levels also seen in the genic region. **(C)** Comparison of WT and AA signals reveals that histone H3 K36 dimethylation is present on both inactive and active rDNA repeats. In contrast to histone H3 K4 dimethylation (Figure 2.6), there is more K36 dimethylation present on active repeats than inactive repeats. The K36 dimethylation on actively transcribed repeats shows a peak at the 3' end of the genic region, as is seen for actively transcribed Pol II-transcribed genes (see Chapter 2 Discussion). Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed two times and average data sets are presented.

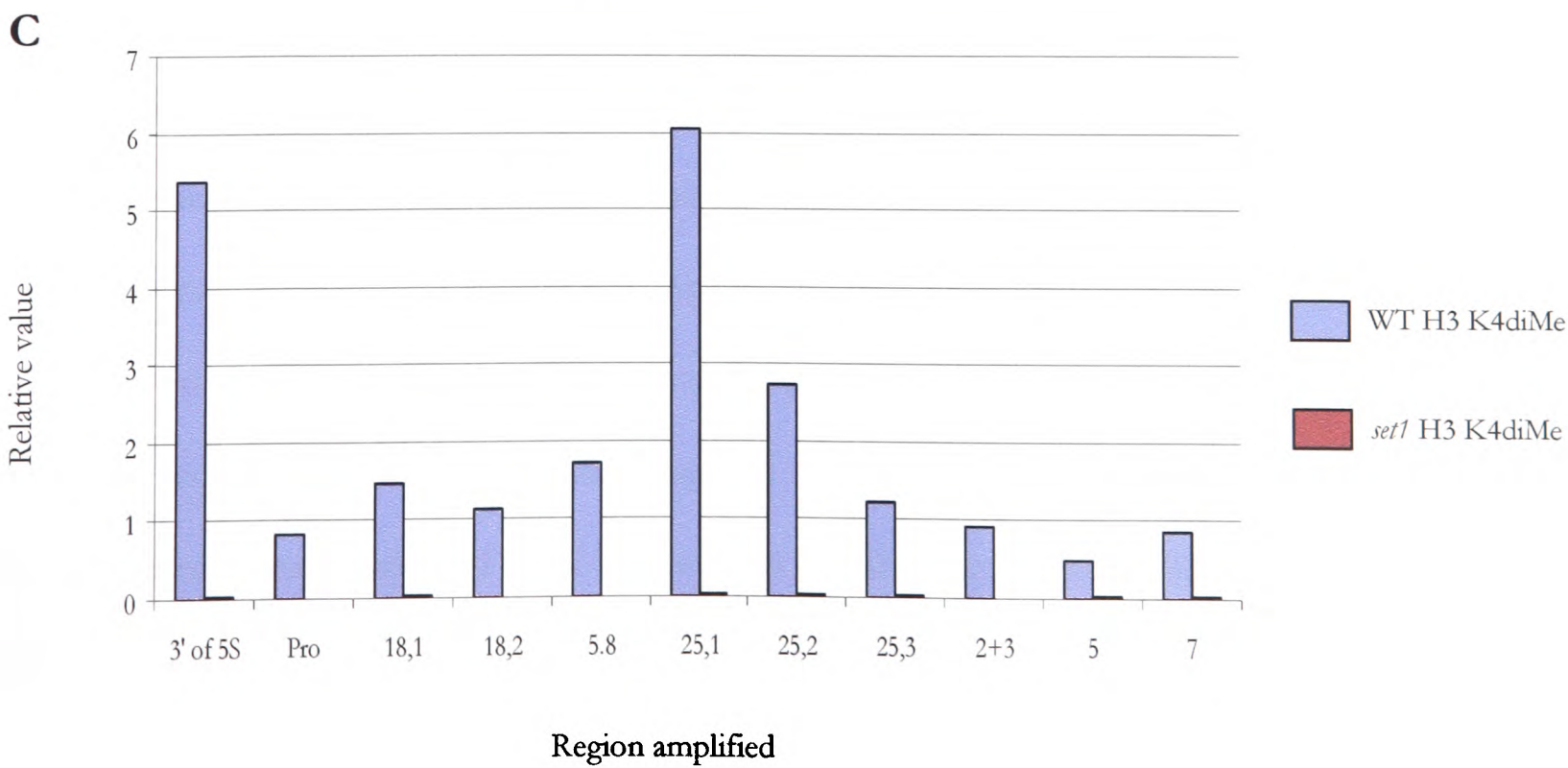
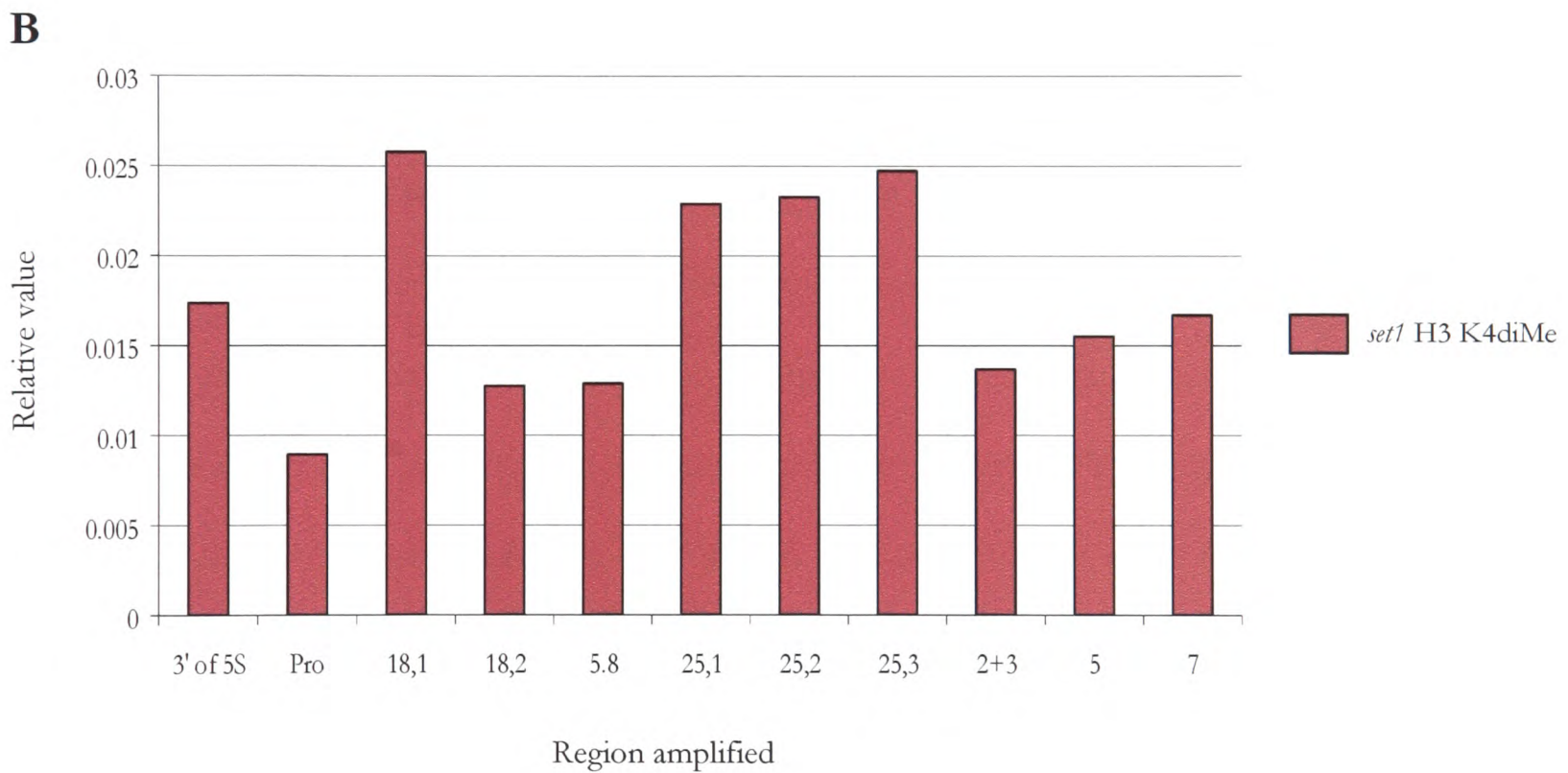
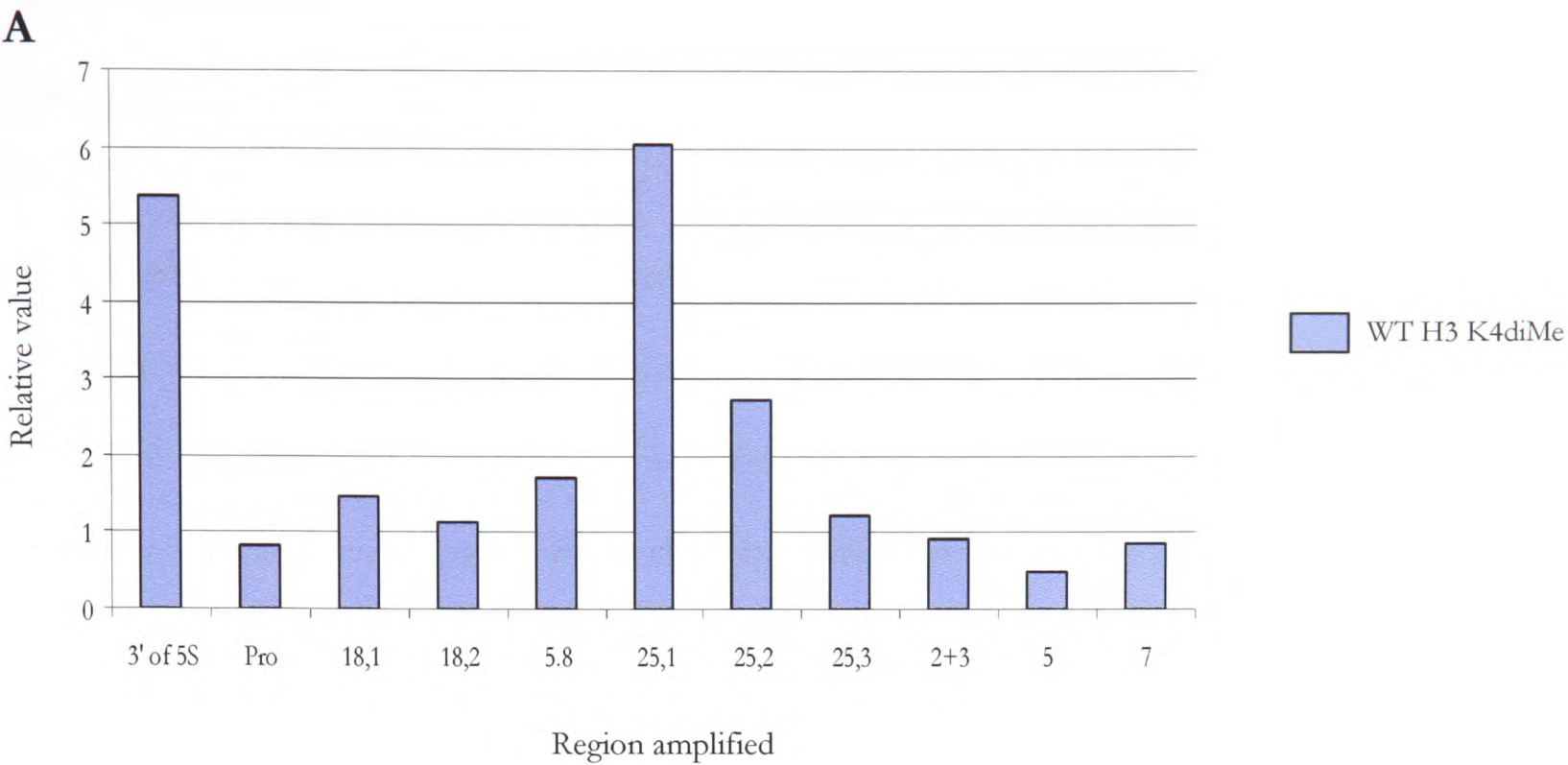


the genic region (18,1-25,3). Dimethylation at K36 is also found in the all-active strain, again with highest levels seen over the genic region (Figure 2.7B). Neither inactive nor active repeats show a pronounced peak of dimethylation, as seen for K4 at inactive repeats (Figure 2.6C). However, both inactive and active repeats show increases in signal across the genic region (Figure 2.7C), followed by a large decrease in signal (2-3 fold) over the termination region (2+3) and downstream (5, 7), compared to the middle and 3' end of the genic region (25,2 and 25,3). Comparing K36 dimethylation at inactive (WT-AA) and active repeats, it is seen that, apart from region 25-3 where inactive and active signals are the same, the active repeats are more heavily dimethylated than the inactive repeats. This is in contrast to K4 dimethylation, where it can be seen that inactive repeats have strong peaks of dimethylation over regions 25,1 and 25,2 (Figure 2.6C).

2.8 Effect of *SET1* deletion on H3 K4 dimethylation at the rDNA locus

At Pol II genes, methylation of histone H3 K4 is carried out by Set1p, the methyltransferase subunit of the COMPASS complex (Briggs et al., 2001; Roguev et al., 2001). H3 K4diMe at inactive repeats is catalysed by Set1p and this methyltransferase activity is a requirement for silencing of Pol II-transcribed genes at the rDNA locus (Briggs et al., 2001; Bryk et al., 2002; Roguev et al., 2001). The above observation that actively transcribed rRNA genes are also associated with K4 dimethylated histone H3 posed the question whether dimethylation at active repeats is dependent on Set1p. To investigate this, ChIP analysis was carried out in a wild-type strain, UCC1001 (Renauld et al., 1993) and a strain deleted for Set1p, UCC1001 $\Delta set1$ (Schramke et al., 2001). In the wild-type strain, H3 K4 dimethylation is seen throughout the rDNA repeat, with a peak of dimethylation again seen over the 25S genic region (Figure 2.8A). Note the different magnitude of signals seen here, compared to the NOY1051 wild-type (Figure 2.6A). The difference between the wild-types is likely due to differences in amenability of the two strains to ChIP analysis and highlights the need to use

Figure 2.8: Histone H3 lysine 4 dimethylation at actively transcribed repeats is dependent on Set1p. (A) Profile of histone H3 K4 dimethylation in the UCC1001 wild-type strain (WT). The profile is comparable to that seen for the NOY1051 wild-type strain (Figure 2.6A) with a strong peak seen over the 25S genic region. The levels of signal seen for the UCC1001 strain are higher than those seen for the NOY1051, indicating the importance of using isogenic strains. **(B)** Analysis of the UCC1001 $\Delta set1$ strain indicates that loss of Set1p results in very low levels of histone H3 K4 dimethylation across the rDNA repeat. **(C)** Comparison of WT and $\Delta set1$ strains reveals that loss of Set1p abolishes histone H3 K4 dimethylation at both inactive and active rDNA repeats. Set1p is therefore responsible for K4 dimethylation on actively transcribed repeats. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and average data sets are presented.



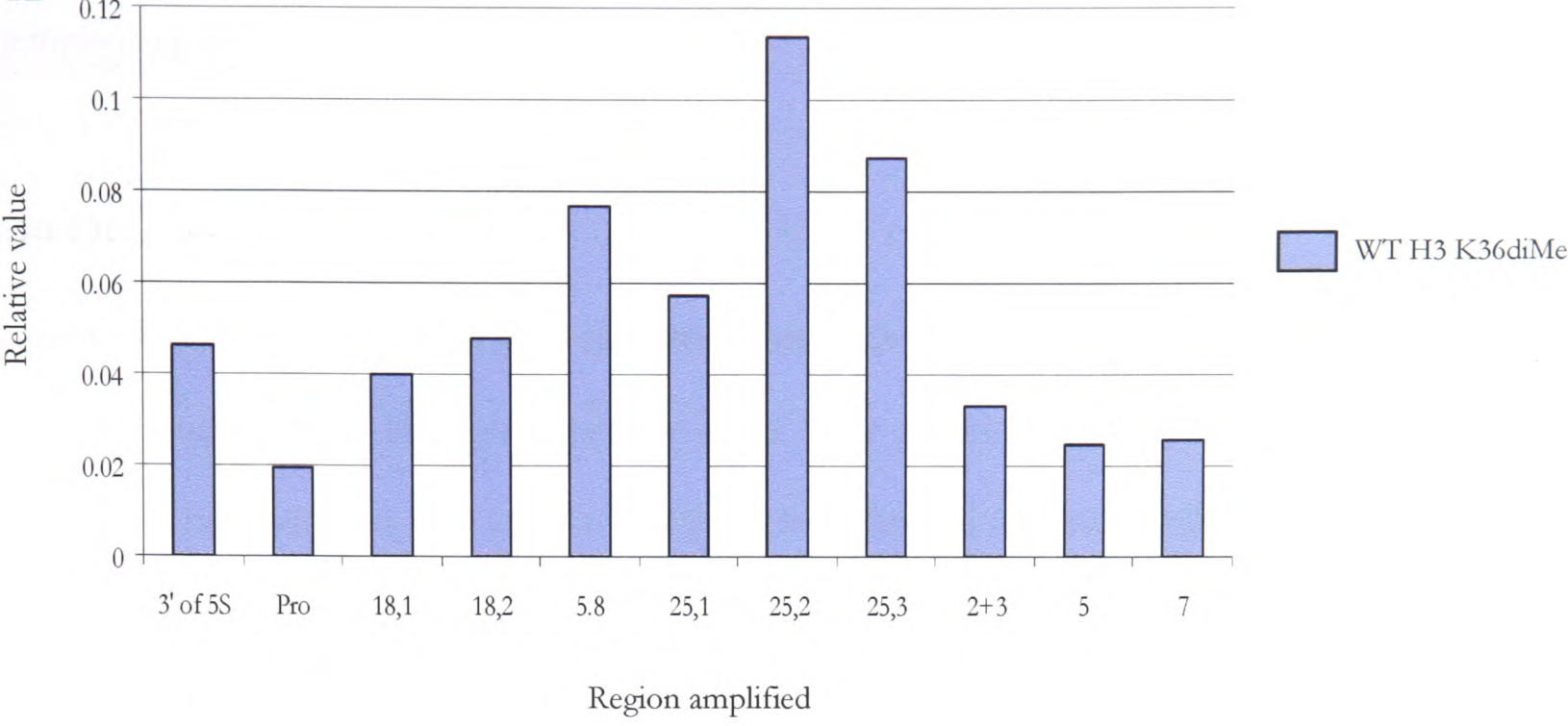
isogenic wild-type strains. It should also be noted that whilst signal levels differ, the same profile is seen for both wild-type strains. The ChIP analysis was next repeated in the strain lacking Set1p. It can be seen that very low levels of signal are obtained (Figure 2.8B). When wild-type and $\Delta set1$ strains are compared, it is clear that only background signals are obtained from the strain lacking Set1p, indicating an absence of dimethylation (Figure 2.8C). This loss of dimethylation is inconsistent with Set1p solely acting to silence rDNA repeats. If this were the case, there should still be dimethylation originating from the active repeats. The complete loss of dimethylation from the rDNA locus therefore indicates that active rDNA repeats are dimethylated at histone H3 K4 in a Set1p-dependent manner.

2.9 Effect of *SET2* deletion on H3 K36 dimethylation at the rDNA locus

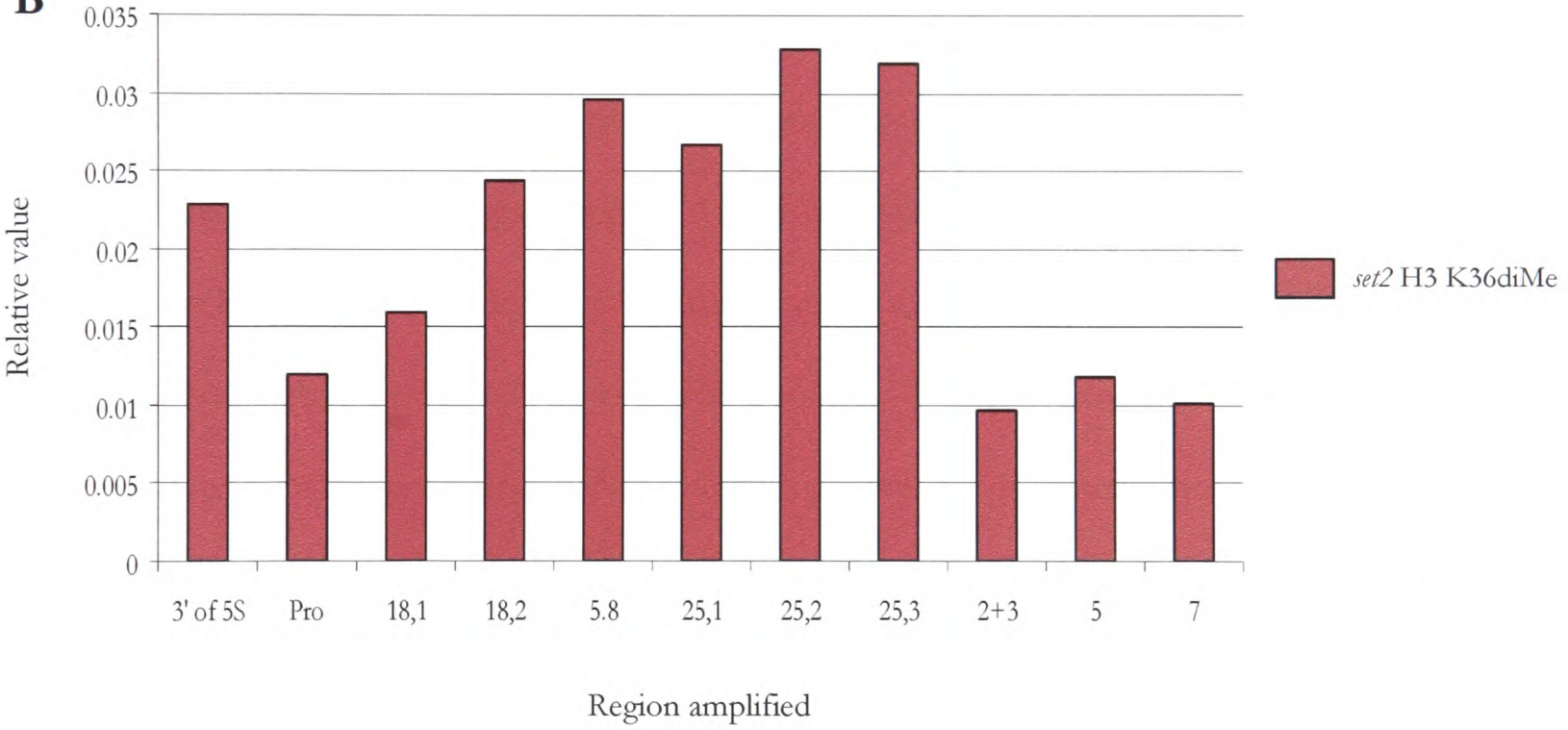
At Pol II loci, dimethylation of K36 is carried out by Set2p (Strahl et al., 2002). The presence of H3 K36 dimethylation at actively transcribed rDNA repeats therefore posed the question whether this dimethylation is dependent on Set2p. To address this, ChIP analysis was performed on a wild-type strain (YTT166) and a strain lacking Set2p (YTT166 $\Delta set2$). In the wild-type strain, K36 dimethylation is seen across the entire repeat, with highest signals present over the genic regions (Figure 2.9A). Loss of Set2p does not, however, lead to very low signals (compared to the isogenic wild-type) as there is still a strong profile present (Figure 2.9B). In both wild-type and $\Delta set2$ strains, the profiles are the same as those seen above in the NOY strains (Figure 2.7). The K36 dimethylation increases across the repeat, peaking at the 3' end of the genic region, before dropping off over the termination region and downstream (2+3-7). Comparing wild-type and $\Delta set2$ profiles, it can be seen that loss of Set2p does not completely abolish K36 dimethylation at rDNA repeats (Figure 2.9C). Whilst signals do decrease in the $\Delta set2$ strain compared to wild-type, dimethylation still persists. The continued dimethylation of rDNA repeats in the absence of Set2p therefore suggests

Figure 2.9: Histone H3 lysine 36 dimethylation at actively transcribed repeats is not solely dependent on Set2p. (A) Profile of histone H3 K36 dimethylation in the YTT166 wild-type strain (WT). The profile is comparable to that seen for the NOY1051 wild-type strain (Figure 2.7A) with the highest signals seen over the genic region. (B) Analysis of the YTT166 $\Delta set2$ strain indicates that loss of Set2p results in an approximately two-to-three-fold decrease in histone H3 K36 dimethylation across the rDNA repeat. (C) Comparison of WT and $\Delta set2$ strains reveals that loss of Set2p results in decreased histone H3 K36 dimethylation at rDNA repeats. Set2p therefore contributes to K36 dimethylation but is not the sole methyltransferase acting at this locus. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and average data sets are presented.

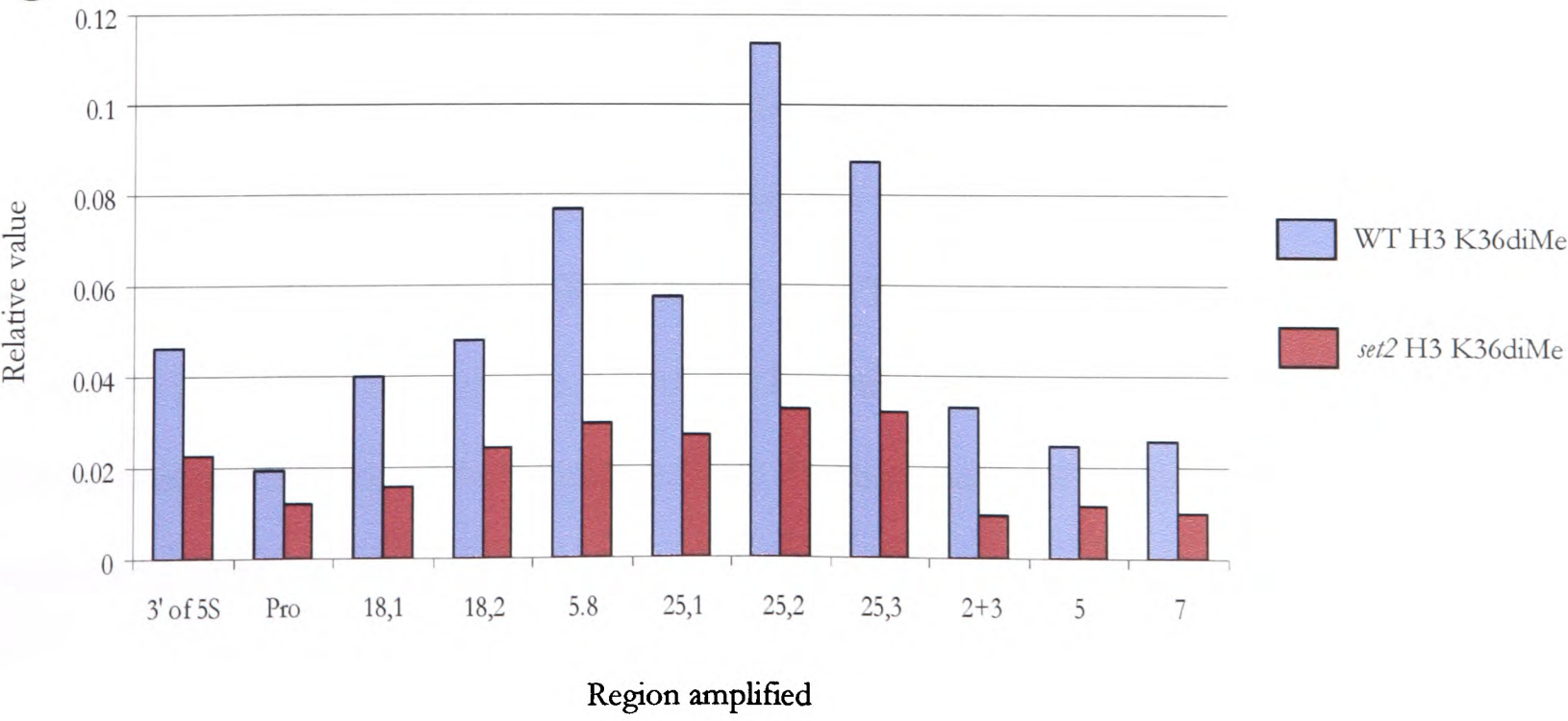
A



B



C



that while this methyltransferase does act at rDNA repeats, it is not the sole methyltransferase.

2.10 Discussion

A number of previous studies have suggested that actively transcribed rDNA repeats are devoid of nucleosomes. However, there is also a body of evidence suggesting that active repeats may be associated with histone and possess chromatin structure. Indeed it has been shown that partial loss of histones has a detrimental effect on Pol I transcription (see Chapter 2 Preface and Chapter 1: Introduction). In this chapter, the technique of ChIP analysis was used to address the question of chromatin and rDNA repeats.

Analysis of wild-type strains using antibodies against core histones H2B and H3 found histone association across the entire repeat. This is predicted as inactive repeats are known to be silenced by being maintained in a heterochromatic state (Santoro et al., 2002), and the signals seen could thus be derived from inactive repeats. This phenomenon of inactive and active rDNA repeats has previously led to studies supporting a chromatin structure on active rDNA repeats being criticised. To overcome this problem, the wild-type data were compared to data from a strain possessing only 42 repeats. Nearly all repeats in this strain are continually transcribed (French et al., 2003; Kobayashi et al., 1998) and this allows one to differentiate between signals derived from inactive and active repeats. Repetition of the ChIP analysis with histone H2B and H3 antibodies in all-active strains led to the discovery that active repeats are clearly associated with these core histones, with association stretching right across the repeat (Figures 2.3B and 2.4B). Thus chromatin structure appears compatible with active transcription by Pol I. The active repeats contribute 40% of total H2B present and 25% of total H3 present. Were the distribution of histones even between inactive and active repeats, one would expect these figures to stand at 50%. The lower values

may reflect an uneven dosage of histones on inactive repeats, compatible with both the heterochromatic nature of inactive, silenced repeats and the highly transcribed nature of active repeats. This high level of transcription has been used to suggest that active repeats cannot accommodate histones. It is known however that Pol II can transcribe through nucleosomes (Hartzog et al., 2002; Studitsky et al., 2004) and the data presented here suggest that Pol I may also possess this ability. The difference in levels seen between H2B and H3 is unexpected. Canonical nucleosomes contain equal amounts of all 4 core histones. The unequal signals seen here may therefore imply that the nucleosomes present on active repeats are dynamic and possibly non-canonical. The presence of H2B is important though, as it counteracts the possible suggestion that only H3 and H4 are present. These histone components are known to be deposited first during nucleosome assembly (Kaufman et al., 1997) and the presence of only H3 or H4 would have suggested that a form of pre-nucleosome were present on active repeats. The data presented here argue against this. It should be noted that a full analysis of chromatin structure on active repeats should include ChIP analyses using antibodies against both H2A and H4. These analyses were attempted with commercial antibodies but were unsuccessful. Antibodies would therefore need to be raised to further this analysis.

The analysis of chromatin structure at active repeats was extended by performing ChIP analysis using antibodies against the histone H3 modifications of K4 and K36 dimethylation in wild-type and all-active (Figures 2.6 and 2.7). These data show that dimethylation of both K4 and K36 residues occurs on active rDNA repeats, although the profiles of dimethylation differ. K4 dimethylation is present on both active and inactive repeats. Inactive repeats show a peak of dimethylation over the 25S genic region and dimethylation is known to be required for silencing of inserted Pol II genes (Briggs et al., 2001; Bryk et al., 2002). Actively transcribed repeats also possess K4 dimethylation but the profile is more even, with no

obvious peak. It does however extend across the entire repeat, suggesting it is required for, or a plays a role in, all stages of transcription (Figure 2.6). K36 dimethylation is present in much lower amounts on inactive genes than on active genes, suggesting it may not be involved in silencing, in contrast to K4 dimethylation. K36 dimethylation of actively transcribed repeats shows a clear increase over the genic region, peaking in the second half of 25S before dropping off over the termination region and downstream. K36diMe at Pol II loci has also been shown to accumulate at the 3' end of genic regions (Morillon et al., 2003a), suggesting similarities in transcription by Pol I and Pol II.

The roles of the methyltransferases Set1p and Set2p were also investigated. Set1p and Set2p catalyse the dimethylation of K4 and K36 respectively at Pol II loci, and it was of interest to determine whether they are also required for the methylation seen at the Pol I locus. Firstly, K4 dimethylation was studied in the presence and absence of Set1p. In the wild-type strain, dimethylation (known to originate from both active and inactive repeats) is seen. Loss of Set1p however, leads to the complete loss of dimethylation, indicating that Set1p is responsible for dimethylation of K4 at both active and inactive repeats (Figure 2.8). The requirement for Set1p at active repeats raises the question as to how Set1p is recruited. At Pol II loci, Set1p binds to the serine 5- phosphorylated CTD (Ng et al., 2002). Whilst Pol I does not possess an analogous domain for Set1p to bind to, Set1p is known to interact with RNA via its RNA recognition motif (Schlichter and Cairns, 2005). This motif may therefore provide a means of Set1p recruitment to the rDNA locus. The effect of loss of Set2p upon K36 dimethylation was next investigated. Comparing wild-type and $\Delta set2$ data, it is seen that dimethylation is not completely abolished in the absence of Set2p (Figure 2.9). The level of dimethylation seen is lower though, indicating that Set2p does act as a methyltransferase at the rDNA locus. However, the continued presence of dimethylation suggests that a further methyltransferase activity may also be acting at this locus. There are known to be 7 SET domain proteins in yeast, with methyltransferase activity assigned to two of them, Set1p and

Set2p. Whilst unrelated activities have been assigned to Set3p, Set5p and Set6p, Set4p and Set7p do not have clear functions assigned. Set7p is known to be a nuclear protein, and thus it would be of interest to investigate whether H3 K36diMe is lost in $\Delta set4$ or $\Delta set7$ deletion mutants. It could however be the case that a methyltransferase unrelated to the SET family is acting at the rDNA locus. Dot1p methylates H3 K79 and does not possess a SET domain. Therefore any search for further methyltransferase activity at the rDNA locus would need to include non-SET domain proteins.

The results from this chapter are summarised in Figure 2.10. Nucleosome-like structures, formed of (at least) histones H2B and H3 are present across the rDNA repeats. The N-terminal histone tails of these histones are methylated at both K4 and K36. K4diMe (shown in green) increases across the rDNA repeat, with a peak seen over the middle of the genic region. This signal then drops off towards the termination region (represented by the “+93” black arrow). K36diMe (shown in blue) increases over the rDNA repeat, with a peak seen at the 3' end of the genic region. As seen for K4diMe, the K36diMe signal then drops off over the termination region and beyond. In both cases, the relative amount of methylation is shown by the different sizes of “K36/K4diMe” lettering. The dimethylation of K4 residues is carried out solely by the Set1p methyltransferase. The dimethylation of K36 residues is carried out by Set2p. In addition, an unidentified methyltransferase also appears to function at this locus (denoted by the “?”).

Figure 2.10: Updated model of the chromatin structure of actively transcribed rDNA repeats. An active rDNA repeat is shown. The black lines represent actively transcribed rDNA organised into nucleosome-like structures composed of, at least, histones H2B and H3 (circles surrounded by dotted lines). The start site of transcription is indicated by the black arrow and the +93 primary termination site is indicated by the “+93” black arrow. The black wavy lines extending from the dotted circles represent the N-terminal histone tails. Black forked structures represent dimethylation of histone tails. Dimethylation of K4 is shown in green as “K4diMe”, whilst dimethylation of K36 is shown in blue as “K36diMe”. K4 dimethylation is seen across the repeat with a peak over the 25S genic region. K36 dimethylation increases over the rDNA repeat and peaks at the 3’ end of the genic region. In both cases, the relative amount of methylation is shown by the different sizes of “K36/K4diMe” lettering. The methyltransferases responsible for dimethylation of histone tail residues are shown in red. Set1p is the sole methyltransferase responsible for the dimethylation of K4 residues. Set2p contributes to dimethylation of K36 residues but is not the sole methyltransferase. The unidentified methyltransferase acting at this locus is represented by “?”.

Set1p



K4diMe

K4diMe

K4diMe

+93

K4diMe



K36diMe

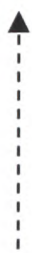
K36diMe

K36diMe

K36diMe

Set2p

?



Chapter 3: Analysis of chromatin remodeling factors at the rDNA locus

Preface

Chromatin remodeling enzymes utilise the energy of ATP hydrolysis to alter DNA:histone interactions and act during all stages of transcription. In addition, chromatin remodeling factors are known to be recruited to chromatin through interaction with modified histone tails (see Chapter 1: Introduction). The presence of histones H2B and H3 on actively transcribed rDNA repeats (Chapter 2) therefore suggested that chromatin remodeling factors may be required during transcription by Pol I. In addition, the presence of both histone H3 K4 and K36 dimethylation suggested mechanisms by which chromatin remodeling factors could be recruited to the locus.

The aim of this chapter was therefore to investigate whether chromatin remodeling factors are associated with the rDNA locus and to investigate their mechanism of recruitment. It has previously been shown that the chromatin remodeling factors Chd1p and Isw1p are required for efficient Pol II transcription (Alen et al., 2002; Kent et al., 2001; Krogan et al., 2003a; Krogan et al., 2002b; Morillon et al., 2003a; Simic et al., 2003). In addition, the K4 dimethylated tails of histone H3 have been shown to recruit these factors (Pray-Grant et al., 2005; Santos-Rosa et al., 2003), suggesting a route by which they could be recruited to rDNA repeats. Furthermore, it has been shown that Chd1p, Isw1p and Isw2p are required for efficient transcription termination of certain Pol II-transcribed genes (Alen et al., 2002). Therefore, ChIP analysis was performed on strains possessing tagged copies of these three remodeling factors. In addition, RNase A treatment was used to determine the role of rRNA in recruitment.

3.1 Profile of Chd1p across the rDNA repeat

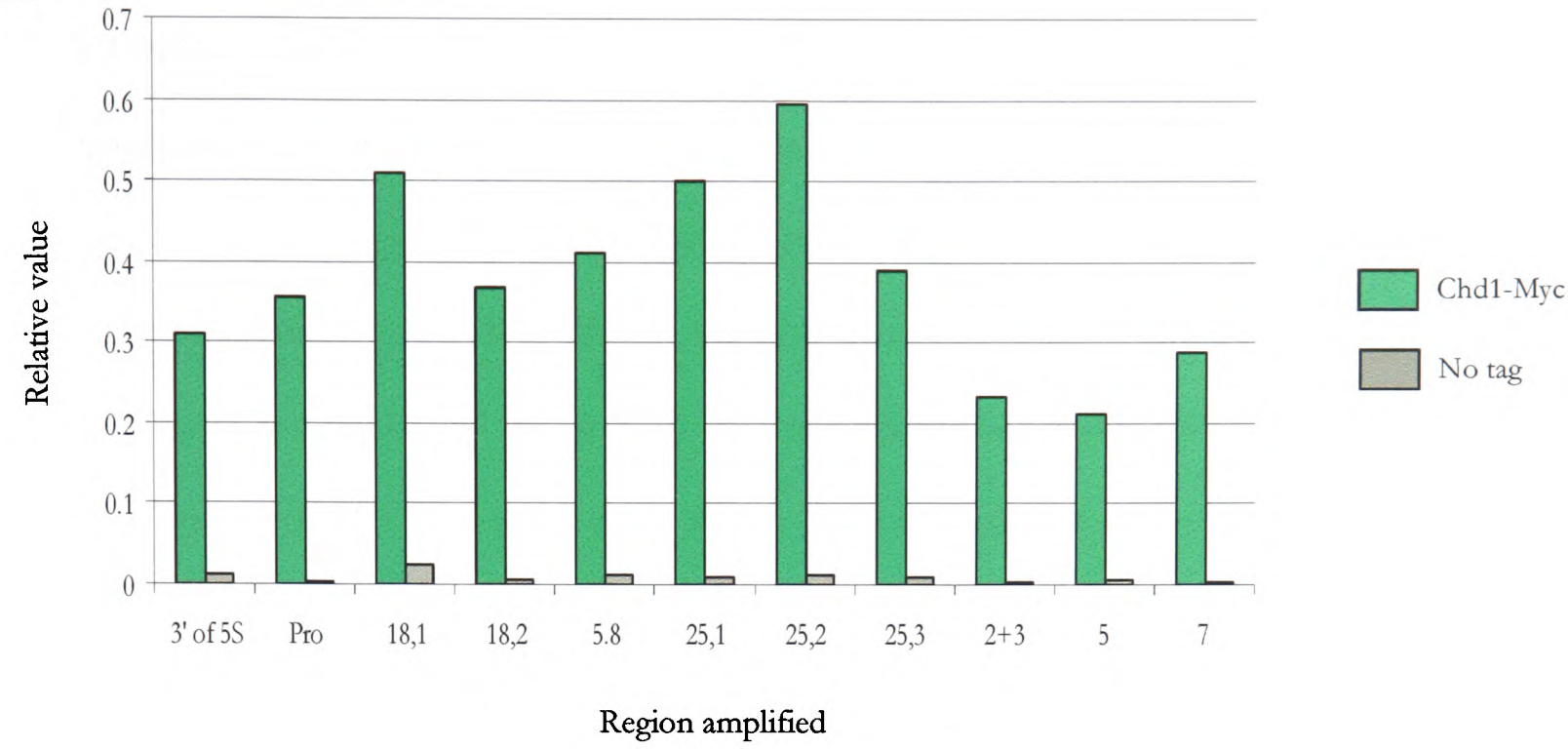
To investigate whether Chd1p is associated with the rDNA locus, ChIP analysis was performed on the strain YHT149, which carries c-Myc-tagged Chd1p (Tran et al., 2000). ChIP analysis was also carried out on a non-tagged strain, YHT117. Addition of anti-c-Myc antibody to this strain should not result in immunoprecipitation of DNA:protein complexes and will therefore determine the level of non-specific signal occurring during the ChIP analysis. As can be seen in Figure 3.1A, Chd1p is associated with the entire rDNA repeat, with the highest level of association seen over the genic regions. The non-tagged strain produces only background signals, indicating the sensitivity of both the ChIP protocol, and the interaction of Chd1p with the rDNA locus. As a further control, the association of Chd1p with Pol II loci was assessed. It has previously been shown that Chd1p is associated with the 3' end of the *CYC1* gene, consistent with its function in transcription termination at this locus (Alen et al., 2002). ChIP analysis of Chd1p association upstream of *CYC1* (*CYC1* 5'), downstream of *CYC1* (*CYC1* 3') and within the genic region of *ISY1* (*ISY1*) (see Figure 2.1C and D) was performed and the immunoprecipitated DNA analysed by real-time PCR. Note the original analysis was carried out by conventional PCR. As can be seen in Figure 3.1B, Chd1p is present across the *CYC1/ISY1* locus, with highest levels found at the 3' end of *CYC1*. These data are consistent with those obtained previously and further confirms the sensitivity of the assay. Finally, by comparing the level of Chd1p association at the rDNA locus with that seen over the 3' end of *CYC1*, it can be seen that there is 3-5-fold more Chd1p present over the genic regions of Pol I-transcribed rRNA genes than at the Pol II-transcribed *CYC1* gene (Figures 3.1A and B).

3.2 Effect of RNase A treatment on the profile of Chd1p

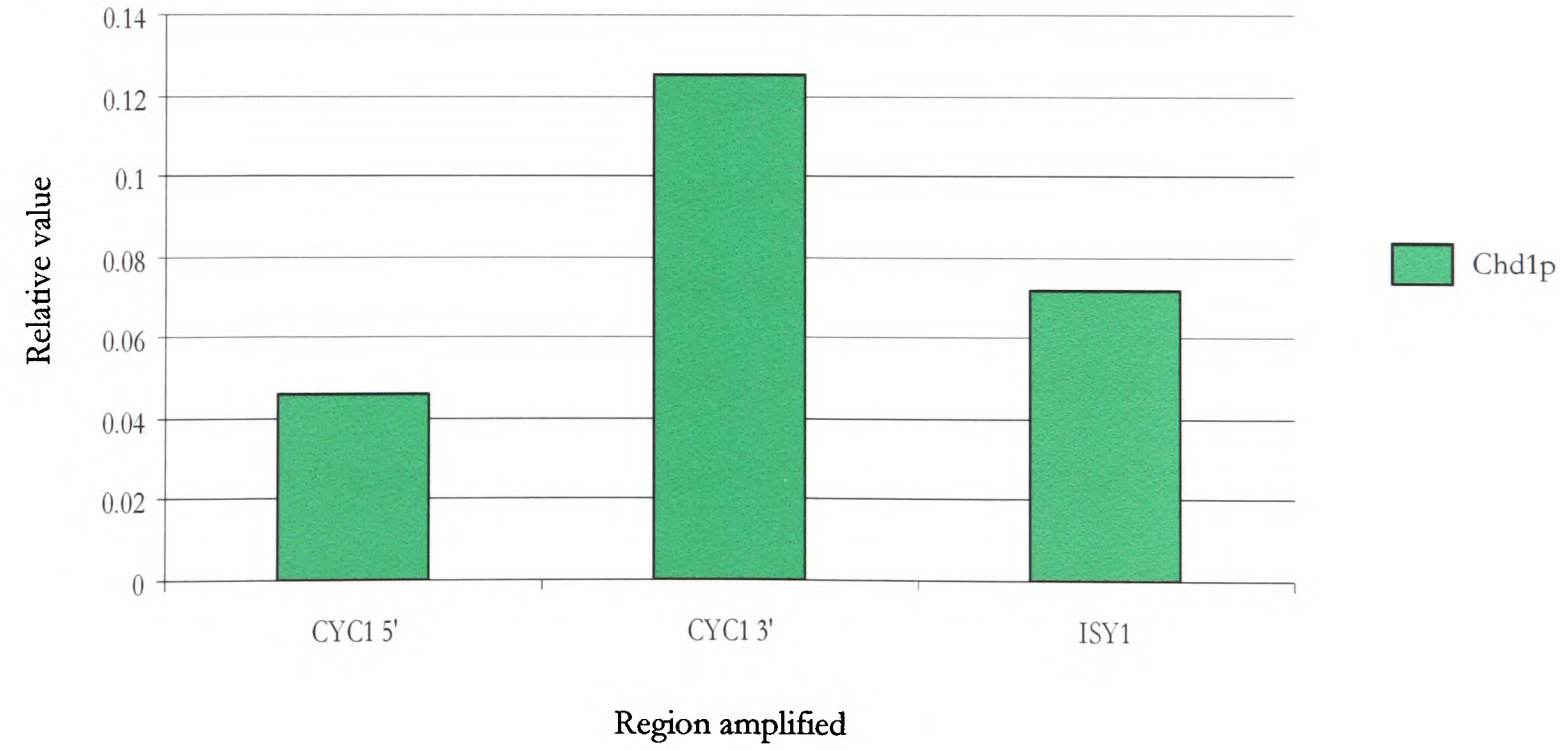
The association of Chd1p with rDNA repeats posed the question of how Chd1p is recruited to the locus. The association of Chd1p could occur through rRNA as it has been shown that

Figure 3.1: Chd1p is present across the rDNA repeat. (A) Analysis of the YHT149 strain using an anti-c-Myc antibody reveals that Chd1p is associated with the entire rDNA repeat, with highest levels seen over the genic regions. Repetition of the analysis in a non-tagged YHT117 control strain produces background signals (grey bars). (B) Analysis of Chd1p association at the Pol II-transcribed *CYC1/ISY1* locus reveals a peak of Chd1p at the 3' end of the *CYC1* gene. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis in (A) was performed three times and in (B) was performed two times. Average data sets are presented.

A



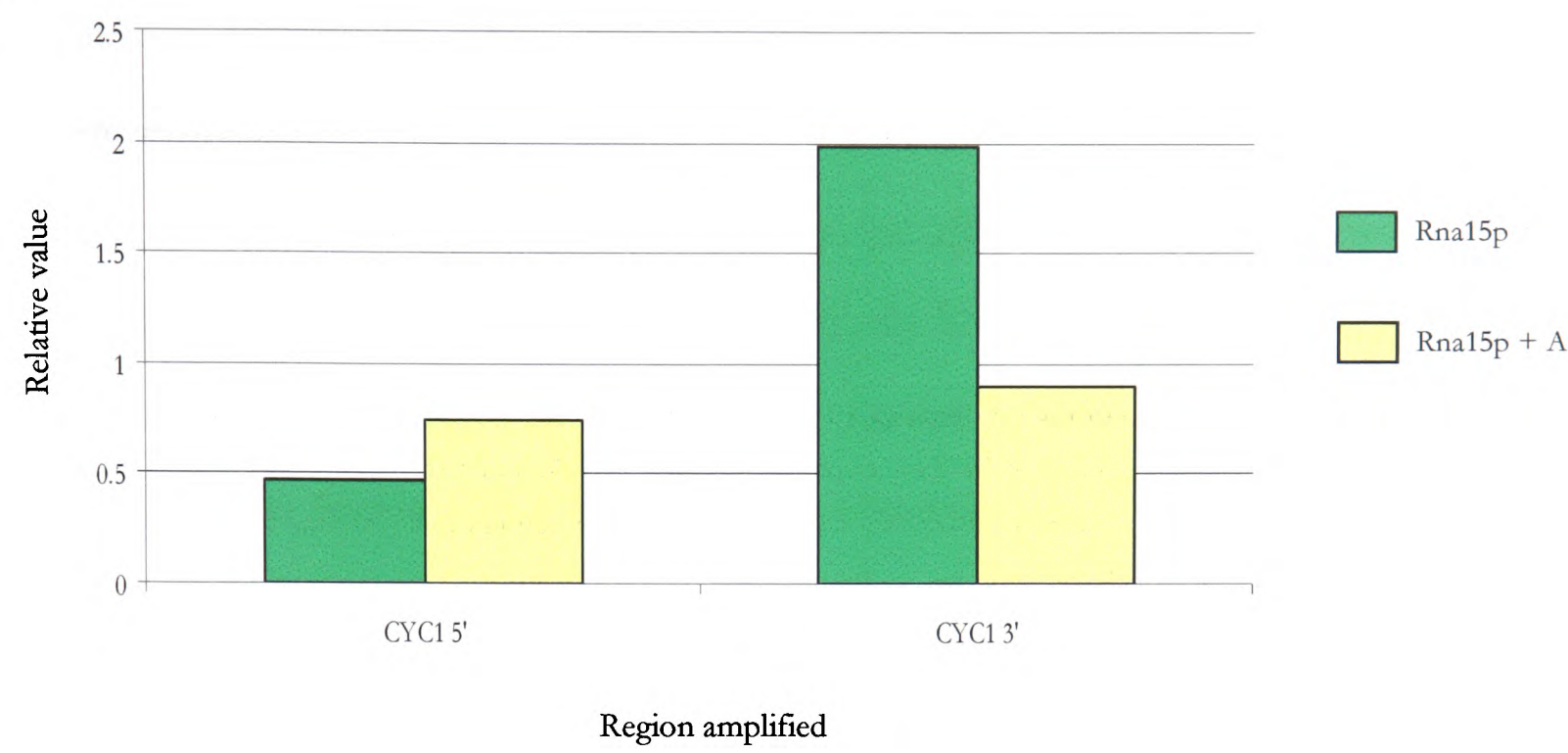
B



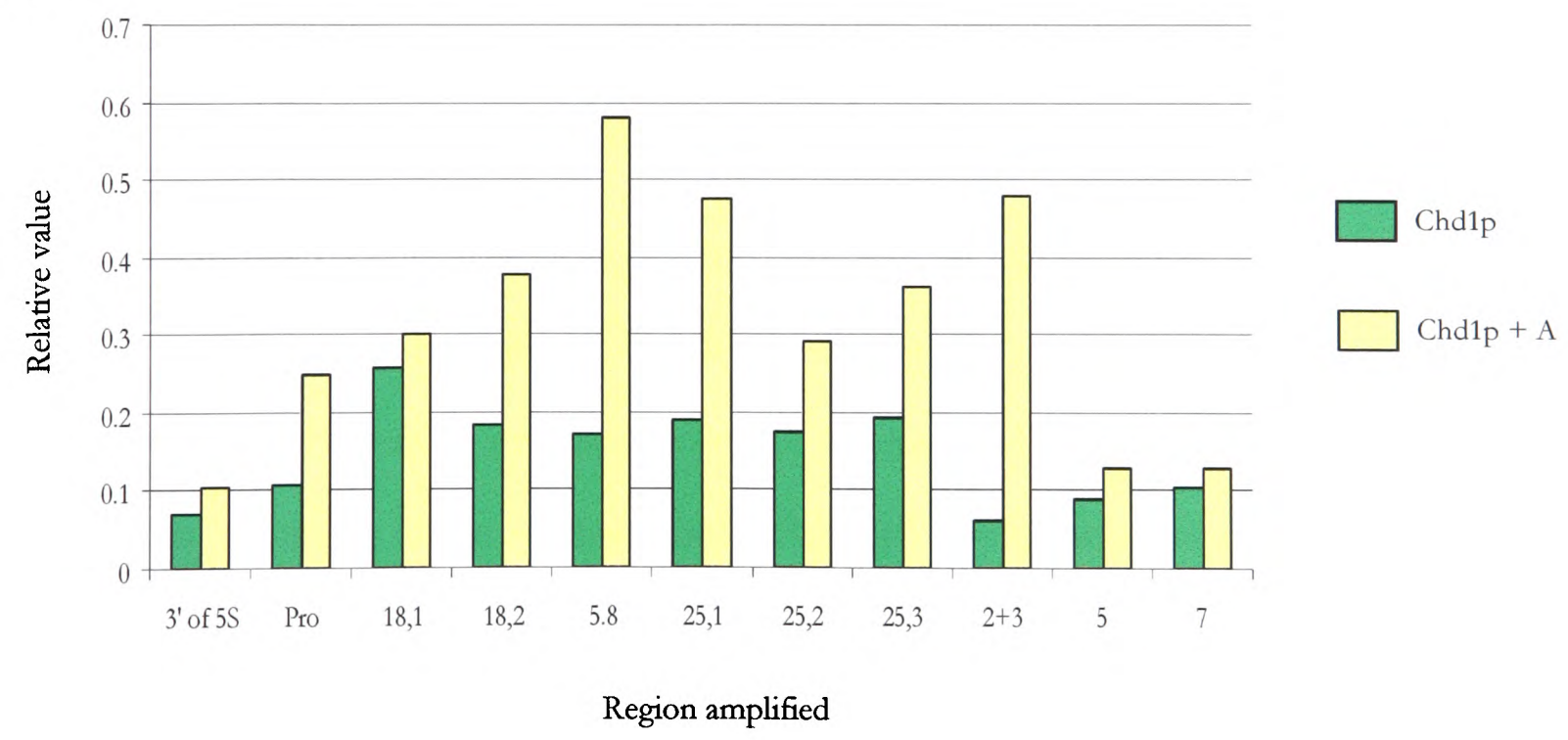
chromodomains can bind RNA (Akhtar et al., 2000) and Chd1p possesses two such domains. Alternatively, recruitment could occur through the K4 dimethylated tails of histone H3 (Pray-Grant et al., 2005). To address this issue, an RNase A treatment step was added prior to antibody addition (Abruzzi et al., 2004). If a factor associates with a given locus via RNA, a drop in signal is expected whilst signals should remain the same if the association occurs via another mechanism. The RNase A protocol was first tested using an antibody against Rna15p, a component of the cleavage and polyadenylation factor CF IA known to interact with the 3' end of *CYC1* (Kim et al., 2004). ChIP analysis was carried out in a wild-type strain (YHT117) using an antibody against Rna15p, with and without RNase A treatment. The untreated sample shows a profile with low signal upstream of *CYC1* (*CYC1* 5') and a strong signal over the 3' end of *CYC1* (*CYC1* 3') (Figure 3.2A). The region *CYC1* 3' covers the poly (A) site and thus is expected to produce a high signal for Rna15p. Comparing untreated with treated samples, it can be seen that RNase A treatment has little effect on the signal upstream of *CYC1* but causes a greater than 2-fold decrease in signal over the 3' end of *CYC1* where Rna15p is known to bind. These data indicate that the RNase A treatment is working, as association of Rna15p, a factor known to bind RNA, is impeded. To investigate the impact of loss of rRNA on Chd1p association with the rDNA locus, the ChIP analysis was repeated. Samples were split into two, with one half untreated and the other treated with RNase A, as detailed in Chapter 8: Methods. Unexpectedly, the addition of RNase A (Figure 3.2B) caused the ChIP signals to increase over the transcribed part of the rDNA repeat (Pro to 2+3), whilst the signals remain almost unchanged upstream of the promoter (3' of 5S) and over the non-transcribed spacer (5- 7). In particular, region 2+3 shows a 7-fold increase in Chd1p signal following RNase A treatment. These data are explicable by considering the availability of the epitope for interaction with the antibody. The high transcription rate of the rRNA genes causes a large amount of rRNA to be produced. This rRNA could interfere with the interaction of c-Myc-tagged Chd1p with the

Figure 3.2: Chd1p is associated with actively transcribed rDNA repeats. Treatment of ChIP samples with RNase A allows the role of RNA in the recruitment of a chromatin-associated factor to be investigated. **(A)** Rna15p, a component of the cleavage and polyadenylation factor CF IA, is associated with the 3' end of *CYC1* (green bars). Addition of RNase A prior to antibody addition confirms that RNA is required for the recruitment of Rna15p to the 3' end of *CYC1* (yellow bars) and indicates that RNase A treatment can be used to investigate the role of RNA in factor recruitment. **(B)** Analysis of c-Myc-tagged Chd1p (YHT149) in the presence (yellow bars) and absence (green bars) of RNase A. Treatment with RNase A prior to antibody addition leads to an increase in Chd1p signal over the promoter, genic and terminator regions (Pro-2+3). No increase in signal is observed upstream of the promoter (3' of 5S) or downstream of the terminator (5, 7). The increase seen is likely due to enhanced antibody binding upon clearance of RNA, indicating that Chd1p interacts with actively transcribed repeats. Representative data sets are shown. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods.

A



B



anti-c-Myc antibody. When rRNA is degraded by RNase A, the epitope becomes more accessible to the antibody, leading to an increase in signal. This hypothesis is consistent with a large increase in signal upon RNase A addition over regions pro to 2+3, whilst the signals do not increase over regions 5 and 7 because Pol I has terminated and the rRNA has been released. The increase in signal seen thus indicates that rRNA is not essential for the recruitment of Chd1p to rDNA repeats. Significantly, the RNase A treatment also allows one to determine whether Chd1p is associated with inactive or active repeats. The ChIP analysis described in Section 3.1 was performed in a wild-type strain. This strain possesses both active and inactive repeats and so the association seen in Figure 3.1A could indicate association with/function at either active or inactive repeats. The increase in signal seen upon RNase A treatment indicates that rRNA is occluding the interaction of the anti-c-Myc antibody with Chd1-Myc. The interference by rRNA thus indicates that Chd1p is associated with active repeats.

3.3 Effect of loss of Set1p and H3 K4 dimethylation on the recruitment of Chd1p to the rDNA locus

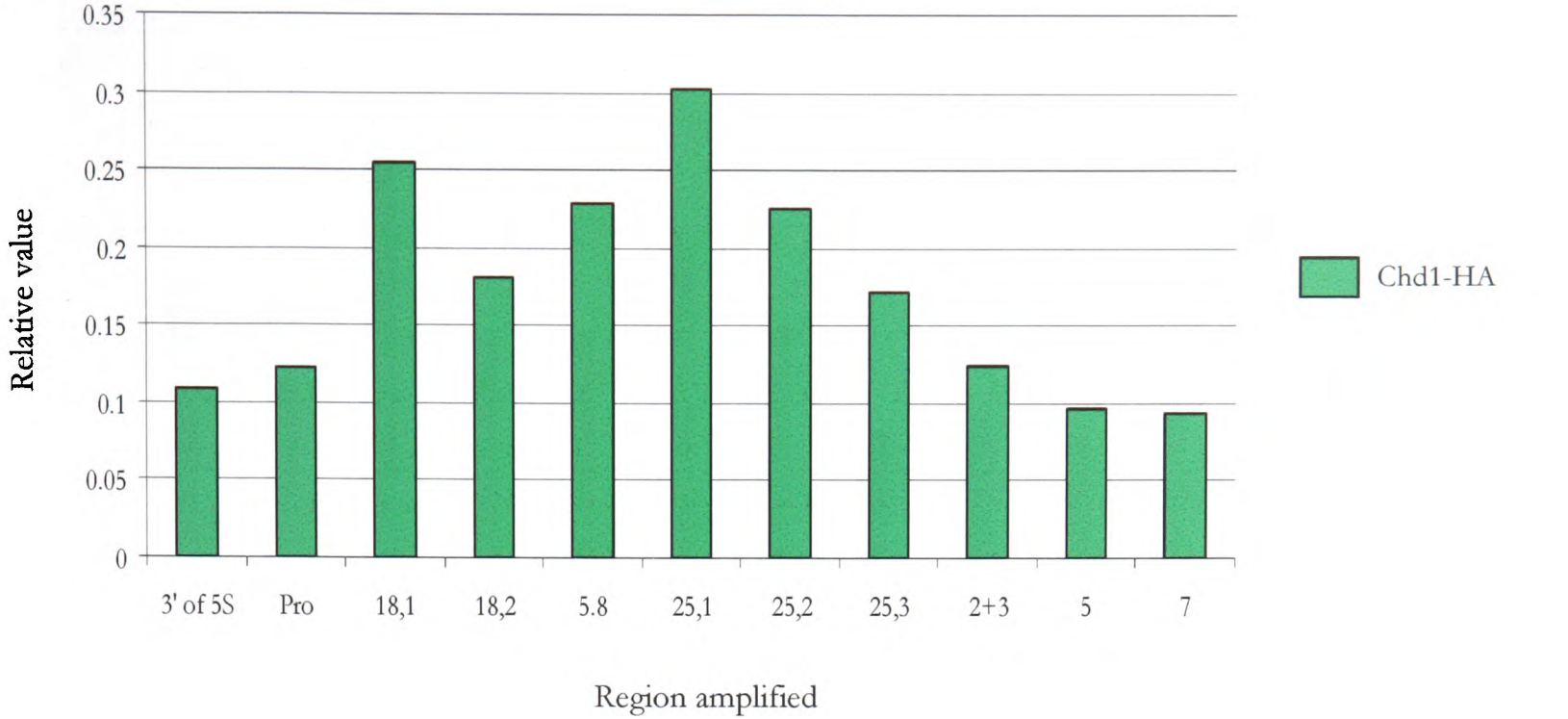
The presence of Chd1p at actively transcribed repeats and the apparent lack of interaction with rRNA posed the question as to how Chd1p is recruited to this locus. It has recently been shown that Chd1p can be recruited to H3 K4 dimethylated chromatin (Pray-Grant et al., 2005). This interaction occurs via the second of Chd1p's two chromodomains. As shown in the previous chapter, actively transcribed rDNA repeats possess H3 K4 dimethylated chromatin (Figure 2.6), thus suggesting a means by which Chd1p could be recruited to the locus. To investigate this hypothesis, ChIP analysis was carried out on a strain lacking Set1p (GHY773 CHD1-HA Δ *set1*) and compared to its isogenic wild-type (GHY773 CHD1-HA; Simic et al. 2003). Both of these strains possess HA-tagged Chd1p, allowing association of Chd1p to be analysed in the presence and absence of Set1p, by addition of anti-HA

antibody. The profile of Chd1p association in the presence of Set1p was first analysed. The profile obtained from this wild-type is very similar to that seen with the YHT149 c-Myc-tagged strain (Figure 3.3A). The profile again shows Chd1p association across the entire repeat, with signals peaking across the genic regions (18,1-25,3). It should be noted that lower signals are obtained in this case, as compared to the c-Myc-tagged YHT149 wild-type previously used (Figure 3.1A). This may suggest that GHY773 is less amenable to ChIP analysis than YHT149. However, it more likely reflects the decreased efficiency of the anti-HA antibody, compared to the anti-c-Myc antibody, in ChIP analysis. This again highlights the need to use isogenic strains in comparative ChIP analysis. The ChIP analysis was next repeated using the $\Delta set1$ strain. This strain lacks Set1p and will therefore lack H3 K4 dimethylation at the rDNA locus (see Figure 2.8). As can be seen in Figure 3.3B, loss of Set1p/H3 K4diMe does not lead to complete loss of Chd1p from the rDNA locus. Instead, Chd1p is still associated, with a profile comparable to that seen in the wild-type (note, for example, the peak in signals over the genic region). Comparing the wild-type and $\Delta set1$ data, it can be seen that whilst there is a 10-20% drop in level of Chd1p association, Chd1p nonetheless remains associated with the rDNA locus (Figure 3.3C). H3 K4 dimethylation is therefore not essential for Chd1p recruitment to the locus.

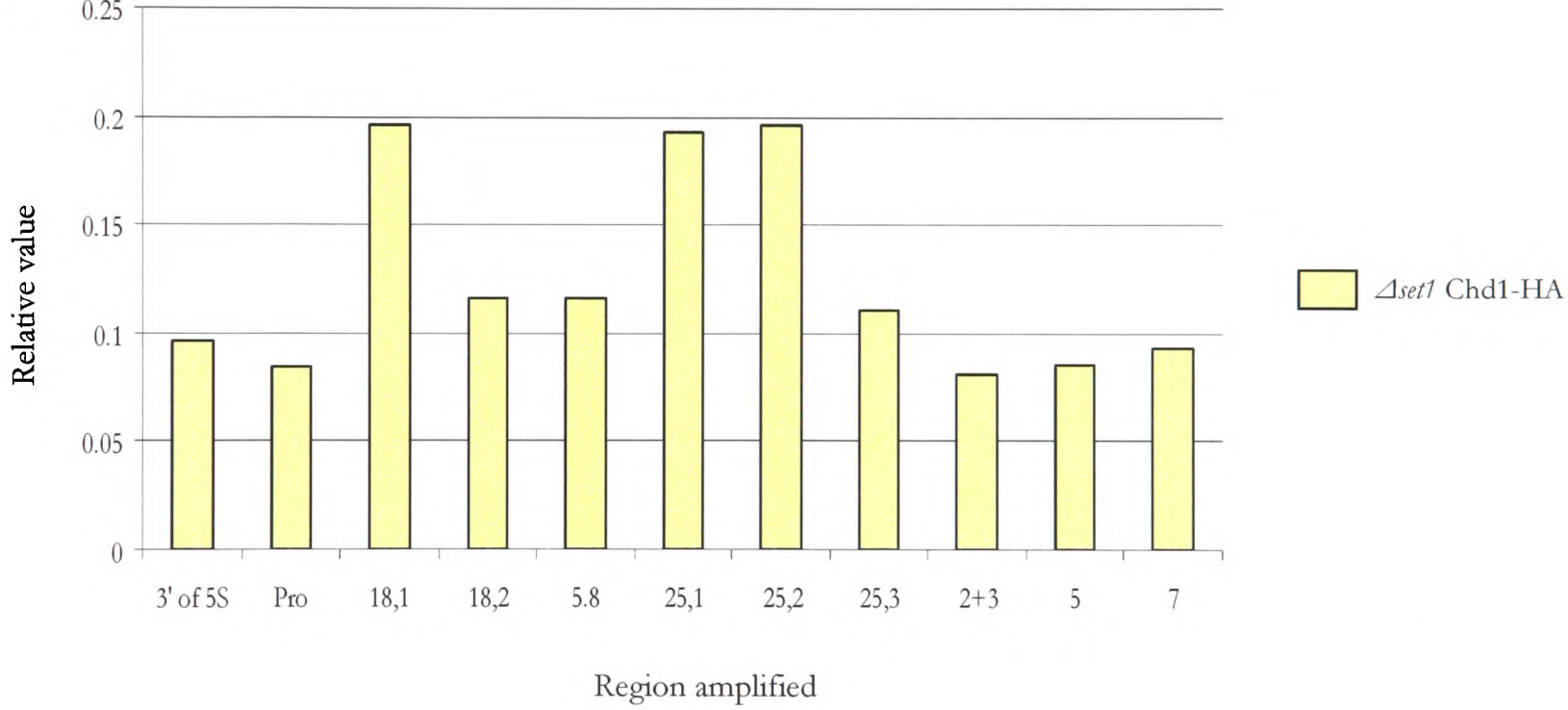
As it has been shown above that, in a wild-type background, loss of rRNA does not lead to loss of Chd1p (Figure 3.2B) and that H3 K4 dimethylation is not essential for Chd1p recruitment, it was unclear how Chd1p is recruited. To address this question, the ChIP analysis of $\Delta set1$ CHD1-HA strain was extended by addition of an RNase A treatment step. As can be seen in Figure 3.3D, addition of RNase A causes a dramatic decrease in association of Chd1p with the rDNA locus. This indicates that in the absence of H3 K4diMe tails, rRNA is involved in Chd1p recruitment. The drop in signal over regions 5 and 7 is unexpected, as the polymerase has terminated and rRNA is absent. The drop in signals seen may be due to a decreased resolution over the termination region, as this region is very

Figure 3.3: Chd1p is recruited to rDNA repeats by two independent mechanisms. (A) Profile of HA-tagged Chd1p. Analysis of Chd1p association with the rDNA repeat was performed using the GHY773 CHD1-HA wild-type strain. The profile obtained is very similar to that obtained using the c-Myc-tagged YHT149 strain (Figure 3.1A). (B) Repetition of the analysis using the GHY773 CHD1-HA $\Delta set1$ strain reveals that Chd1p is still recruited to rDNA repeats in the absence of Set1p/H3 K4 dimethylation, with only a small loss in signal seen. (C) Comparison of wild-type (Chd1-HA) and $\Delta set1$ ($\Delta set1$ Chd1-HA) strains reveals that Chd1p is still recruited to rDNA repeats in the absence of Set1p/H3 K4 dimethylation and with a profile similar to that seen for the wild-type. (D) Treatment of samples from the $\Delta set1$ CHD1-HA strain with RNase A leads to a large decrease in Chd1p recruitment to the locus. This indicates that rRNA is required for the recruitment of Chd1p to the rDNA locus when H3 K4 dimethylation is absent. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and average data sets are presented, except Figure 3.3D where a representative data set is shown.

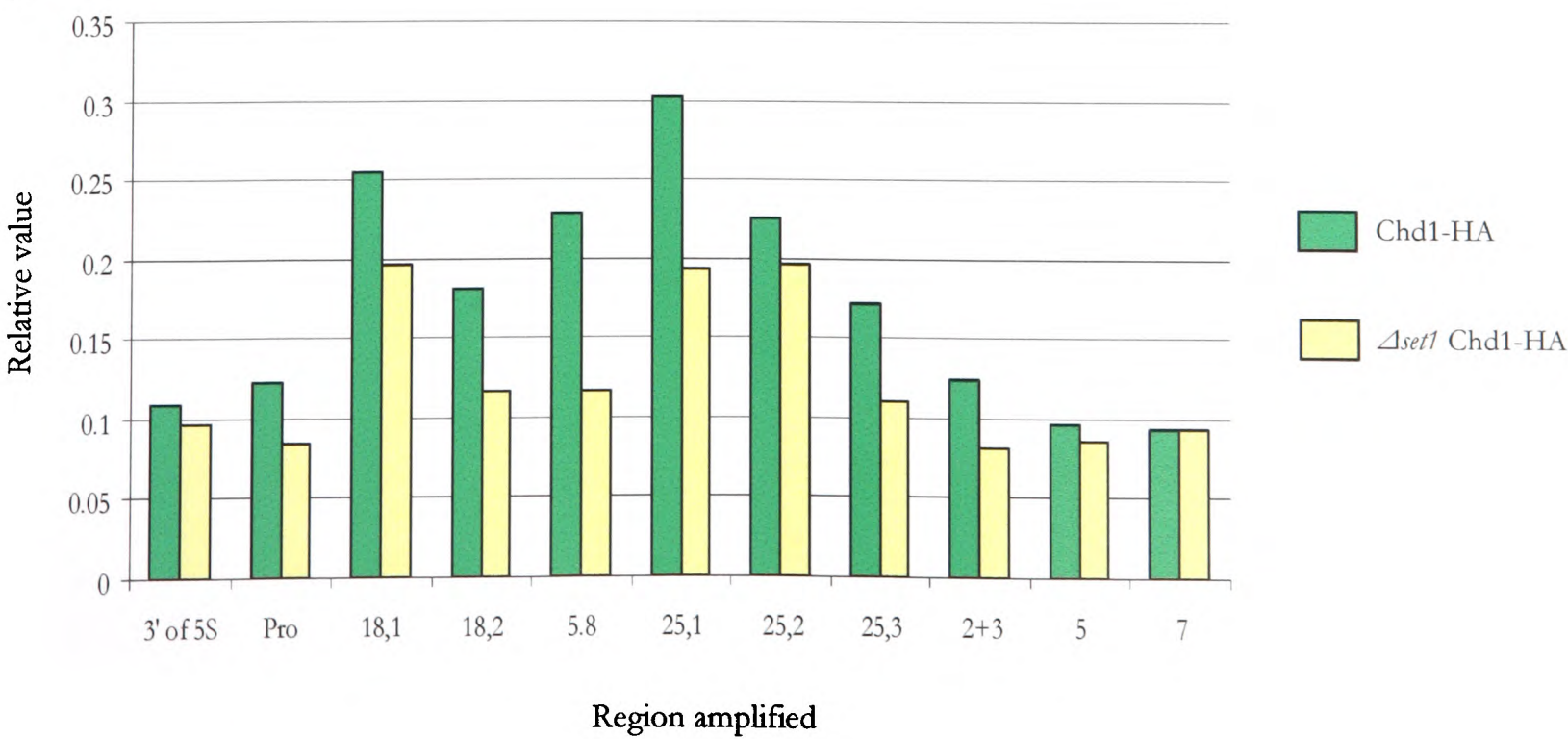
A



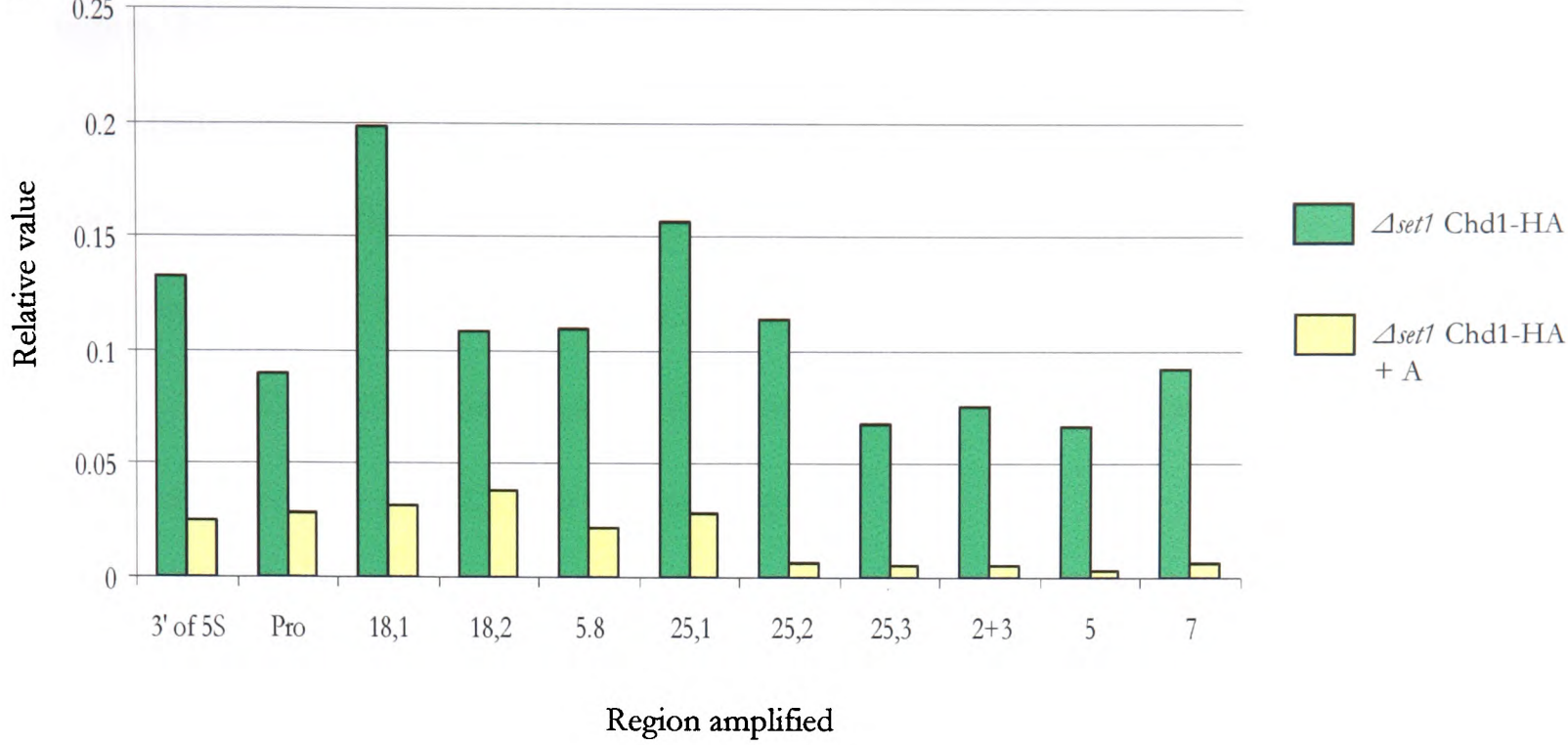
B



C



D



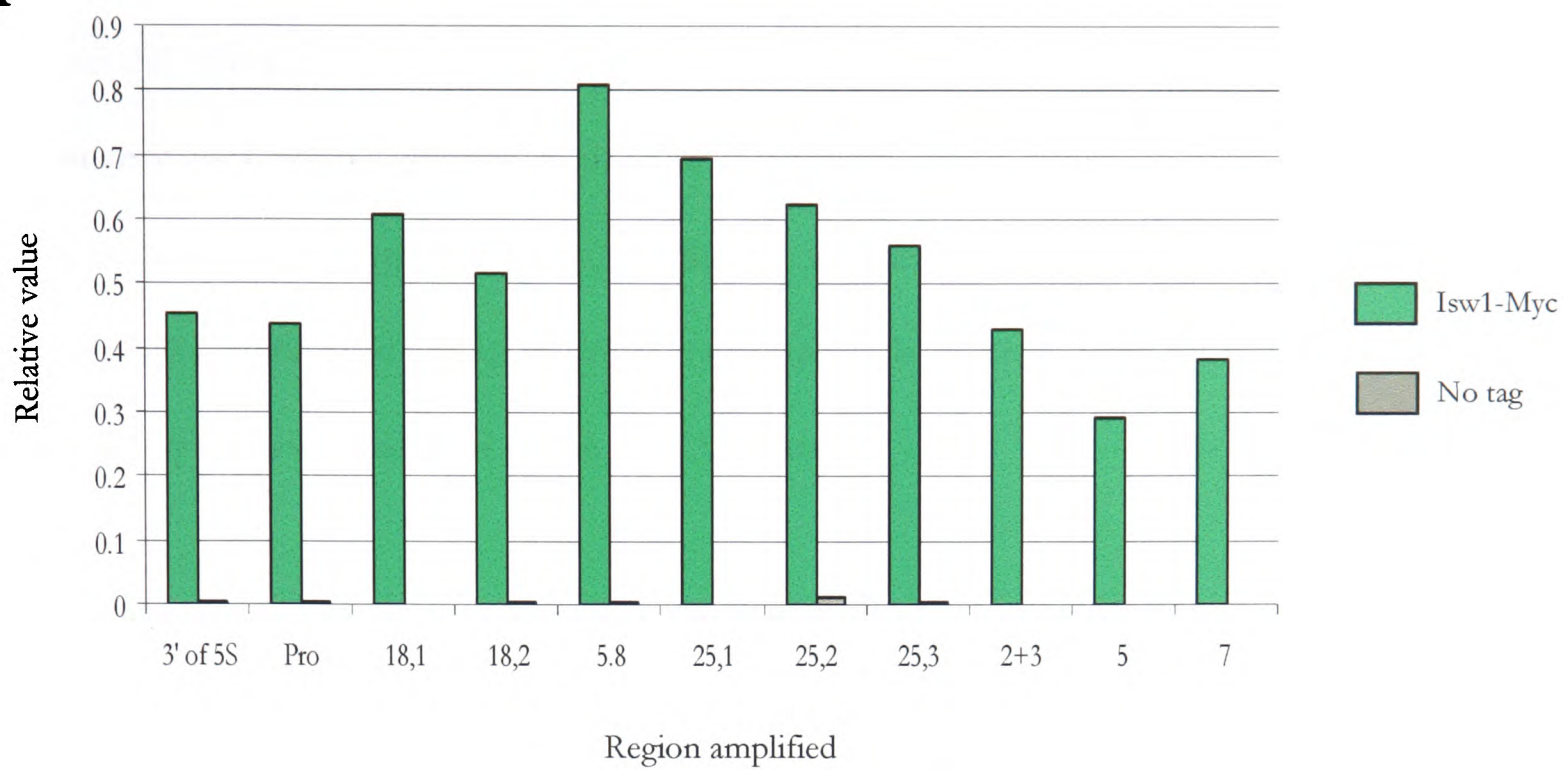
small and necessitates primer pairs being close together. Thus the drop may reflect that seen over region 2+3. Overall, the drop in Chd1p upon RNase A treatment suggests a role for rRNA in Chd1p recruitment, in addition to the proposed role of H3 K4 dimethylation. The chromodomains of Chd1p may be involved in both methods of recruitment, as interactions of chromodomains with both H3 K4diMe and RNA are known (Akhtar et al., 2000; Pray-Grant et al., 2005). It therefore appears that both H3 K4 dimethylation and rRNA are involved in the recruitment of Chd1p to active repeats.

3.4 Profile of Isw1p across the rDNA repeat

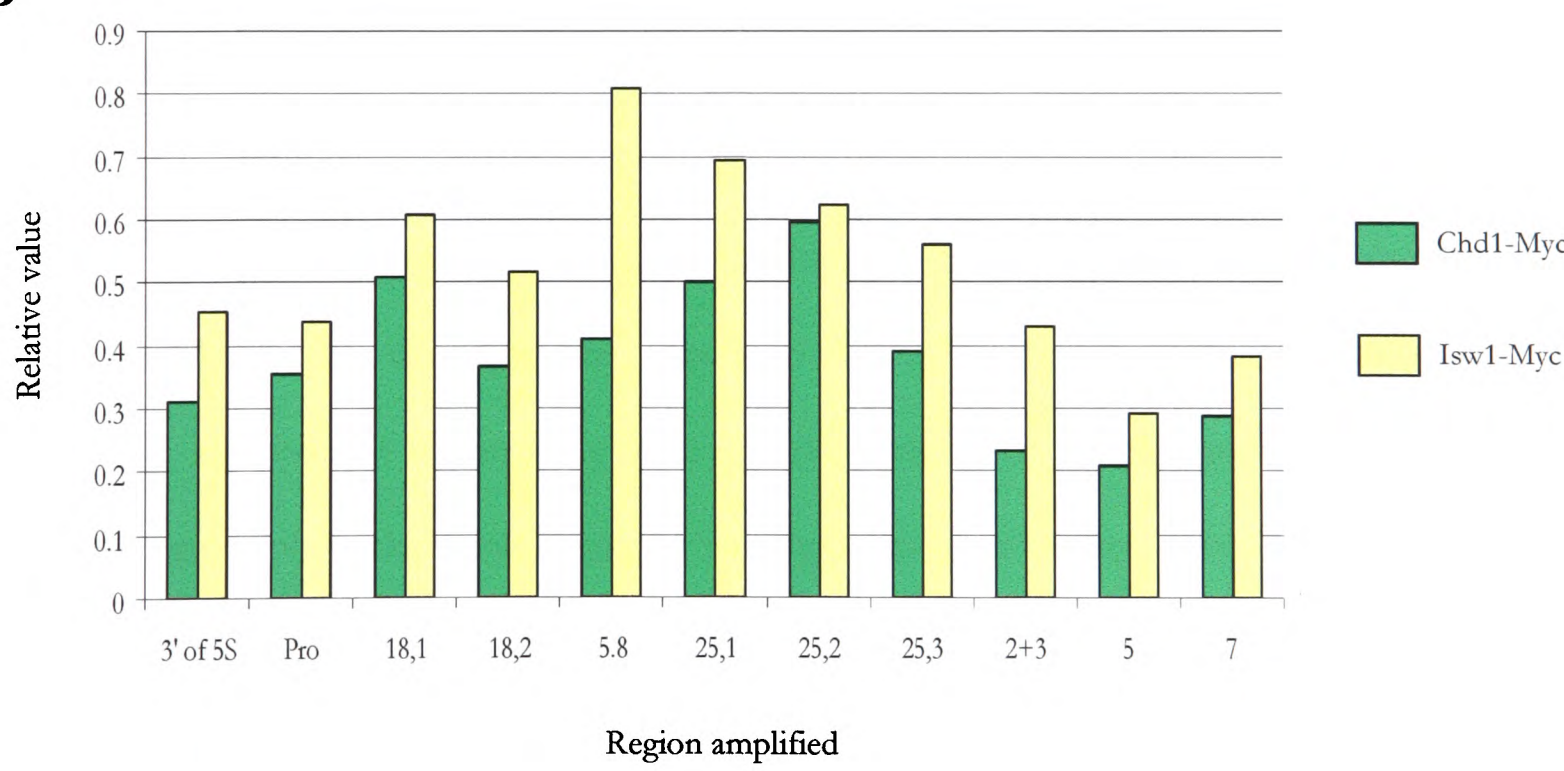
It was next of interest to investigate whether the chromatin remodeling factor Isw1p is associated with rDNA repeats. Isw1p has been shown to bind H3 K4diMe tails (Santos-Rosa et al., 2003) and interacts with Chd1p both physically and genetically (Gavin et al., 2002; Tsukiyama et al., 1999). To investigate whether it is associated with the rDNA locus, ChIP analysis was carried out on a strain carrying Myc-tagged Isw1p, Isw1-Myc (Kent et al., 2001). This analysis shows that Isw1p is associated with the entire rDNA locus. Highest signals are seen across the genic region (Figure 3.4A). The association seen is specific as a non-tagged strain gives only background signals (grey bars). Comparing the profiles of Chd1p and Isw1p, it can be seen that the profiles are very similar, both in the level of association of these factors with the rDNA locus, and in terms of the profile across the locus (Figure 3.4B). It should be noted that ChIP analysis combined with RNase A treatment was attempted on the Isw1-Myc strain. However, it was not possible to use the data obtained from real-time PCR analysis following this ChIP experiment. Immunoprecipitated samples (IP) gave very high signals, above those seen for the total (TOT) sample. However, the signals from NO (no antibody control) samples ran with those from IP samples. Such data have not been seen before and are highly unexpected. It would suggest that addition of RNase A causes very high, non-specific binding of molecules within

Figure 3.4: Isw1p is associated with the entire rDNA repeat. (A) Analysis of the Isw1-Myc strain with an anti-c-Myc antibody reveals that Isw1p is associated with the entire rDNA repeat, with highest levels seen over the genic region. Repetition of the analysis in a non-tagged CEN.PK2 control strain produces background signals (grey bars). **(B)** Comparison of the profiles of Chd1p and Isw1p reveals that their association with the rDNA locus is very similar, both in terms of level of association with the locus and with regard to their profile across the locus. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed three times and an average data set is presented.

A



B



the NO samples to the beads during the isolation of chromatin:antibody complexes. It is unclear why this happens. However, this phenomenon occurred on multiple occasions and meant that the effect of loss of rRNA on Isw1p association could not be assessed.

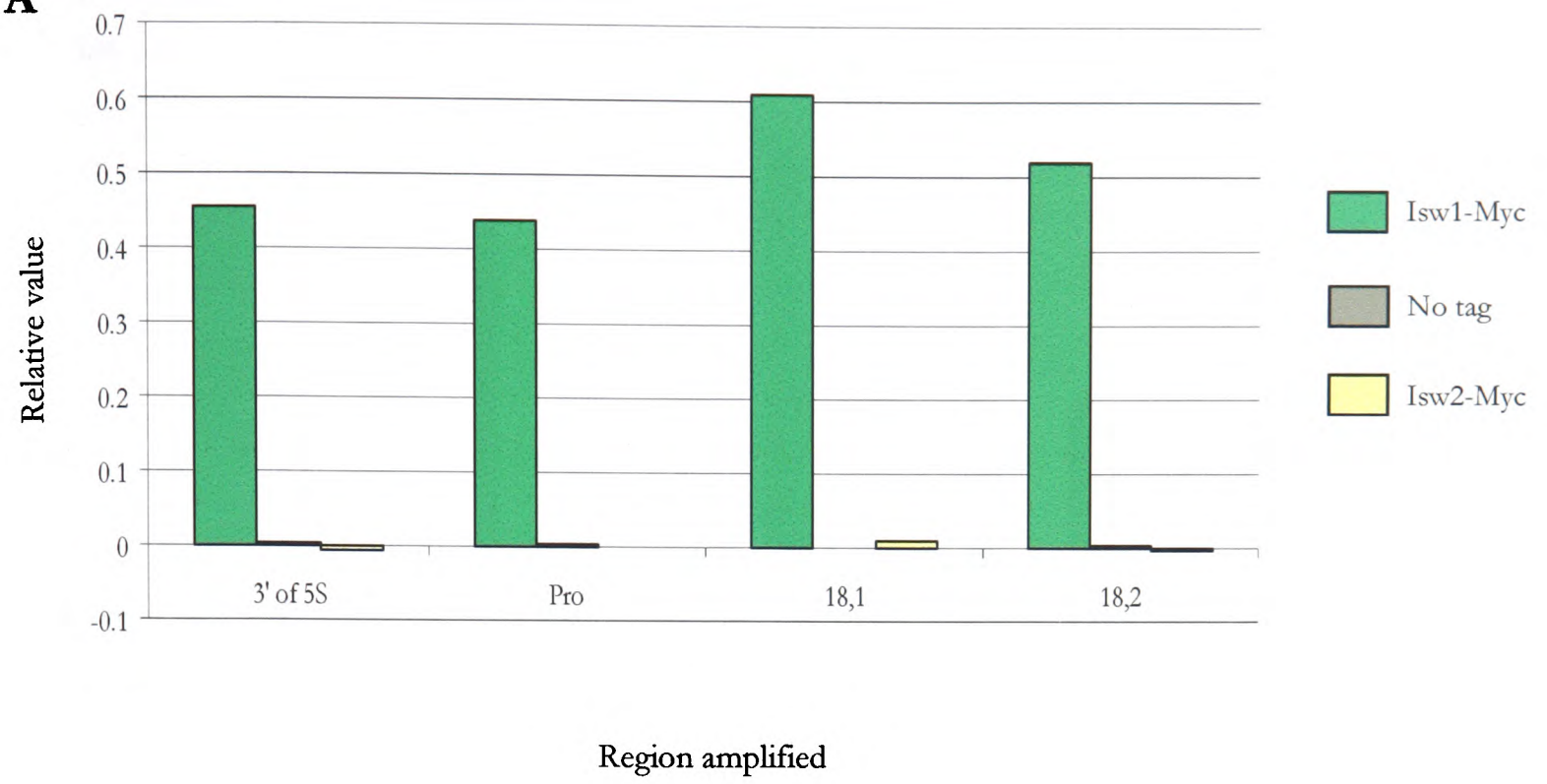
The similarity in Chd1p and Isw1p profiles described above, coupled with the known interactions between Chd1p and Isw1p, the known interactions of Isw1p with H3 K4diMe and the known functions of Isw1p in Pol II transcription is striking. Thus these data suggest that Isw1p may act with Chd1p at active rDNA repeats.

3.5 Profile of Isw2p across the rDNA repeat and effect of RNase A treatment

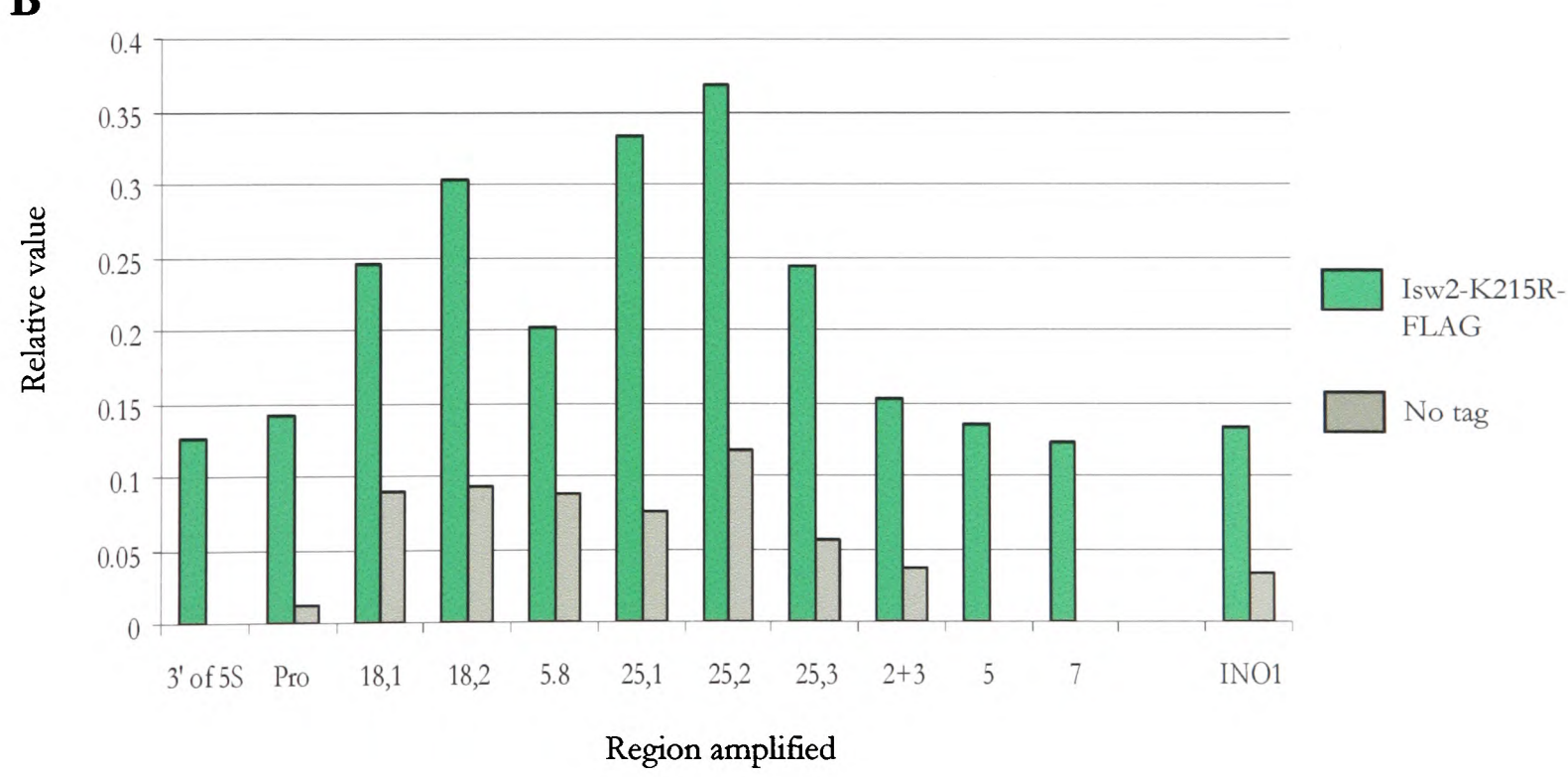
It has previously been shown that Isw2p functions redundantly with Chd1p and Isw1p at the *GAL10-7* locus to ensure efficient transcription termination. In light of the presence of both Chd1p and Isw1p at the rDNA locus, it was of interest finally to investigate whether Isw2p is also associated with this locus. ChIP analysis was initially carried out using an Isw2-Myc strain (Kent et al., 2001). The signals seen for the first four regions of the rDNA repeat probed are shown in yellow, compared to the average data for Isw1p (green) over the same regions (Figure 3.5A). It can be seen that, in comparison to Isw1p signals, Isw2p signals are at background level. This would, at first, appear to suggest that Isw2p is absent from the rDNA repeat. However, it has recently been reported that ChIP analyses performed with tagged Isw2p give only background signals. It was suggested that the lack of Isw2p detection is due to the transient nature of interaction of Isw2p with DNA. To overcome this problem, the Tsukiyama group produced a FLAG-tagged point mutant of Isw2p (Isw2-K215R-FLAG; Gelbart et al. 2005). The point mutation resides in the catalytic site of Isw2p and is thought to increase the dwelling time of the remodeling factor, thus enabling detection in ChIP analysis. This strain (YTT2164) was therefore employed in ChIP analysis in this study and compared to the non-tagged isogenic wild-type (YTT1401). By using this strain, it can

Figure 3.5: Isw2p is associated with the entire rDNA repeat. (A) Analysis of the Isw2-Myc strain with an anti-c-Myc antibody. By comparing the signals obtained for Isw2-Myc (yellow bars), Isw1-Myc (green bars) and the non-tagged CEN.PK2 control strain (grey bars) over the first four regions of the rDNA repeat probed, it can be seen that Isw2p signals are at background level. **(B)** Use of a FLAG-tagged Isw2 strain carrying a catalytic point mutation (Isw2-K215R-FLAG; YTT2164) reveals Isw2p association across the rDNA repeat, with highest levels seen over the genic region. As a positive control, a known Isw2p locus was also probed (INO1, right-hand data set), revealing association of Isw2p and confirming the use of this strain in determining Isw2p association at the rDNA locus. Repetition of the analysis in a non-tagged YTT1401 control strain produces background signals (grey bars). **(C)** Treatment of samples from the Isw2-K215R-FLAG strain with RNase A leads to a decrease in Isw2p recruitment across the entire locus, indicating a role for rRNA in the recruitment of this remodeling factor. Quantitation of all real-time PCR data was carried out as detailed in Chapter 8: Methods. ChIP analysis was performed two times in (B) and an average data set is presented, except (C) where a representative data set is shown.

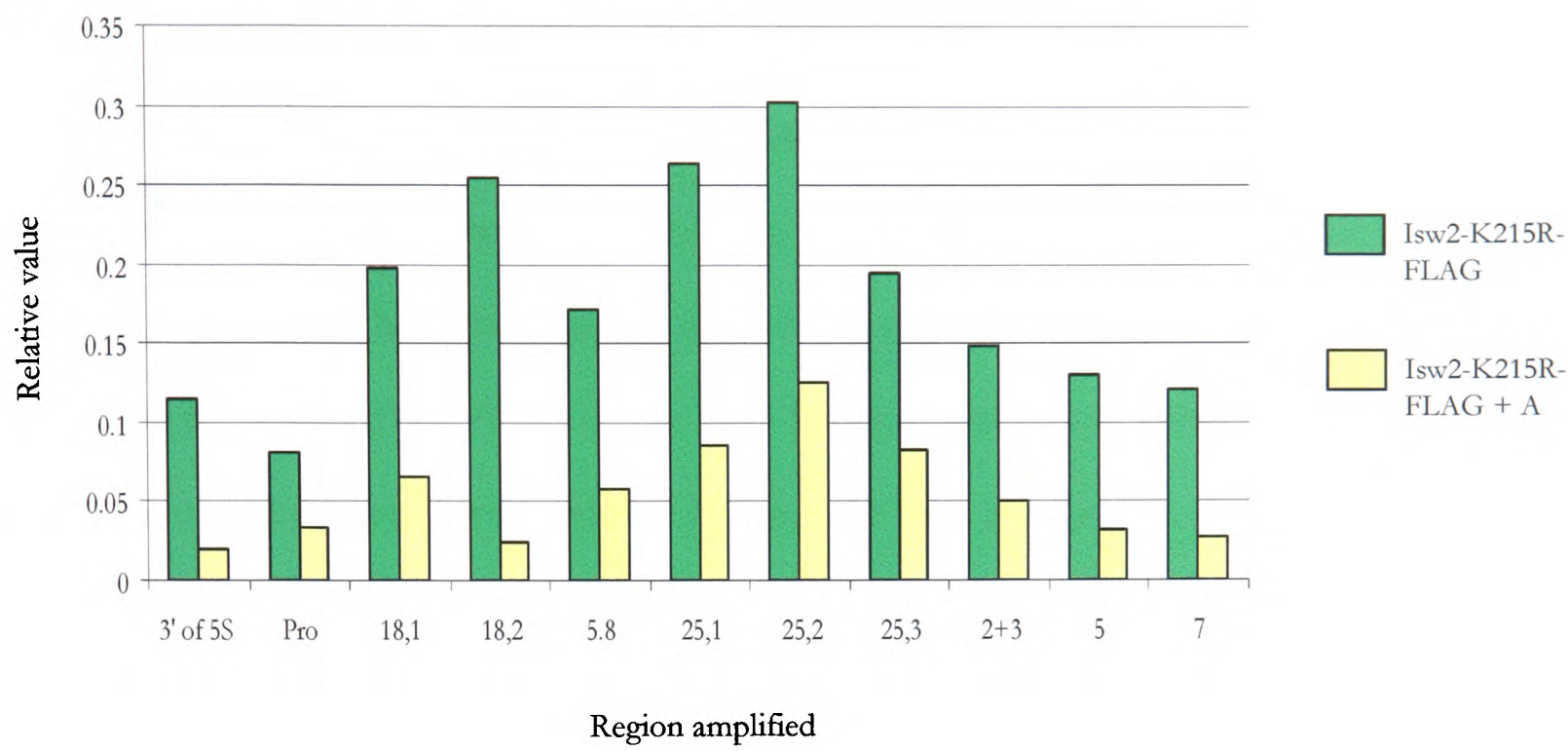
A



B



C



be seen that Isw2p is present across the rDNA repeat (Figure 3.5B). As for Chd1p and Isw1p, Isw2p shows highest levels of association across the genic regions. The non-tagged control gives much higher signals over certain regions than was seen for Chd1p and Isw1p non-tagged controls, indicating the FLAG tag produces more non-specific interactions than the c-Myc tag. Taking this higher level of non-specific signals into account, it can be seen that Isw2p is present at the rDNA locus at lower levels than Chd1p and Isw1p. To further confirm that the Isw2-K215R-FLAG can be used to detect Isw2p association with DNA, a further region of the genome was analysed. Primer pairs were designed to amplify a region upstream of the *INO1* gene (Figure 2.1D and Methods). It has previously been shown that Isw2p is recruited to the *INO1* locus through its interaction with Ume6p (Goldmark et al., 2000). Primer pairs were designed to span the Isw2p binding site, acting as a positive control for Isw2p association. Considering Figure 3.5B again, the right-hand data set shows association of Isw2p at the *INO1* locus. The binding is specific (four-fold enrichment over background) and indicates that Isw2-K215R-FLAG can be used to probe for association of Isw2p at the rDNA locus.

As discussed above in Section 3.2, RNase A treatment of ChIP samples can be used to investigate whether a given factor associates with active or inactive repeats. Isw2-K215R-FLAG ChIP samples were therefore treated with RNase A to investigate this question. As can be seen in Figure 3.5C, treatment with RNase A leads to a decrease in Isw2p signal over all regions analysed, with on average a 4-fold decrease seen. The decrease in signal upon loss of rRNA would suggest that either Isw2p interacts with rRNA at the rDNA locus, or that Isw2p is recruited by another factor, whose recruitment is affected by loss of rRNA. Apart from the catalytic ATPase subunit, Isw2p possesses a SANT domain which is thought to interact the histone tails (Boyer et al., 2004). This domain is not thought to interact with RNA. Thus it may be more likely that Isw2p is recruited by an unknown factor. The recruitment of Isw2p by Ume6p is seen at the *INO1* locus (Kent et al., 2001) and thus it

would not be surprising if Isw2p were recruited via another factor to the rDNA locus. Thus at present, it is unclear how Isw2p is recruited. However, the drop in recruitment seen upon loss of rRNA would suggest that Isw2p is recruited to active rDNA repeats.

3.6 Discussion

The presence of (modified) histones on actively transcribed rDNA repeats, coupled with previous studies detailing the role of chromatin remodeling factors in Pol II transcription, suggested that chromatin remodeling factors may also play a role in transcription by Pol I. The aim of this chapter was therefore to investigate whether chromatin remodeling factors are associated with rDNA repeats. As the remodeling factors Chd1p, Isw1p and Isw2p are implicated in Pol II transcription, this research focussed on these three factors.

Chromatin remodeling factors act to aid the passage of Pol II through its chromatin template. It was therefore of interest to determine whether these factors also act during Pol I transcription. ChIP analysis of a c-Myc-tagged Chd1p strain showed that Chd1p is present across the entire rDNA locus, with the highest signals seen over the genic region (Figure 3.1A). Addition of RNase A led to an increase in signal, indicating that rRNA impedes the interaction of the anti-c-Myc antibody with the c-Myc tag on Chd1p (Figure 3.2B). This allows two conclusions to be drawn: rRNA is not essential for Chd1p recruitment under wild-type conditions; and that Chd1p is associated with active repeats. The presence of Chd1p at high levels across all regions probed would suggest that Chd1p is involved in all stages of transcription by Pol I. A number of studies have shown roles for Chd1p in both elongation and termination of transcription by Pol II (Alen et al., 2002; Krogan et al., 2002b; Simic et al., 2003). The presence of this remodeling factor across the entire repeat thus parallels the known roles in Pol II transcription and highlights further similarities between Pol I and Pol II transcription.

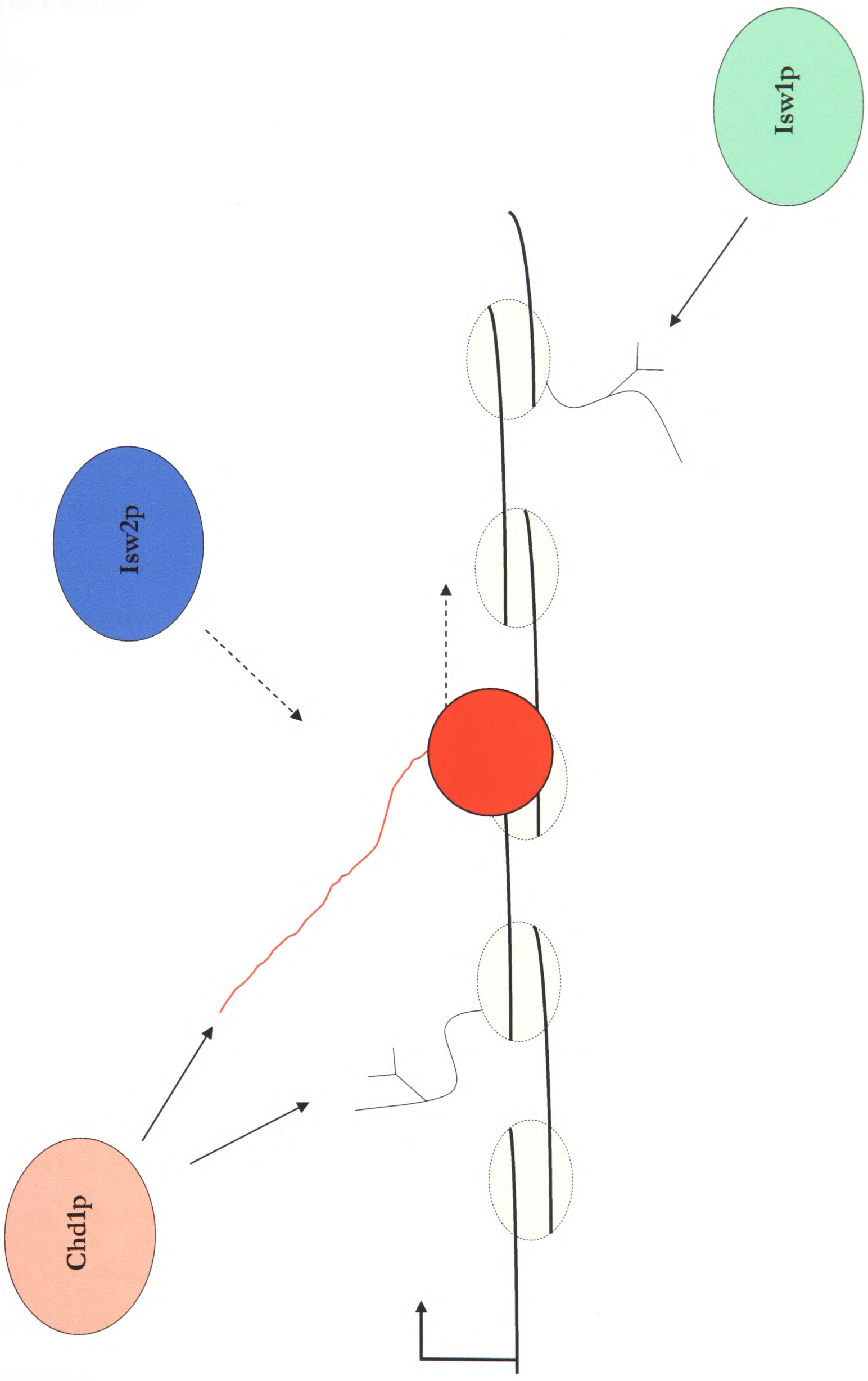
To further address the question of Chd1p recruitment to Pol I chromatin, the impact of loss of Set1p/H3 K4diMe on the association of Chd1p with the rDNA locus was studied. It has been shown that Chd1p can interact with dimethylated K4 residues via its chromodomain (Pray-Grant et al., 2005), suggesting a mechanism of recruitment. Loss of H3 K4diMe however did not lead to complete loss of Chd1p (Figure 3.3C). The observed small drop in signal seen suggests that H3 K4 dimethylation is involved in recruitment of Chd1p, but is not essential. To investigate how Chd1p is still recruited at high levels in the absence of H3 K4 dimethylation, an RNase A treatment step was also included in the ChIP analysis (Figure 3.3D). Unexpectedly, loss of rRNA leads to almost complete loss of Chd1p from the rDNA locus, indicating that rRNA plays a role in Chd1p recruitment in the $\Delta set1$ strain. Thus there appear to be two independent mechanisms of Chd1p recruitment. In a wild-type strain, H3 K4 dimethylation is present across active repeats and may serve to recruit Chd1p (as shown by the continued presence of Chd1p at the rDNA locus in a wild-type strain treated with RNase A, Figure 3.2B). If H3 K4 dimethylation at the rDNA locus is lost however, rRNA becomes involved in Chd1p recruitment, as evidenced by the loss of Chd1p from the locus in the $\Delta set1$ /RNase A experiment (Figure 3.3D). It should also be noted that Chd1p is thought to interact with histone H3 dimethylated at K36 (Hampsey and Reinberg, 2003). This histone mark is present on actively transcribed rDNA (Chapter 2) and could therefore serve as an additional mechanism of Chd1p recruitment. To address this issue, Chd1p would need to be tagged in a $\Delta set2$ background and ChIP analysis performed.

The association of Chd1p with actively transcribed rDNA repeats suggests a role for chromatin remodeling factors in Pol I transcription. The analysis was therefore extended to Isw1p and Isw2p. It was found that both Isw1p and Isw2p are present across the rDNA locus (Figures 3.4 and 3.5). It was not possible to study the effect of RNase A treatment on Isw1p recruitment. This analysis was however possible for Isw2p and it was found that loss

of rRNA leads to loss of Isw2p from the locus. This suggests that rRNA is involved either in the recruitment of Isw2p itself, or that Isw2p is recruited by an additional factor which is sensitive to the loss of rRNA. All three remodeling factor profiles show similarities, with peaks of signal seen over the genic regions. This accumulation of chromatin remodeling factors may indicate their role in facilitating efficient transcription elongation, possibly by remodeling or chaperoning histones during the passage of Pol I. A second, not mutually exclusive, theory is that the accumulation of these factors over the genic region represents loading of the factors onto rDNA to facilitate efficient termination of transcription. This could occur by the remodeling of nucleosomes into a structure that prohibits progression of Pol I downstream. Overall, the presence of remodeling factors across the entire rDNA repeat implies a role for these factors at all stages of transcription.

The results of this chapter are summarised in Figure 3.6. Chromatin remodeling factors Chd1p, Isw1p and Isw2p are recruited to rDNA repeats, with all three factors thought to act during transcription of active repeats. Recruitment of Chd1p occurs by two independent mechanisms. The first mechanism involves the recruitment of Chd1p by histone H3 tails dimethylated at K4; a mechanism that is likely facilitated by one or both of the chromodomains of Chd1p. In the absence of this mark, recruitment can still occur through the rRNA, possibly again facilitated by the chromodomains. The association of Isw1p with the rDNA locus parallels that of Chd1p, suggesting recruitment of Isw1p to active rDNA repeats. This association may be facilitated by the interaction of Isw1p with H3 K4diMe tails. Finally, Isw2p is recruited to rDNA by a mechanism involving rRNA. Whilst Isw2p may directly interact with rRNA to facilitate its recruitment, it appears more likely that Isw2p is recruited by an unidentified factor.

Figure 3.6: Updated model of involvement of chromatin remodeling factors in Pol I transcription. An active rDNA repeat is shown. The black lines represent actively transcribed rDNA organised into nucleosome-like structures composed of, at least, histones H2B and H3 (circles surrounded by dotted lines). The start site of transcription is indicated by the black arrow. The black wavy lines extending from the dotted circles represent the N-terminal histone tails. Black forked structures represent dimethylation of histone tails. Chd1p, Isw1p and Isw2p are recruited to rDNA repeats, with all factors thought to act at active repeats. Chd1p (pink circle) is recruited to active rDNA repeats by two independent mechanisms. The first involves the recruitment of Chd1p via histone H3 tails dimethylated at K4 (black forks). In the absence of K4 dimethylation, recruitment is facilitated by rRNA (red line). Isw1p (green circle) is recruited to rDNA repeats with a profile very similar to that seen for Chd1p, suggesting recruitment of this factor to active rDNA. The known interaction of Isw1p with histone H3 K4 dimethylated tails suggests a mechanism for the recruitment of this factor. Isw2p (blue circle) is recruited to rDNA repeats by a mechanism, possibly involving an unidentified factor, that is sensitive to rRNA (dashed arrow).



Chapter 4: Functional studies of chromatin remodeling factors

Preface

The presence of core histones H2B and H3 across the rDNA locus, combined with the presence of modified histone H3 suggested that chromatin remodeling factors may act at this locus. This was investigated in the previous chapter and it was found that Chd1p, Isw1p and Isw2p are present across the rDNA locus. It was next of interest to investigate the possible functions of these remodeling factors at the rDNA locus. The aim of this chapter was therefore to use the technique of *in vivo* chromatin analysis to investigate chromatin structure over the rDNA locus. The analysis was first performed in wild-type and all-active strains to investigate potential changes in chromatin structure during active transcription. To study the effects of remodeling factors, the analysis was then performed in both wild-type and mutant strains that lack one or more of the remodeling factors.

4.1 *In vivo* chromatin analysis

This technique analyses chromatin structure by studying micrococcal nuclease (MNase) cleavage patterns (Nedospasov and Georgiev, 1980; Wu, 1980), visualised by indirect end-labelling (Livingstone-Zatchej and Thoma, 1999; Wu, 1989). MNase cuts double stranded DNA within the linker or non-nucleosomal sequences, leading to the characteristic ladder pattern seen for chromatin.

Yeast cells are sphaeroplasted by treatment with yeast lytic enzyme. Following this, chromatin is digested by addition of MNase. The fragments produced are digested to completion by a chosen restriction enzyme, size-fractionated by agarose gel and blotted onto a membrane. The membrane is then probed using a radioactive end-label (Figure 4.1A). This end-label is a small piece of DNA that abuts the restriction site. Hybridisation of the radioactive end-label to the membrane-bound fragments thus maps the location of MNase cleavage relative to the chosen restriction site (Figure 4.1B).

Figure 4.1A: Schematic of the *in vivo* chromatin analysis protocol. 100ml cultures were grown overnight and used as described in the schematic. For a more detailed description of the protocol, see Chapter 8: Methods.

A



Harvest *S. cerevisiae* cells



Sphaeroplast with yeast lytic enzyme



Digest with MNase, purify chromatin fragments
and run on agarose gel



Southern blot to a membrane overnight



Random primer end-label



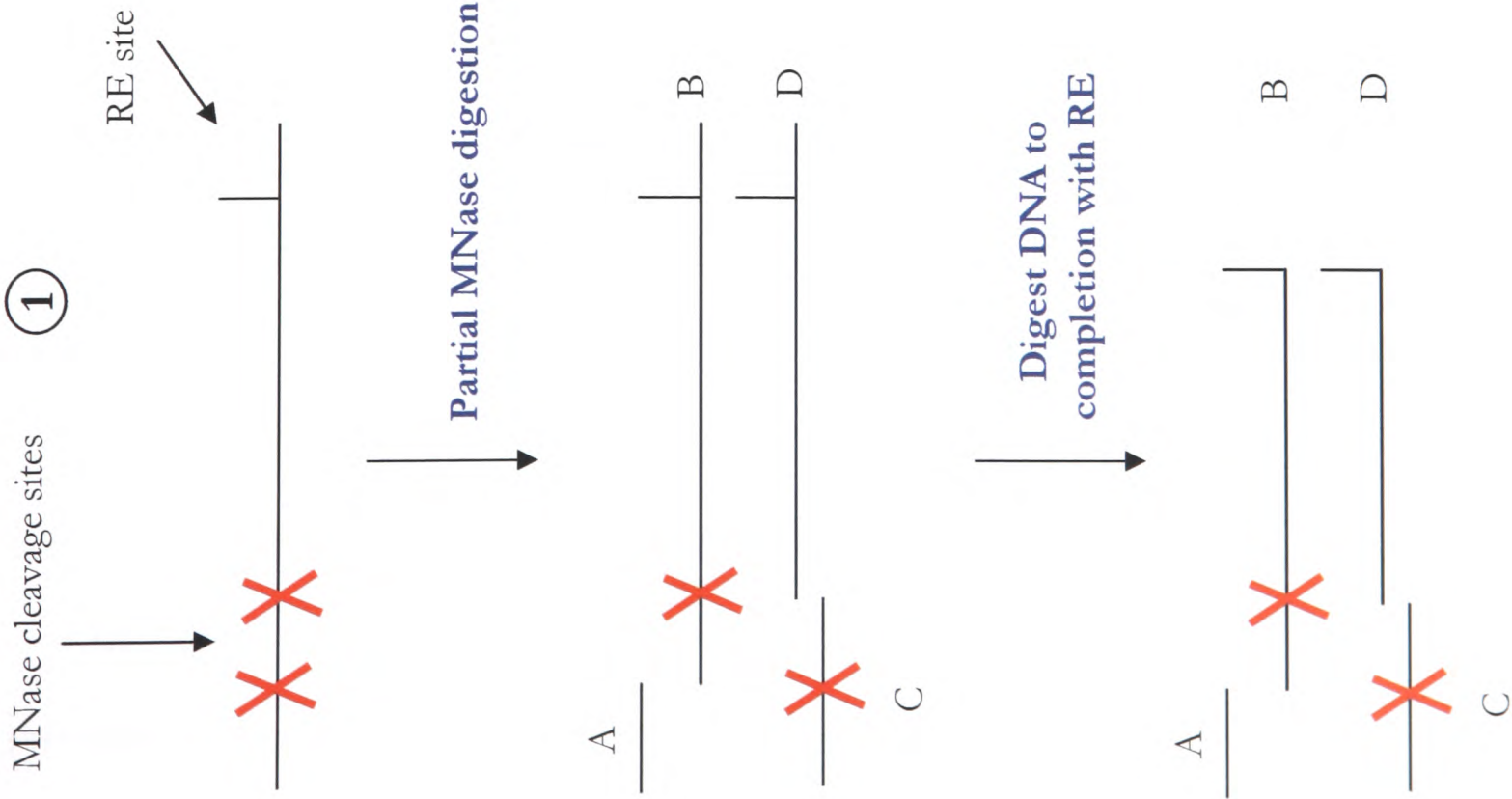
Hybridise end-label to membrane



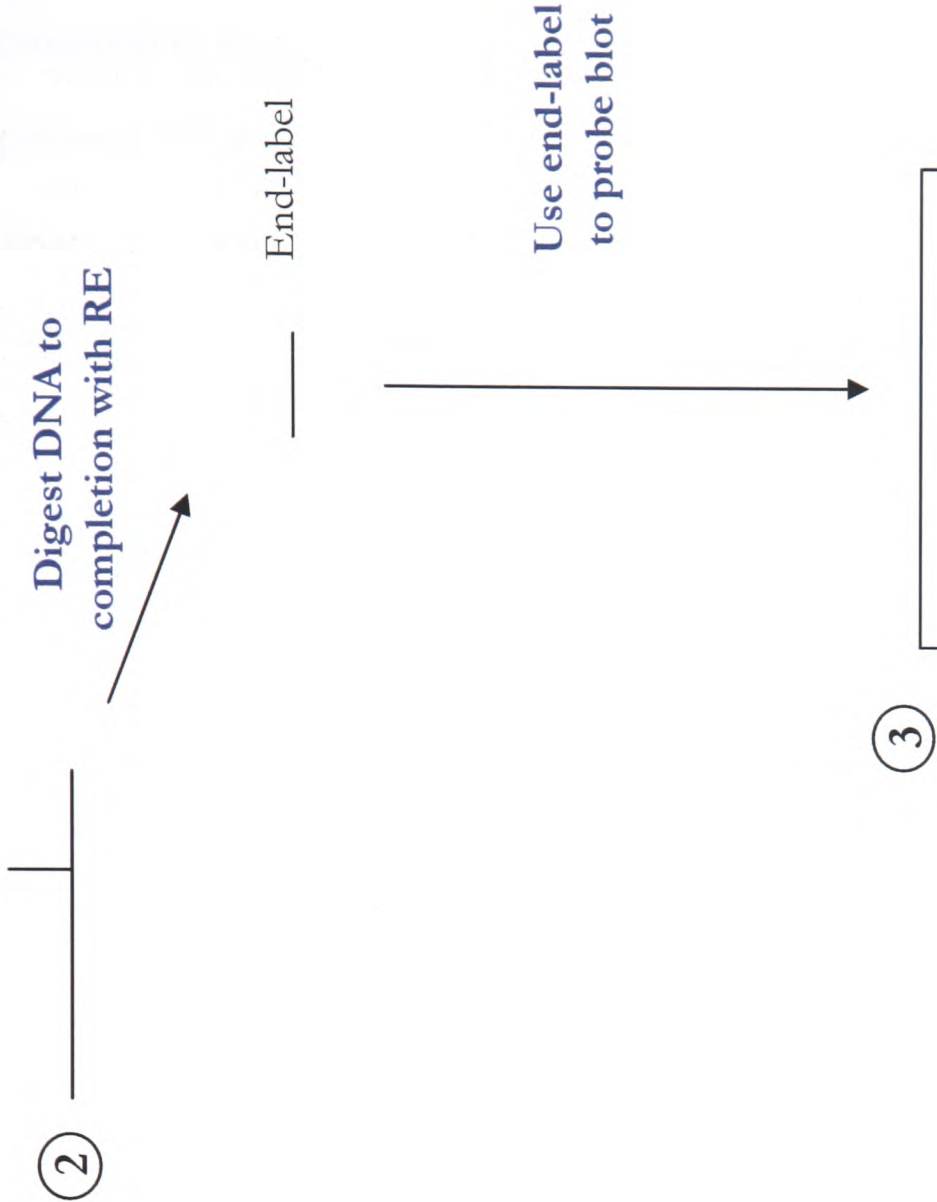
Wash membrane and expose to film

Figure 4.1B: Further schematic of the *in vivo* chromatin analysis protocol. (1) The black line represents chromatin containing micrococcal nuclease (MNase, red crosses) and a restriction enzyme (RE) cleavage site. Cleavage with MNase produces a series of fragments which are isolated and digested to completion with the restriction enzyme. Fragments are run on an agarose gel. (2) A DNA fragment, containing the restriction site, is produced by PCR. Digestion with the restriction enzyme produces two unequal sized fragments. The smaller fragment is gel purified and (3) used as an end-label to probe the blot. Hybridisation of the end-label to the membrane-bound fragments reveals a ladder of fragments and maps the location of MNase cleavage relative to the chosen restriction enzyme site. Protection of ~146bp region suggests the presence of a nucleosome.

B



Run digested fragments on gel



In the analyses described below, the MNase cleavage patterns in all-active and mutant strains are compared to those obtained in wild-type strains. In addition, all patterns are compared to the pattern of cleavage seen in naked DNA. This naked control is carried out because of the preference of MNase for AT-rich sequences. Comparison of naked and wild-type chromatin cleavage patterns should therefore reveal the protection of sites from cleavage by nucleosomes. This technique has been used to show both the presence of chromatin structure at Pol II-transcribed loci and the dependence of these structures on chromatin remodeling factors (Alen et al., 2002; Kent et al., 2001; Kent et al., 2004). Here it is used to study the rDNA termination region.

4.2 Analysis of chromatin structure in an all-active strain

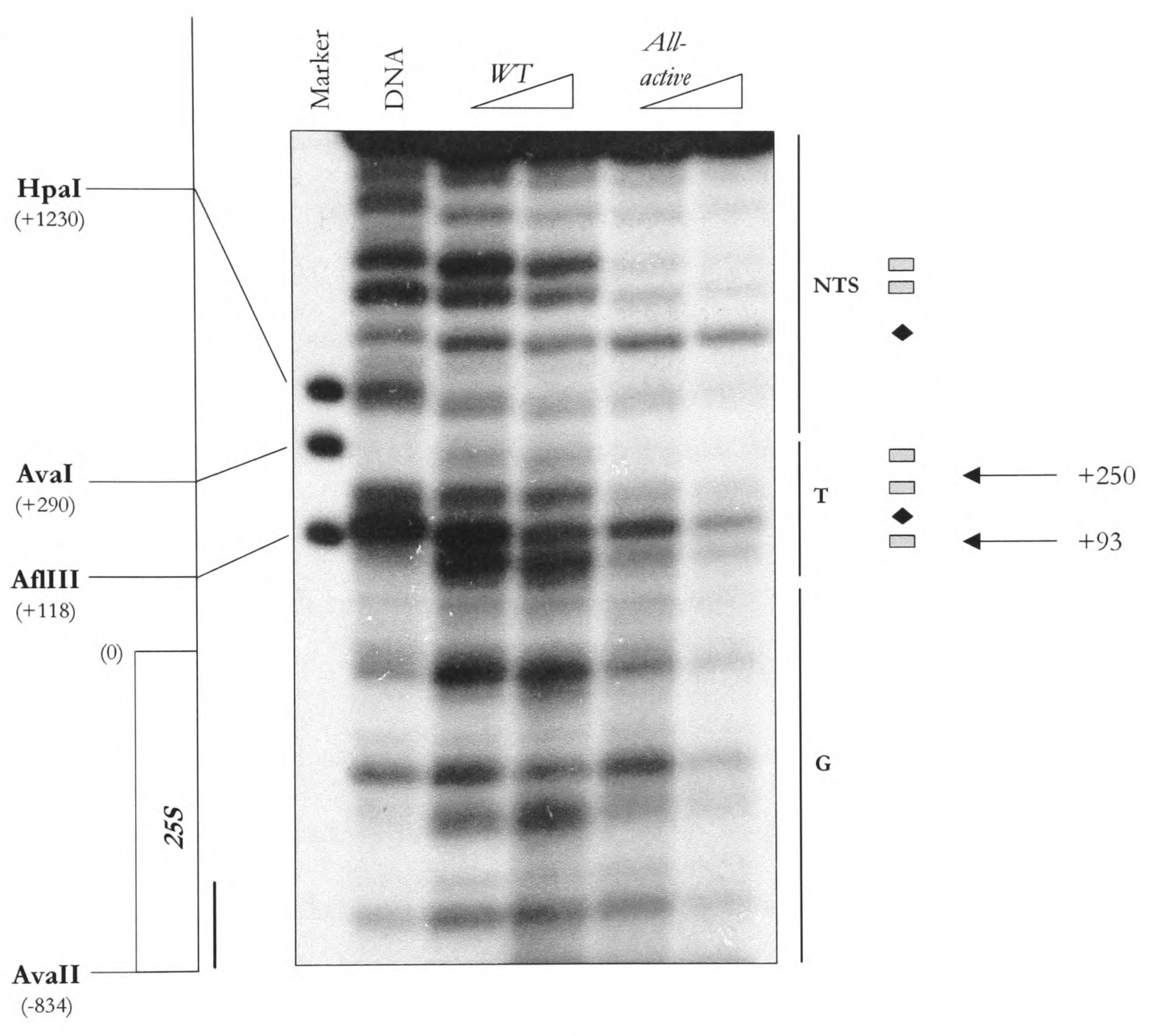
The chromatin structure of the rDNA termination region was first analysed in NOY1051 wild-type and NOY886 all-active strains (Figure 4.2). The location of the end-label within the 3' end of the 25S gene means that the region visualised extends from within the 25S gene, through the termination region and into the non-transcribed spacer. Comparing wild-type and all-active cleavage patterns, it can be seen that the all-active strain shows significant differences in cleavage pattern over the termination region and also over the downstream non-transcribed spacer. Over the termination region, both protection from cleavage (grey rectangles) and enhancement of cleavage (black diamonds) are seen, indicating that the chromatin environment of this region undergoes a number of changes during active transcription.

4.3 Analysis of chromatin structure in single mutants

4.3.1 $\Delta chd1$

The presence of chromatin structure and chromatin remodeling factors at the rDNA locus posed the question as to the function of these factors at the locus. To investigate this, *in vivo*

Figure 4.2: Analysis of chromatin structure in an all-active strain. The chromatin environment of the rDNA termination and non-transcribed spacer regions undergo a number of changes, relative to the environment of the isogenic wild-type. Chromatin digestions were performed in isogenic NOY1051 wild-type (WT) and NOY886 all-active (AA) strains with 150 and 300 units/ml MNase (open triangles denote increasing MNase concentration). The region probed is divided into genic (G), termination (T) and non-transcribed spacer (NTS) regions by black bars. Within the termination and NTS regions in the all-active strain, both increased cleavages by MNase (black diamonds) and protections from MNase cleavage (grey rectangles) are seen as compared to the wild-type strain. Specific restriction digests (Marker) are shown relative to a schematic map of the locus. The map shows the cleavage sites of the three restriction enzyme sites used to produce the markers (AflIII, AvaI, HpaI) in relation to the 25S genic region and the location of cleavage by AvaII, the end-digest enzyme. The +93 and +250 termination sites are indicated by black arrows. The indirect end-label probe is shown as a black bar towards the 3' end of the 25S gene. "DNA" denotes de-proteinised DNA control.



chromatin analysis was carried out in wild-type and $\Delta chd1$ mutants strains (Figure 4.3A). It can be seen that loss of Chd1p does not alter the chromatin environment of the rDNA genic/termination region when compared to the wild-type strain. This may suggest that Chd1p does not act at the rDNA locus. However, as has been shown for the *GAL10-7* locus, this result may indicate that remodeling factors are acting redundantly, with loss of one factor being compensated for by the continued presence of other factors (see below).

4.3.2 $\Delta isw1$

The role of Isw1p at the rDNA locus was next investigated by analysing chromatin structure in wild-type and $\Delta isw1$ strains. As can be seen in Figure 4.3B, loss of Isw1p does not alter the chromatin structure over the rDNA termination region. As above, this suggests that Isw1p, if it is acting at this locus, may be acting redundantly.

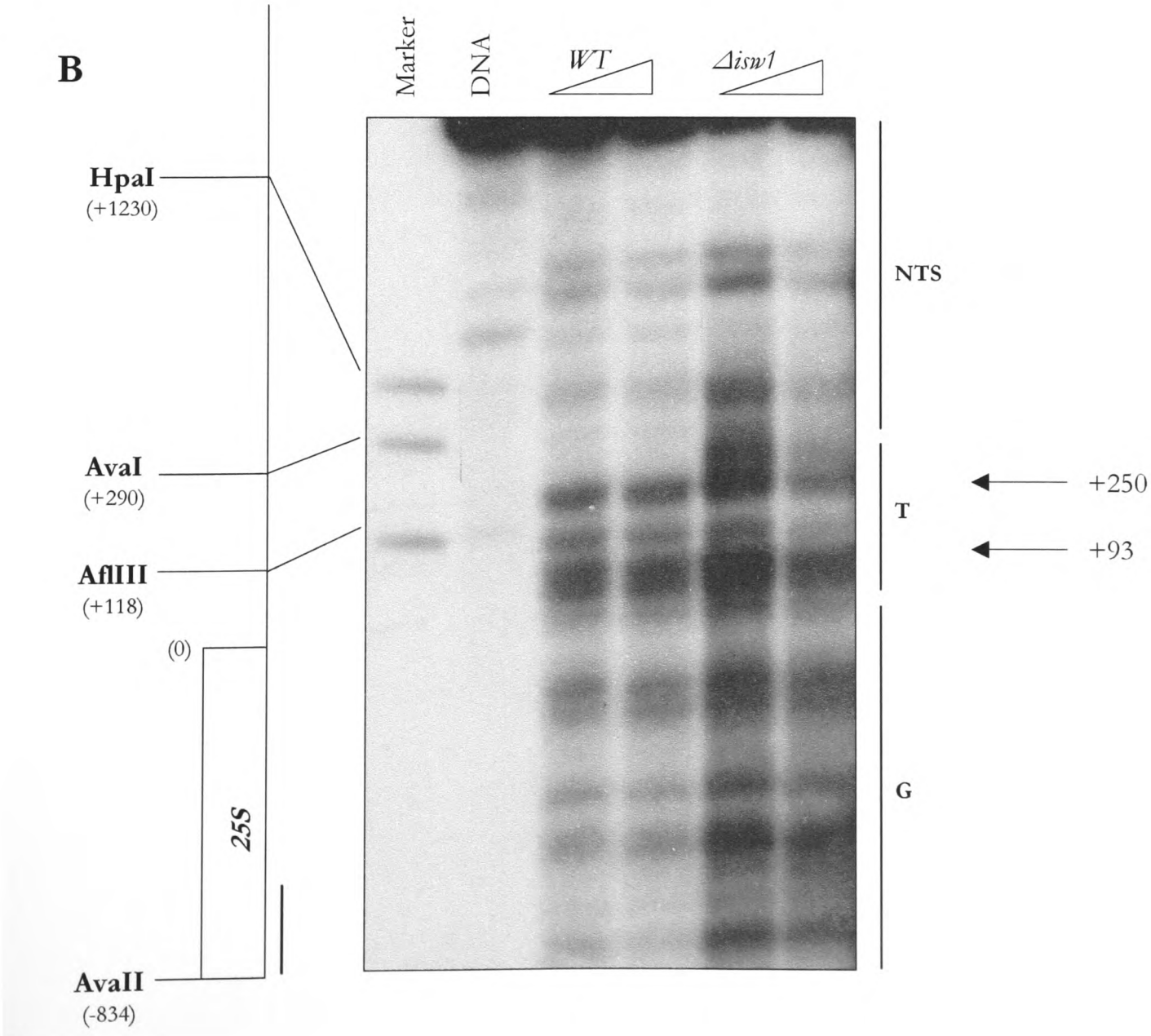
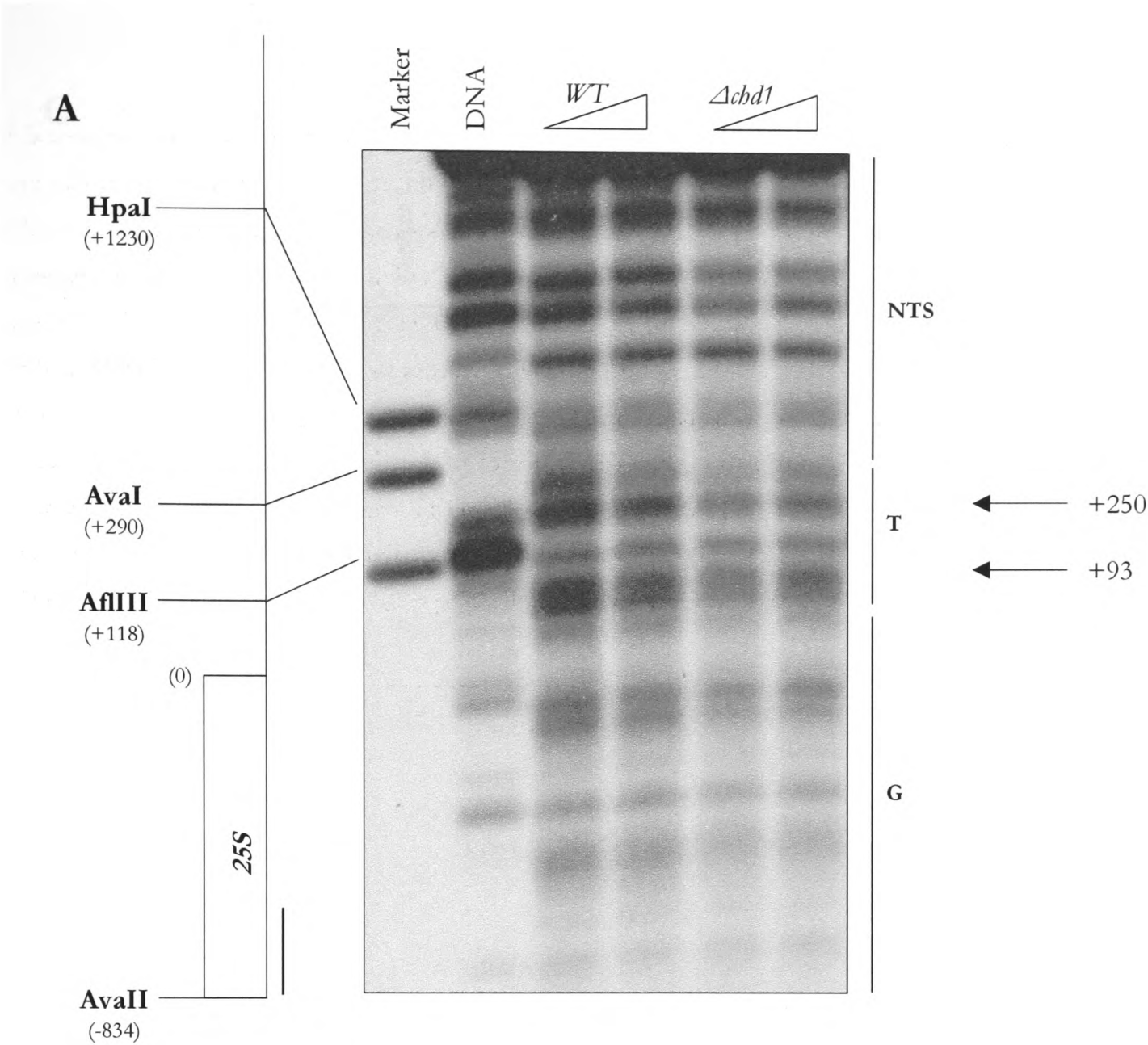
4.3.3 $\Delta isw2$

The final single mutant strain to be analysed was $\Delta isw2$. As shown in Figure 4.3C, loss of Isw2p does also not alter chromatin structure over the rDNA termination region. Thus, all single mutant strains fail to show alterations, indicating that chromatin structure over the rDNA termination is not determined by the sole action of any of these factors.

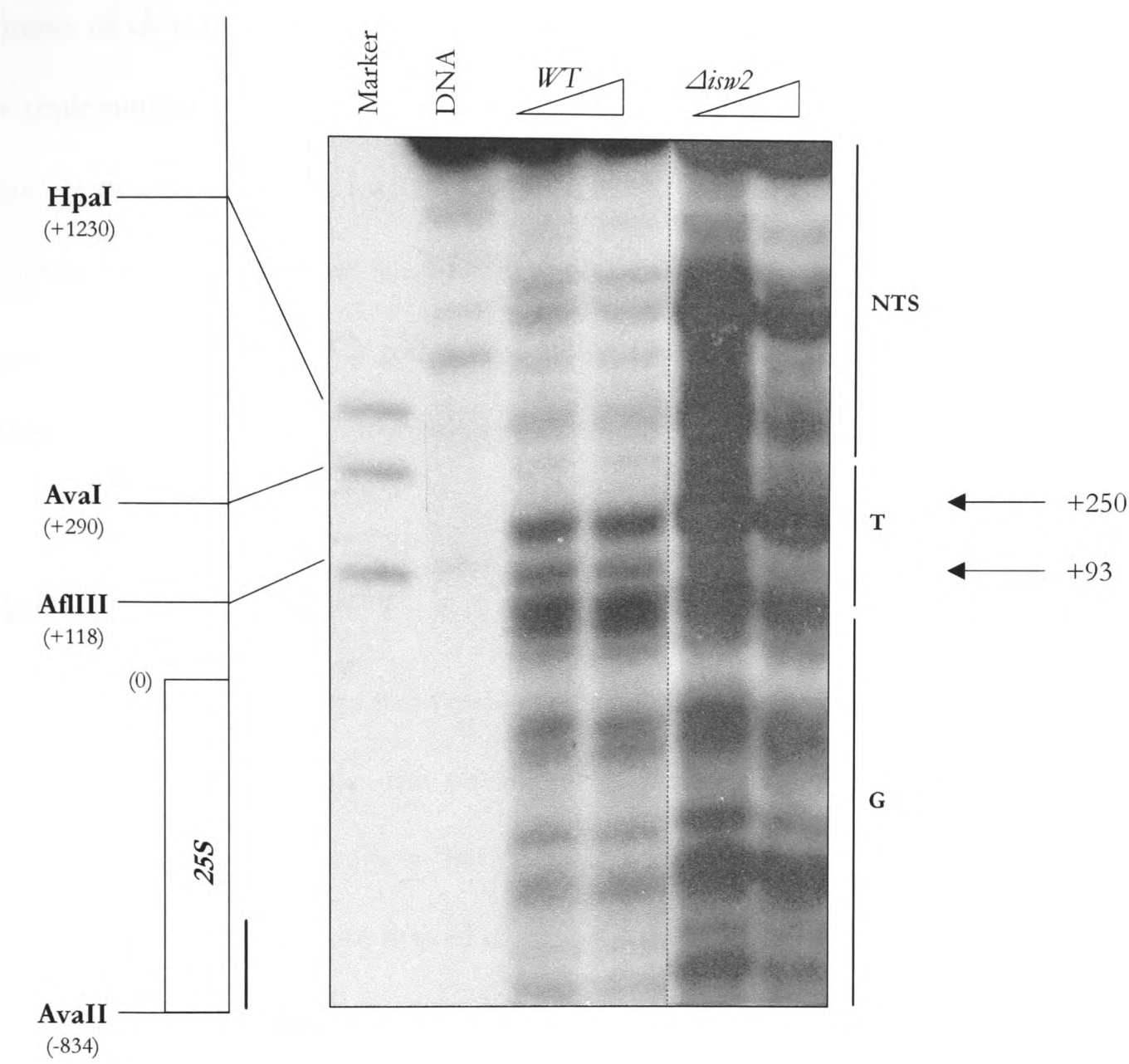
4.4 Analysis of chromatin structure in a $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ triple mutant

To address the question of redundancy in chromatin remodeling factors at the rDNA locus, chromatin structure was analysed in double mutant strains. Neither double mutant strain analysed ($\Delta chd1$, $\Delta isw1$ and $\Delta chd1$, $\Delta isw2$) showed altered chromatin structure over the rDNA termination region (data not shown). Therefore the analysis was repeated in a $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ triple mutant strain. In contrast to the single and double mutant strains, loss of

Figure 4.3: Analysis of chromatin structure in single mutant strains. Loss of a single chromatin remodeling factor does not lead to alterations in the chromatin structure over the rDNA termination region. Chromatin digestions were performed in (A) $\Delta chd1$, (B) $\Delta isw1$, (C) $\Delta isw2$ and isogenic wild-type strains with 150 and 300 units/ml MNase (open triangles denote increasing MNase concentration). Note that the overall pattern of cleavage seen in the $\Delta isw2$ strain (C) is the same as that in the wild-type. The higher signals reflects a greater amount of material loaded onto the gel. The region probed is divided into genic (G), termination (T) and non-transcribed spacer (NTS) regions by black bars. Specific restriction digests (Marker) are shown relative to a schematic map of the locus. The map shows the cleavage sites of the three restriction enzyme sites used to produce the markers (AflIII, AvaI, HpaI) in relation to the 25S genic region and the location of cleavage by AvaII, the end-digest enzyme. The +93 and +250 termination sites are indicated by black arrows. The indirect end-label probe is shown as a black bar towards the 3' end of the 25S gene. "DNA" denotes de-proteinised DNA control.



C

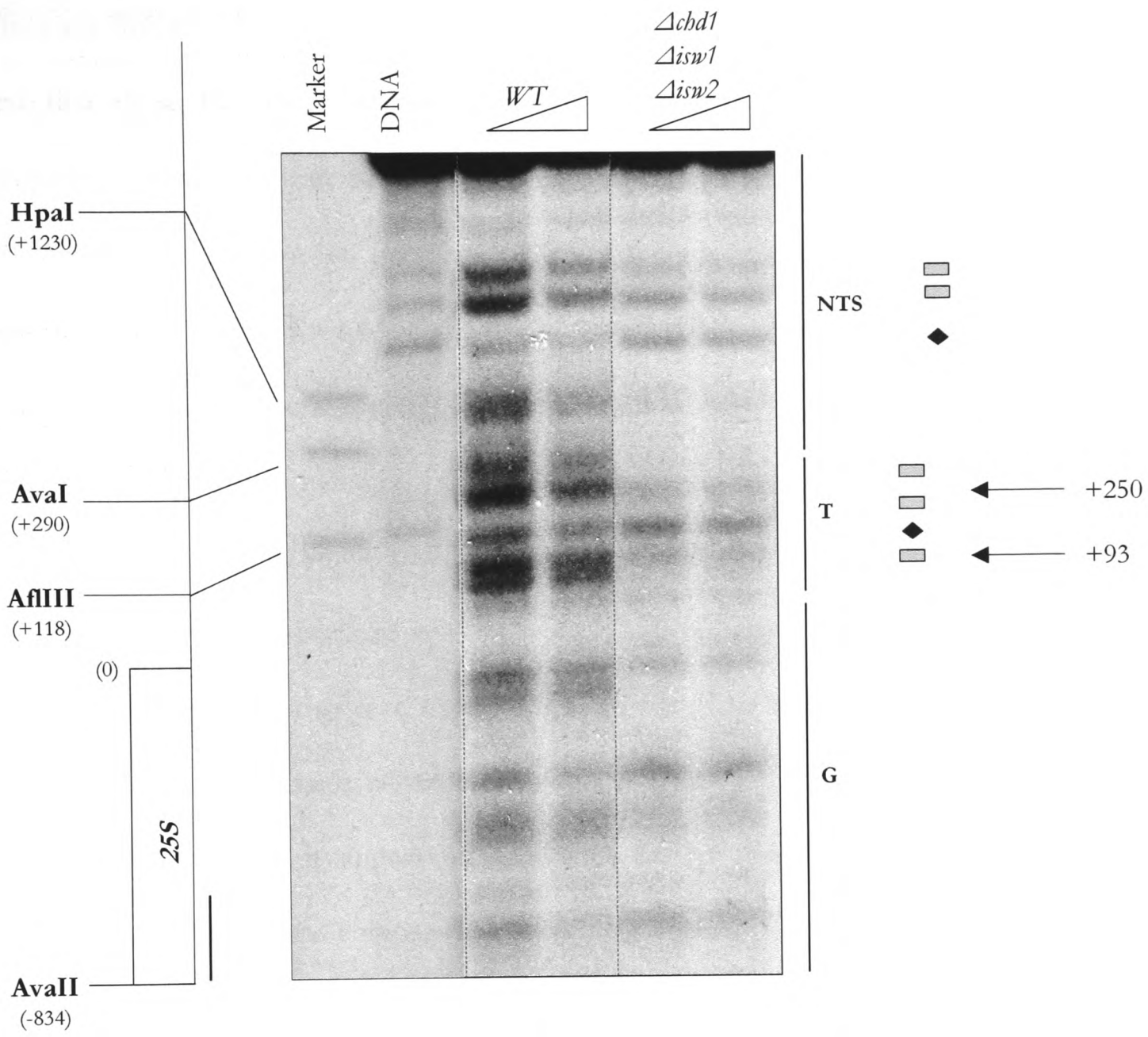


all three remodeling factors leads to alterations in chromatin structure over the termination region and also within the downstream non-transcribed spacer (Figure 4.4). As wild-type patterns of cleavage were seen in single and double mutants, the alterations seen here with the triple mutant suggest that these remodeling factors are acting redundantly at the rDNA locus. It can be seen that loss of Chd1p, Isw1p and Isw2p leads to a pattern of MNase cleavage which mimics that seen for the all-active strain over the termination and spacer regions (compare Figure 4.2 and 4.4). Loss of Chd1p, Isw1p and Isw2p thus results in all repeats adopting an active chromatin environment.

4.5 Discussion

The aim of this chapter was to investigate the chromatin structure of the rDNA genic and termination regions. Firstly, the chromatin structure at active repeats was analysed by comparing an all-active strain to the isogenic wild-type (Figure 4.2). The current view of active repeats is that they are devoid of chromatin. If this is the case, one would expect the all-active strain to be identical to the naked DNA lane, which reflects the cutting pattern of MNase on DNA lacking chromatin structure. However, the all-active strain shows a number of differences in cleavage pattern compared to the naked lane. In addition, the all-active cleavage pattern is similar to that seen in a wild-type strain (compare Figures 4.2 and 4.4). Half the signal seen in the wild-type strain is derived from inactive, heterochromatic repeats. The similarity in all-active and wild-type profiles, coupled with the ChIP data described above (Chapters 2 and 3), suggests that the all-active repeats possess chromatin structure. This structure is very similar to that seen for the wild-type but does differ over the termination region, suggesting either that transcription termination causes an altered chromatin structure, or that this altered chromatin structure facilitates transcription termination.

Figure 4.4: Analysis of chromatin structure in a triple mutant strain. Loss of Chd1p, Isw1p and Isw2p leads to alterations in the chromatin structure over the rDNA termination and downstream non-transcribed spacer regions that mimic those seen in the all-active strain. Chromatin digestions were performed in isogenic wild-type and $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ triple mutant strains with 150 and 300 units/ml MNase (open triangles denote increasing MNase concentration). The region probed is divided into genic (G), termination (T) and non-transcribed spacer (NTS) regions by black bars. Within the termination and NTS regions in the triple mutant strain, both increased cleavages by MNase (black diamonds) and protections from MNase cleavage (grey rectangles) are seen as compared to the wild-type strain. Specific restriction digests (Marker) are shown relative to a schematic map of the locus. The map shows the cleavage sites of the three restriction enzyme sites used to produce the markers (AflIII, Aval, HpaI) in relation to the 25S genic region and the location of cleavage by AvaII, the end-digest enzyme. The +93 and +250 termination sites are indicated by black arrows. The indirect end-label probe is shown as a black bar towards the 3' end of the 25S gene. "DNA" denotes de-proteinised DNA control.



The function of chromatin remodeling factors over the rDNA genic and termination regions was next investigated. Loss of a single chromatin remodeling factor was not found to have an effect on MNase cleavage patterns, as compared to wild-type (Figure 4.3). This could suggest that these factors do not act to remodel chromatin over the region analysed. However, it could also indicate that these factors are acting redundantly, with the loss of one factor compensated for by the presence of other factors. To test this possibility, *in vivo* chromatin analysis was performed in $\Delta chd1$, $\Delta isw1$ and $\Delta chd1$, $\Delta isw2$ mutant strains. Neither of these strains showed alterations to chromatin structure over the region analysed (data not shown). The analysis was therefore extended and a $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ triple mutant was analysed. In contrast to the single and double mutants, loss of Chd1p, Isw1p and Isw2p lead to an altered chromatin structure over the rDNA termination region (Figure 4.4). This structure is very similar to that seen in an all-active strain (Figure 4.2), indicating that loss of these remodeling factors causes all repeats to adopt an active chromatin structure over the termination region. Thus it appears that Chd1p, Isw1p and Isw2p act redundantly to maintain a specific chromatin environment at inactive repeats.

The cleavage of naked DNA with MNase allows determination of the underlying cleavage pattern of this enzyme. In theory, comparison of naked and wild-type lanes should show the presence of ~150bp protected sites in the wild-type strain, indicative of positioned nucleosomes. It should however be noted that neither wild-type nor all-active strains show clear sites of protection indicative of regularly positioned nucleosomes as compared to the naked DNA lane. This would be at least expected in the wild-type strain where half the repeats are bound up in heterochromatin. The absence of such protected sites in the wild-type strain suggests that this analysis may be confused by the presence of multiple repeats with slightly different chromatin and transcription states in all strains analysed, possibly leading to an average signal being seen.

Chapter 5: Nascent analysis of transcription termination by RNA Polymerase I

Preface

The presence of chromatin structure and chromatin remodeling factors at actively transcribed rDNA repeats suggested that these remodeling factors are playing an active role in facilitating transcription by Pol I. In addition, it has previously been shown that Chd1p, Isw1p and Isw2p act redundantly to ensure efficient transcription termination at the Pol II-transcribed *GAL10-7* locus (Alen et al., 2002). The aim of this chapter was therefore to study the role of remodeling factors in termination by performing transcription run-on analysis (TRO) on single, double and triple mutant strains.

5.1 Transcription run-on analysis

TRO analysis is a technique allowing one to measure nascent transcription in terms of polymerase density across the probed region. Yeast cells are harvested under conditions that cause the stalling of polymerases on the DNA template. The cells are then permeabilised and incubated in a buffer containing [α - 32 P] rUTP. This buffer supports further transcription, with polymerases transcribing on for $\sim$ 150bp. The presence of radioactive rUTP causes transcripts to be radiolabelled. RNA is then harvested, hydrolysed and hybridised to filter-bound single-stranded M13 probes. The signals obtained from the filters are quantified and the magnitude of signal obtained is proportional to the density of transcriptionally engaged polymerase present on the gene, allowing determination of the profile of nascent transcription across the gene. Profiles obtained from mutant strains are compared to that of the isogenic wild-type, allowing diagnosis of termination defects. TRO analysis was carried out using probes spanning the rDNA repeat, from the promoter region up to the Pol III-transcribed 5S gene (Prescott et al., 2004). Figure 5.1A and B show a schematic representation of the TRO protocol and Figure 5.1C shows the location of the

Figure 5.1A: Schematic of the transcription run-on protocol. 100ml cultures were grown overnight and used as described in the schematic. For a more detailed description of the protocol, see Chapter 8: Methods.

A



Harvest and permeabilise
S. cerevisiae cells at 4°C



Resuspend cells in TRO buffer plus
ATP, GTP, CTP and [$\alpha^{32}\text{P}$] UTP



Transcribe at 30°C for 5 minutes



Extract rRNA at 65°C



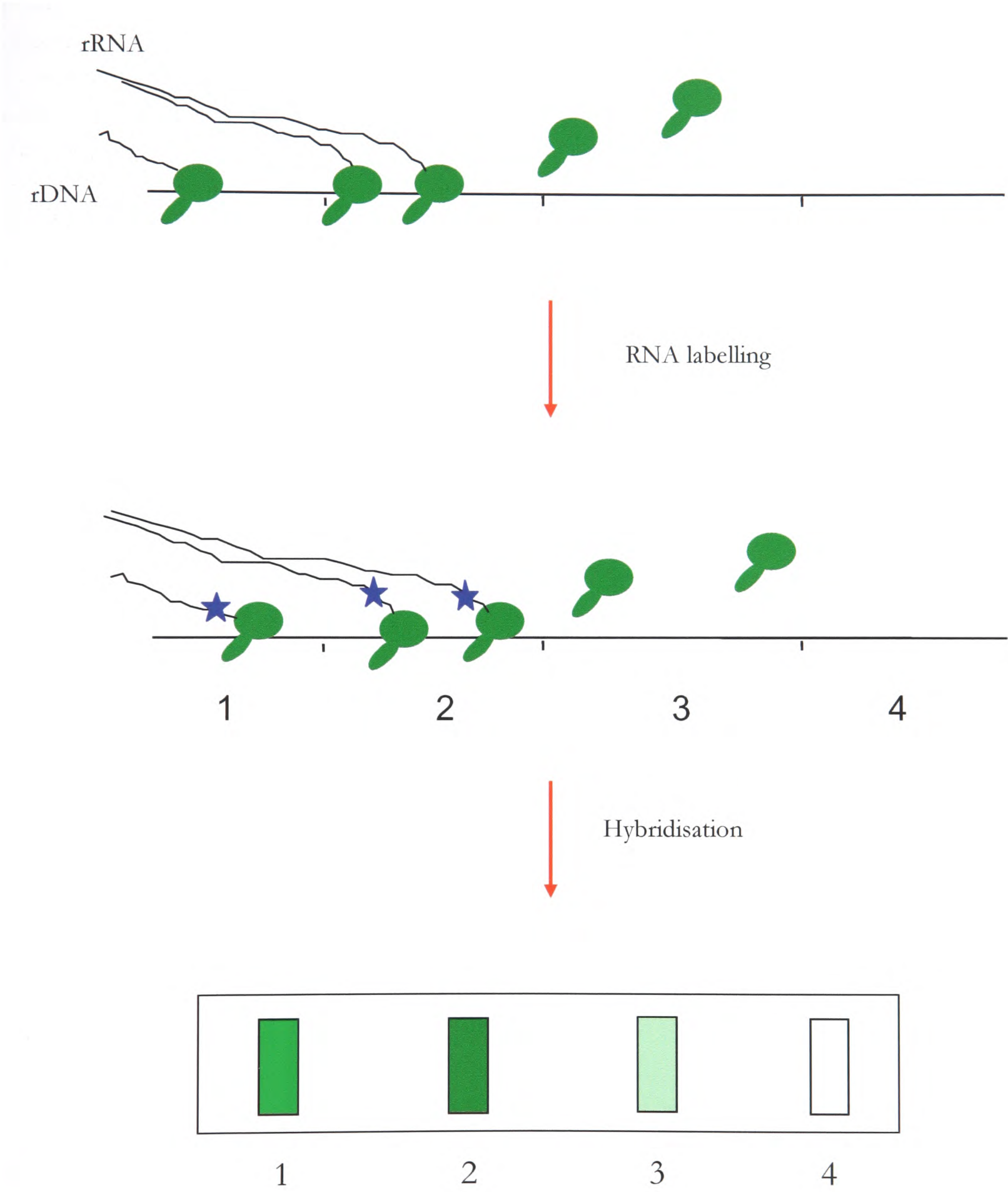
Hydrolyse rRNA



Hybridise rRNA to filters, wash filters
and expose to film and Phosphor screen

Figure 5.1B: Schematic of the transcription run-on analysis. The rDNA repeat is indicated by a black line. The small black lines separate the different regions probed in the analysis. The green shapes represent Pol I complexes transcribing the repeats. Black lines extending from the Pol I complexes denote rRNA. Labelling of rRNA with [$\alpha^{32}\text{P}$] rUTP is shown by blue stars. The numbers “1-4” represent four consecutive regions probed in a run-on analysis. In the final panel, the filters are shown and numbered 1-4. The density of polymerase transcribing a given region correlates with the level of signal obtained for that region. Thus region 1 is occupied by one polymerase and produces a certain level of signal (deep green). Region two has a greater polymerase density and thus produces a higher signal (very dark green). The dissociation of polymerase from region three results in a low signal (light green) whilst no signal is produced in region four as polymerase is absent. Signals obtained from the filters are quantified and corrected as detailed in Chapter 8: Methods.

B



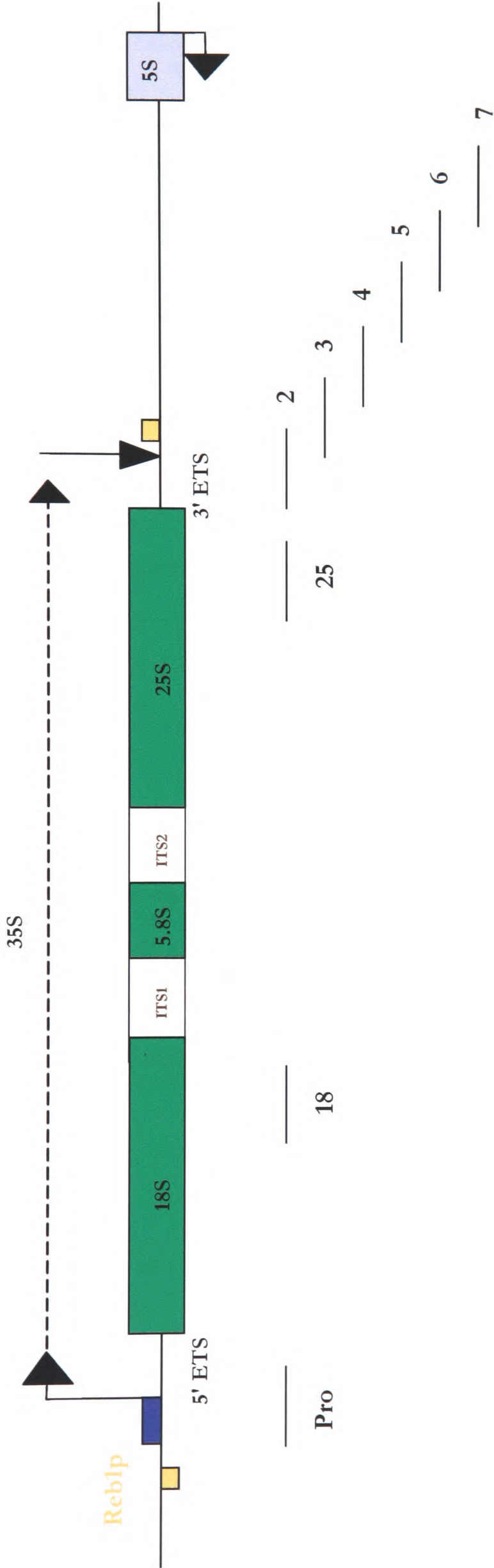


Figure 5.1C: Location of regions probed within the rDNA repeat during transcription run-on analysis. Black lines denote regions spanned by M13 probes. Regions are named according to their location within the rDNA repeat except the probes 2-7 which are simply numbered in order. The exact locations of the M13 probes, their sizes and U contents are listed in Chapter 8: Methods. For a full description of the elements found within the rDNA repeat, see Figure 1.1 of Chapter 1: Introduction. The diagram is representative and is not drawn to scale.

D

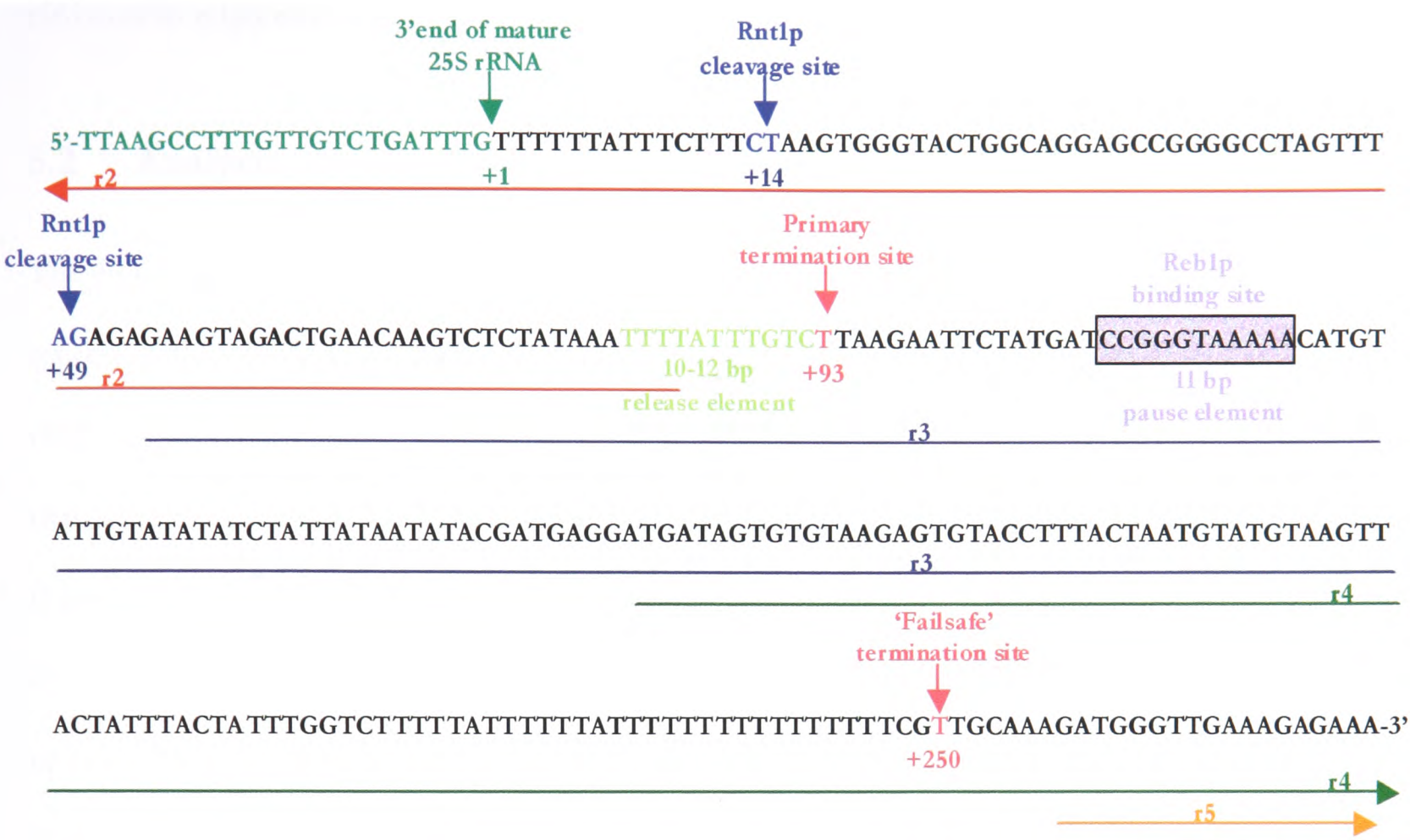


Figure 5.1D: Sequence detail of the rDNA repeat indicating the locations of M13 probes relative to key rDNA elements. The sequence shown covers the 3' end of the 25S gene (green) and downstream sequence spanning the +93 and +250 termination sites (pink). In addition, the 11 bp Reb1p binding and pause element are shown (purple box). The yellow coloured sequence indicates the 10-12 bp release element. The two Rnt1p cleavage sites at +14 bp and +49 bp are identified in blue. Probe 2 is shown in red (r2), probe 3 in blue (r3), probe 4 in green (r4) and probe 5 in orange (r5). Reproduced with permission from E.M. Prescott, D. Phil thesis, University of Oxford, 2002.

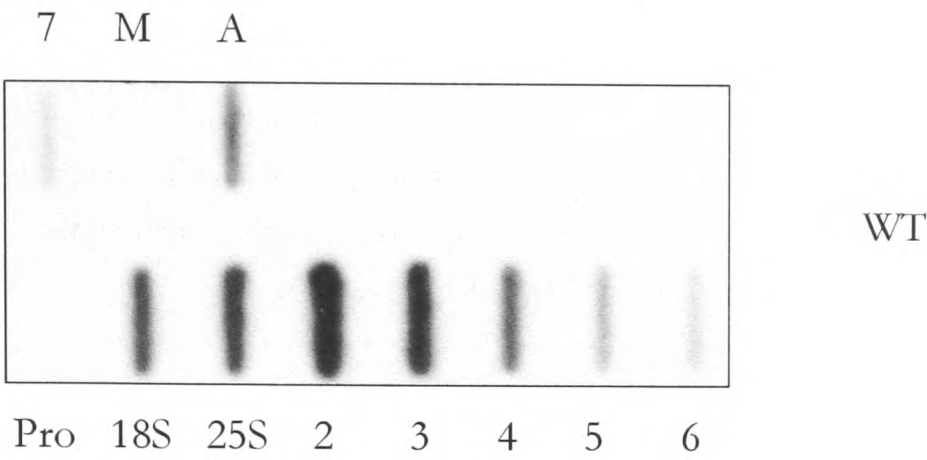
TRO probes. Finally, Figure 5.1D shows the location of key termination region sequence elements in relation to TRO probe locations.

5.2 Analysis of wild-type transcription termination

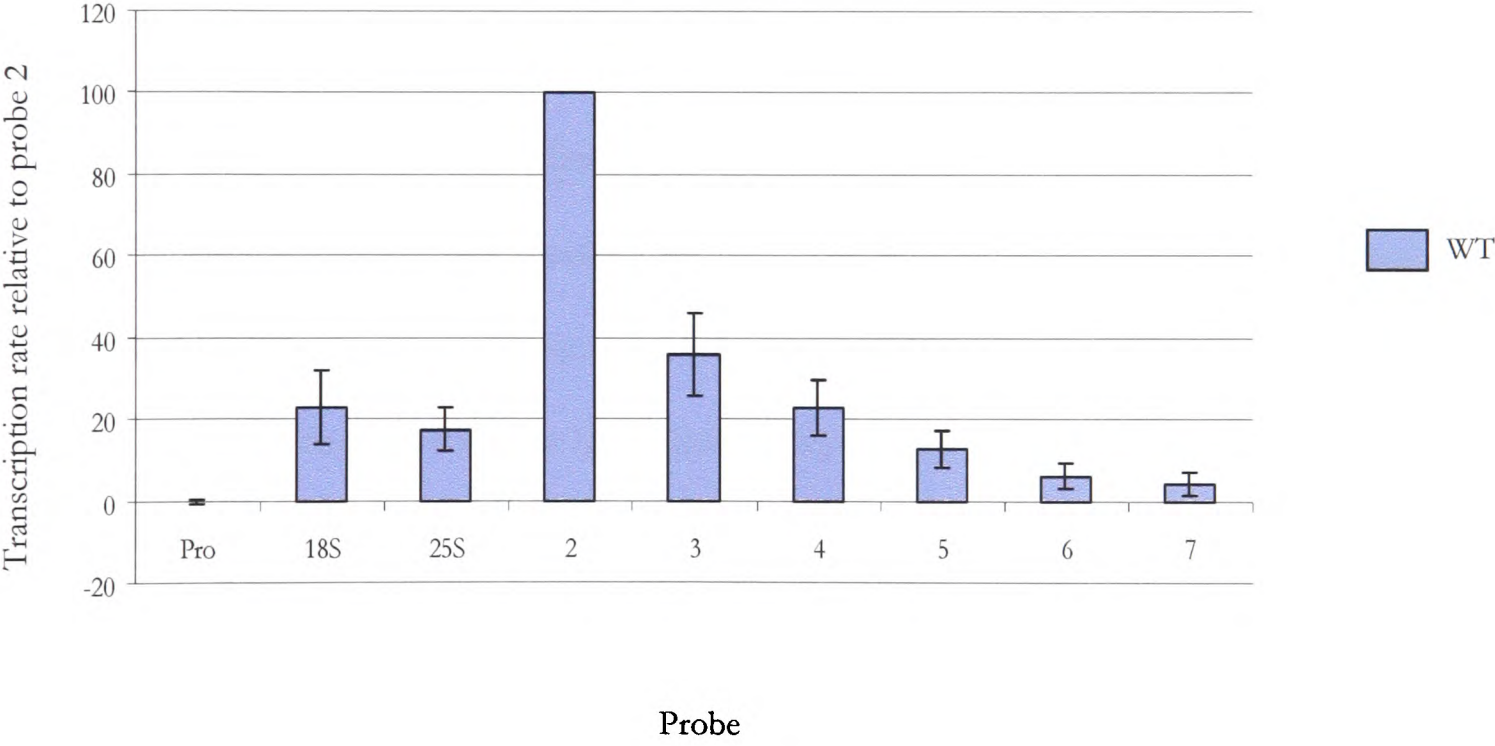
The Pol I nascent transcription profile was determined in the YTT166 wild-type strain. A representative raw data set is presented and shows the profile of transcription across the rRNA gene (Figure 5.2A). Average quantitated data are shown in Figure 5.2B. It can be seen that there is no signal over the promoter region, although Pol I is present in this region (Figure 2.2), indicating that Pol I is not engaged in transcription. The signal then increases over the genic regions (18S and 25S) before peaking over probe 2. Probe 2 ends just upstream of the +93 primary termination site and covers the T-rich element (Figure 5.1D). The high signal thus represents polymerase that is paused upstream of the Reb1p, prior to terminating. The signal then drops off considerably over probes 3, 4 and 5. Probe 4 spans the +250 failsafe terminator and it can be seen that ~80% of polymerases terminate before this site. Further downstream, over probes 6 and 7, signals are very low, indicating that the polymerase has terminated and is disengaging from the template. The figure of 80% of polymerases terminating before the +250 failsafe terminator (probe 4) is slightly lower than the generally accepted figure of 90%. However, some strain variation is known to occur (Prescott, E. M., 2002) and highlights the importance of using isogenic strains. In conclusion, the overall termination profile seen here is consistent with previous studies based on both TRO and steady-state rRNA analyses (Johnson and Warner, 1991; Prescott et al., 2004; Reeder et al., 1999).

Figure 5.2: Transcription profile across the rDNA repeat in a YTT166 wild-type strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. **(B)** Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B



5.3 Analysis of transcription termination in single mutants

In order to study the role of chromatin remodeling factors in Pol I transcription, mutant strains were employed. First, strains deleted for single chromatin remodeling factors were studied.

5.3.1 $\Delta chd1$

The first single mutant strain to be analysed was YTT400, in which Chd1p is deleted. A raw data set (Figure 5.3.1A) and average quantitated data (Figure 5.3.1B) are shown. It can be seen that loss of Chd1p does not significantly affect transcription by Pol I, with a normal transcription profile seen. Thus it appears that loss of Chd1p alone does not alter the profile of Pol I transcription.

5.3.2 $\Delta isw1$

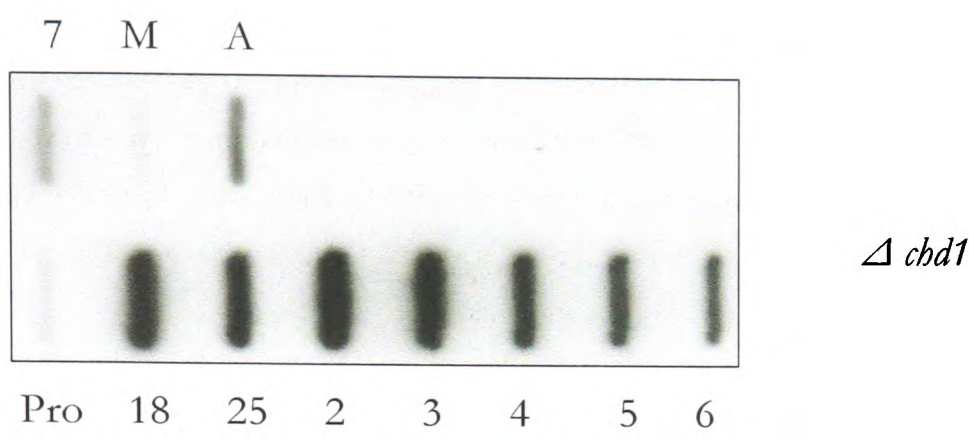
The next single mutant strain to be analysed was YTT186, in which Isw1p is deleted. A raw data set (Figure 5.3.2A) and average quantitated data (Figure 5.3.2B) are shown. It appears that loss of Isw1p leads to a small increase in polymerase density over probes 3 and 4. However the error bars for this data are large, indicating that any phenotype seen upon loss of Isw1p is not consistent.

5.3.3 $\Delta isw2$

The final single mutant to be considered was YTT196, in which Isw2p is deleted. A raw data set (Figure 5.3.3A) and average quantitated data (Figure 5.3.3B) are shown. Once again, it can be seen that the $\Delta isw2$ profile is very similar to that of the wild-type, indicating that loss of Isw2p does not affect Pol I transcription.

Figure 5.3.1: Transcription profile across the rDNA repeat in a YTT400 $\Delta chd1$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. (B) Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B

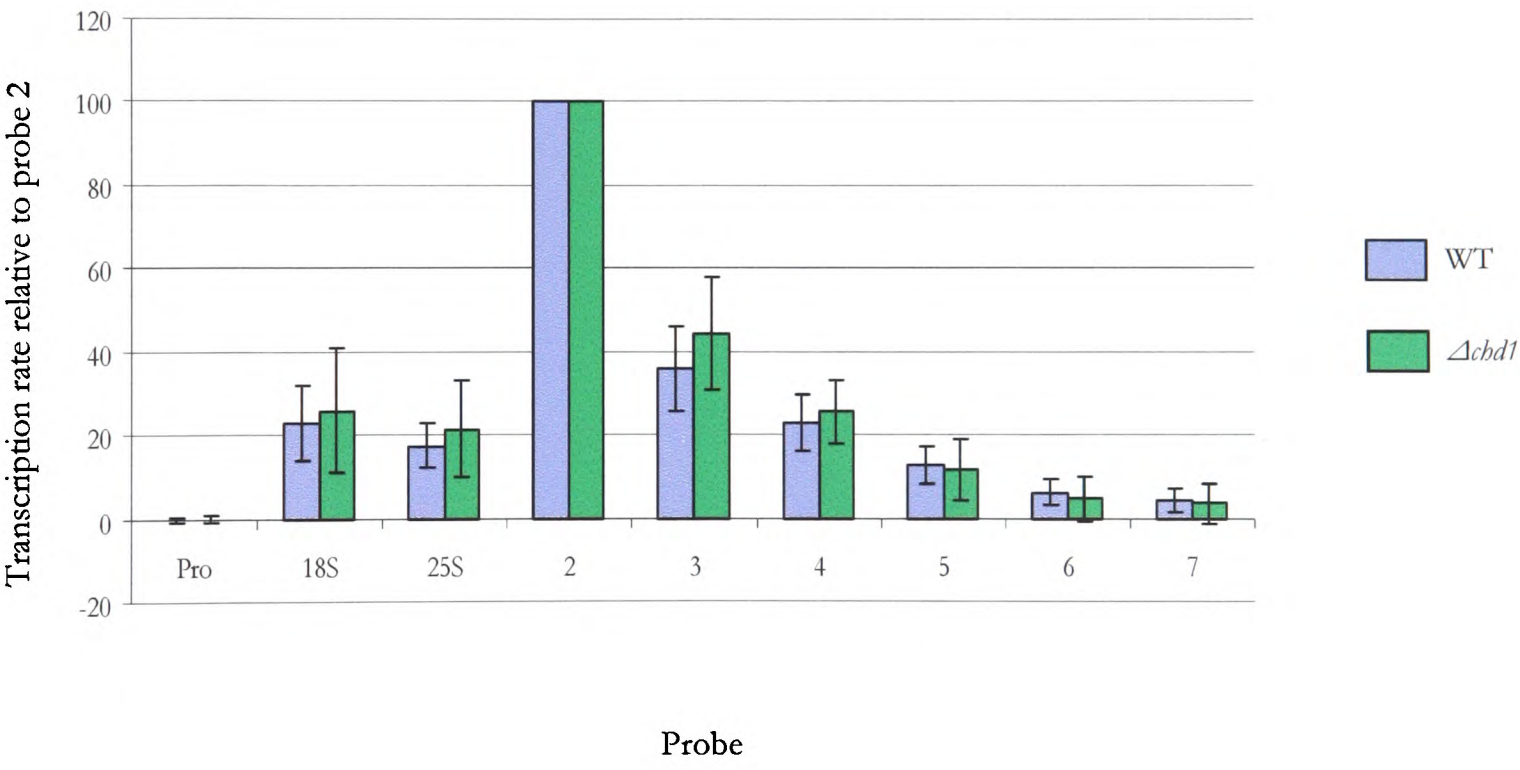
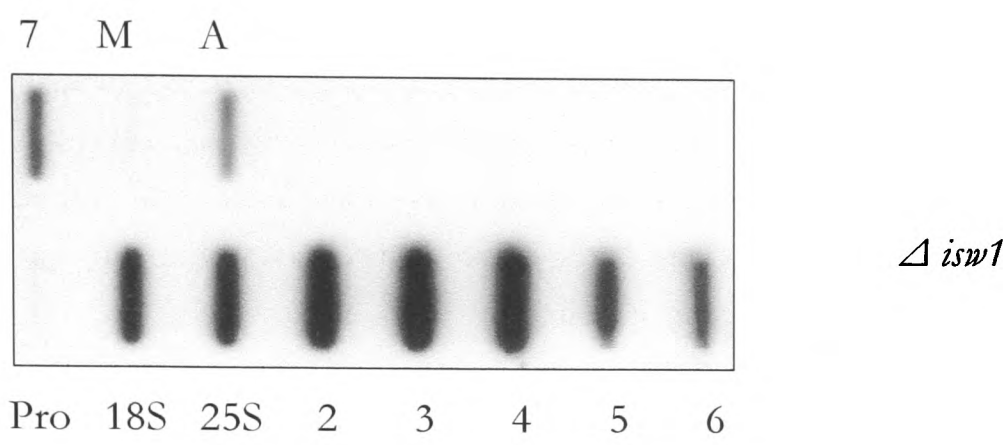


Figure 5.3.2: Transcription profile across the rDNA repeat in a YTT186 $\Delta isw1$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. **(B)** Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B

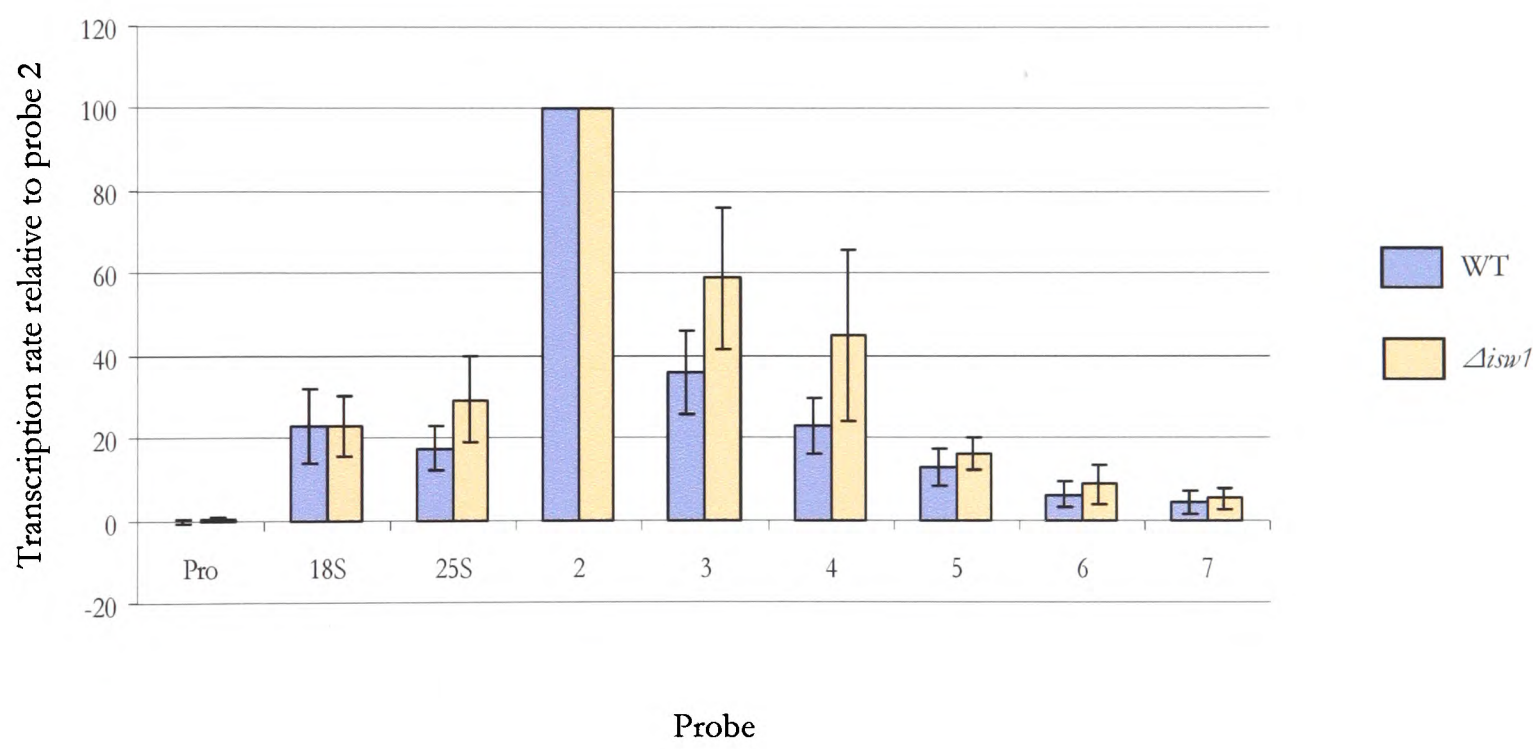
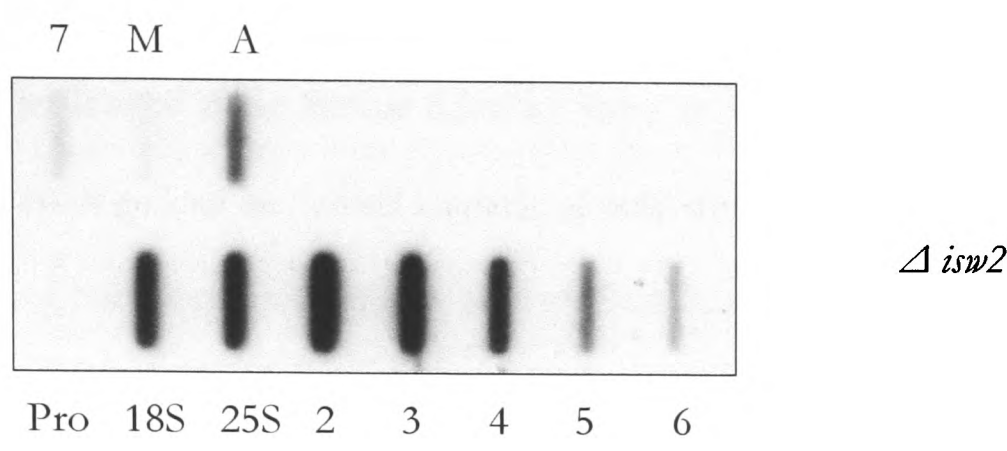
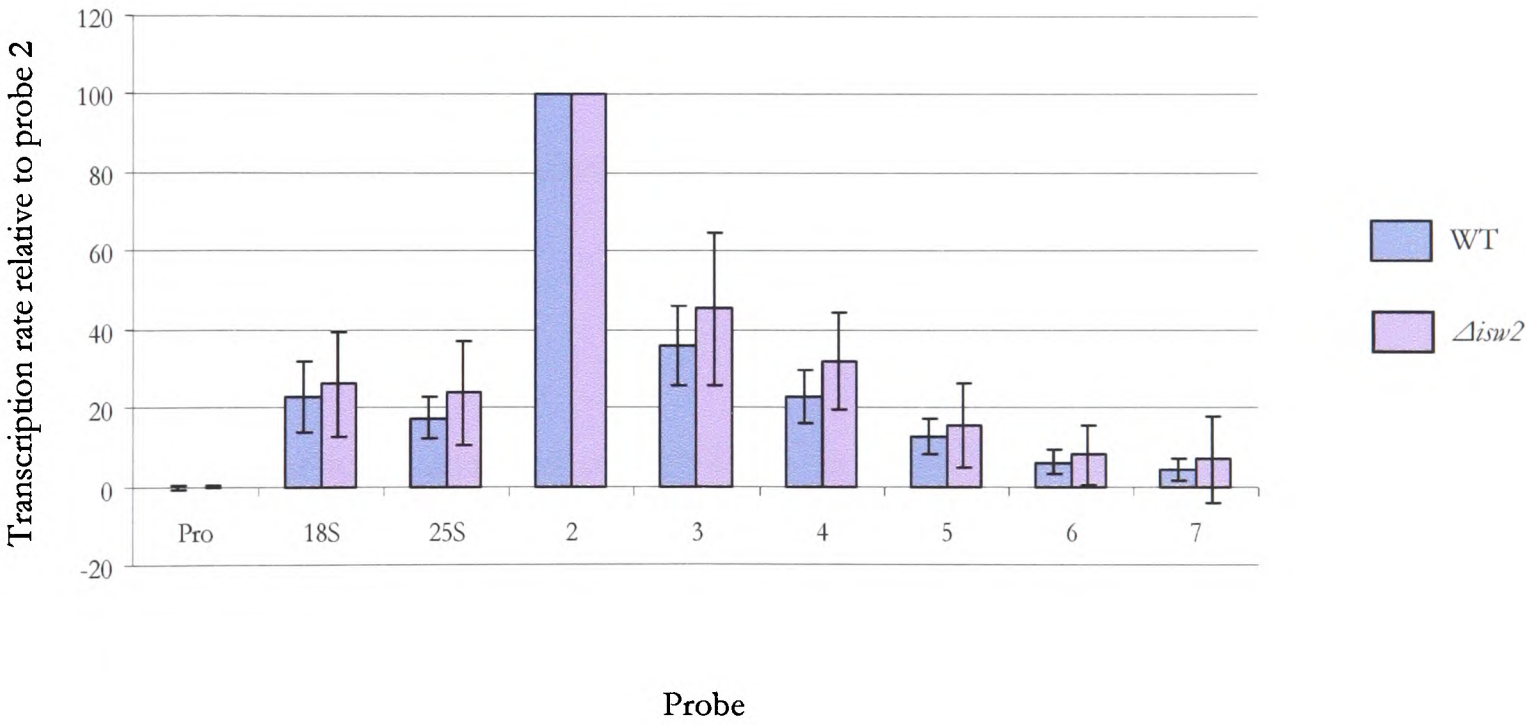


Figure 5.3.3: Transcription profile across the rDNA repeat in a YTT196 $\Delta isw2$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. (B) Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B



5.4 Summary of single mutant transcription run-on analysis

Figure 5.4 shows a composite graph of all single mutant profiles compared to that of the wild-type. Both Chd1p and Isw2p show very similar profiles to that of the wild-type, indicating that loss of these factors does not have an adverse effect on Pol I transcription. Loss of Isw1p does lead to a small amount of read-through over probes 3 and 4. However, the large error bars over these probes indicate that there is a great deal of variation in signal over these probes, suggesting that any effect seen upon loss of Isw1p is not consistent.

5.5 Analysis of transcription termination in double mutants

The lack of phenotypes seen in the single mutant strains could suggest that these factors do not play an active role in Pol I transcription. However, it could also suggest that these factors are redundant, with loss of one factor being compensated for by the continued presence of another factor. To study this potential phenomenon, the TRO analysis was extended and performed on double mutant strains.

5.5.1 $\Delta chd1, \Delta isw1$

The first double mutant to be analysed was YTT223, in which both Chd1p and Isw1p are deleted. As before, a raw data set (Figure 5.5.1A) and average quantitated data (Figure 5.5.1B) are shown. It can be seen that loss of both Chd1p and Isw1p does not lead to a distortion in transcription profile. Rather, the profile is very similar to that of the wild-type over all regions probed.

5.5.2 $\Delta chd1, \Delta isw2$

The second double mutant to be analysed was YTT225, in which both Chd1p and Isw2p are deleted. A raw data set (Figure 5.5.2A) and average quantitated data (Figure 5.5.2B) are shown. Whilst the profile shows slightly more variation over probes 3-5 than was seen for

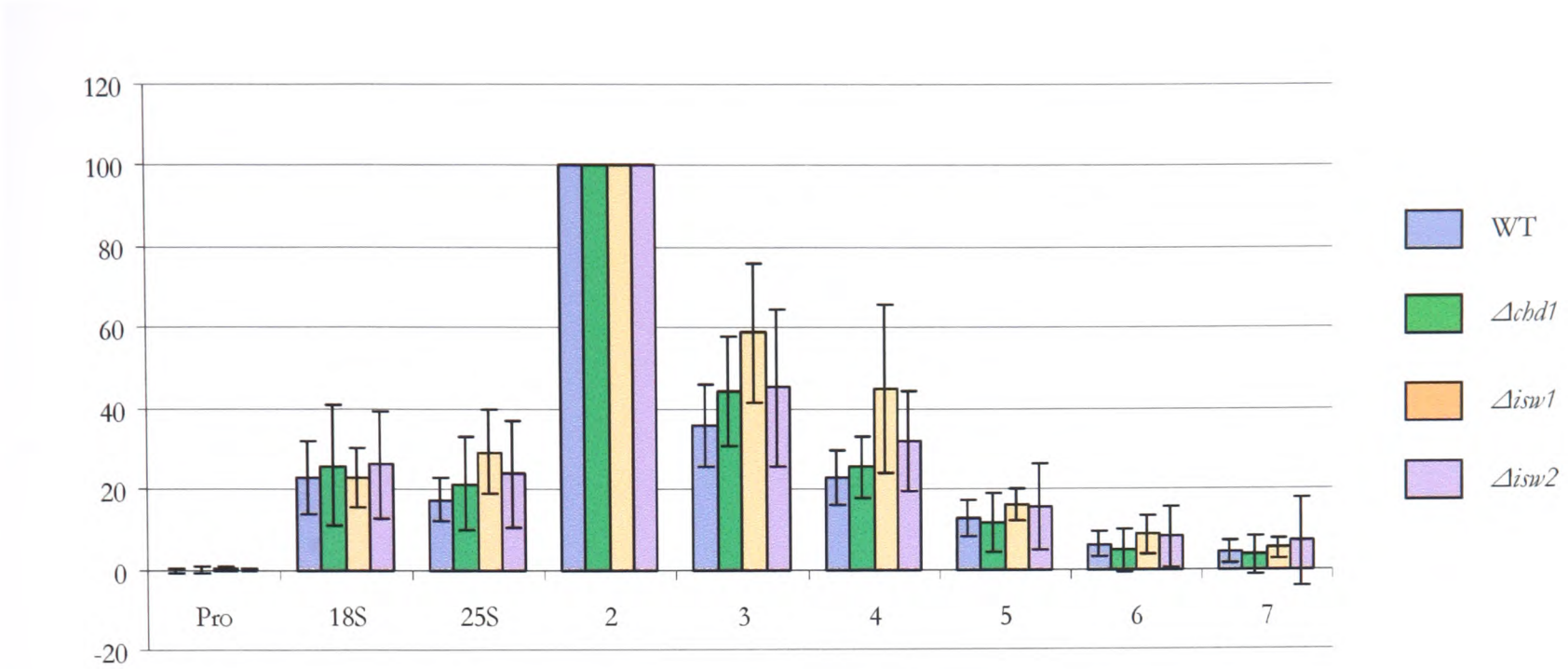
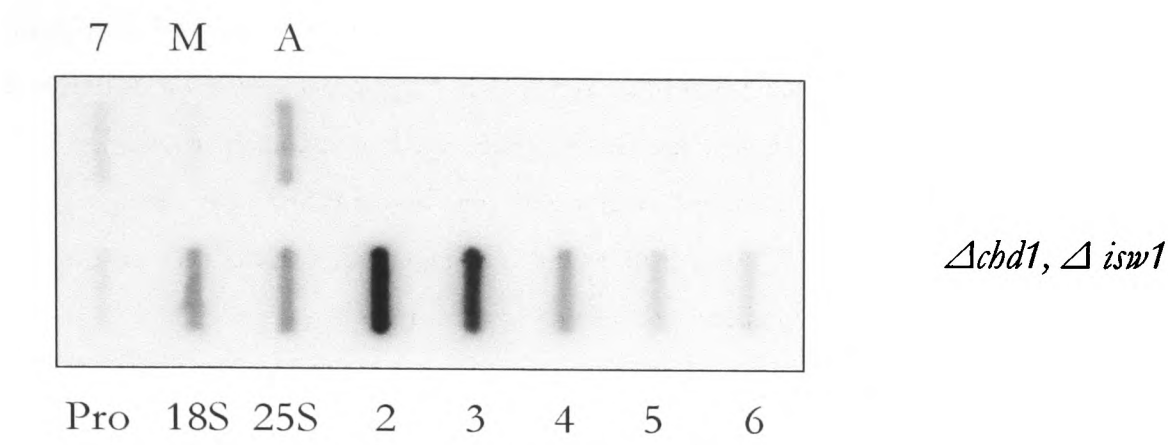


Figure 5.4: Composite graph of quantitated transcription run-on data from wild-type and single mutant strains. Transcription run-on profiles obtained from single mutant strains are not significantly different to the profile obtained from an isogenic wild-type strain, indicating that loss of a single chromatin remodeling factor does not affect the transcription profile of RNA Polymerase I. Average quantitation of multiple data sets is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

Figure 5.5.1: Transcription profile across the rDNA repeat in a YTT223 $\Delta chd1$, $\Delta isw1$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. (B) Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B

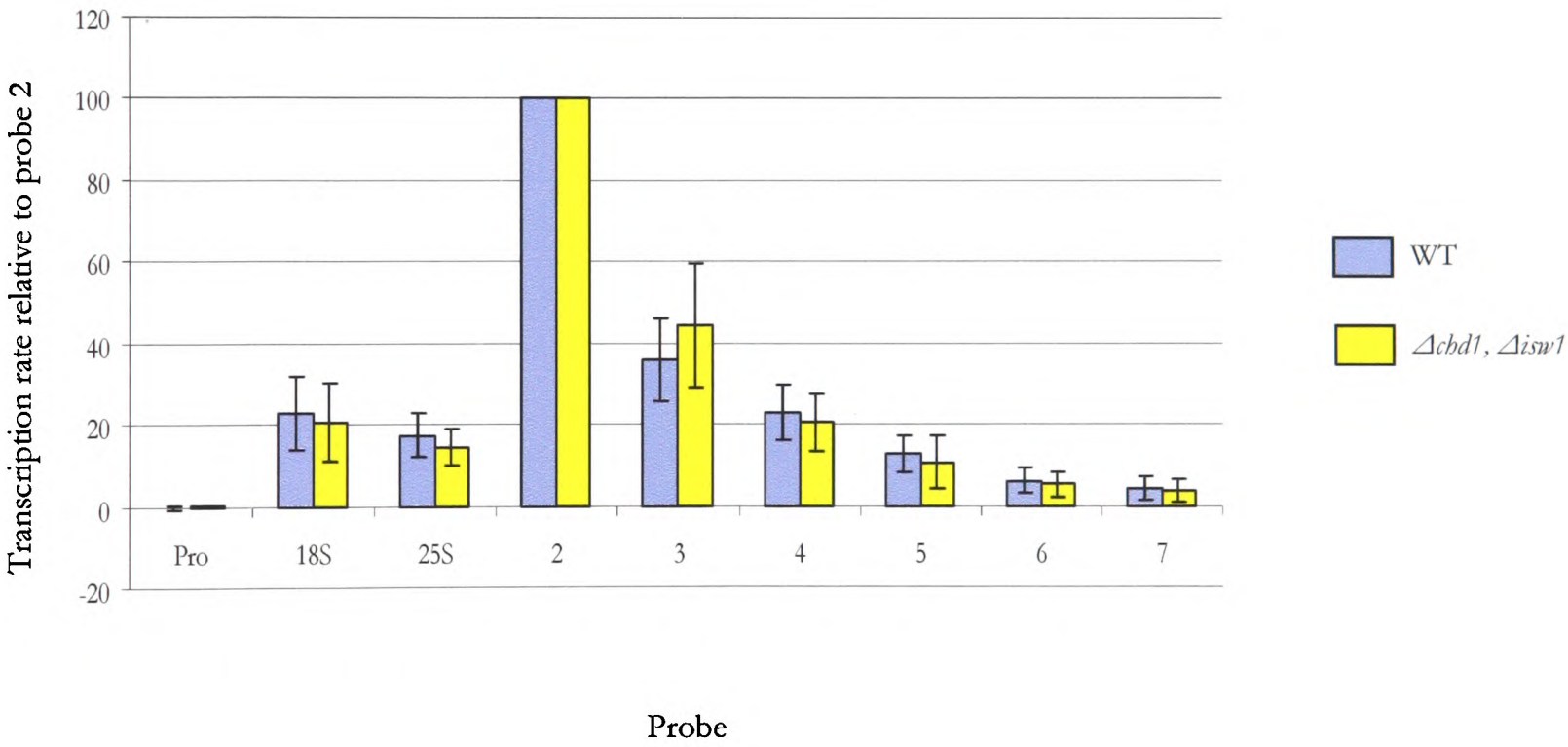
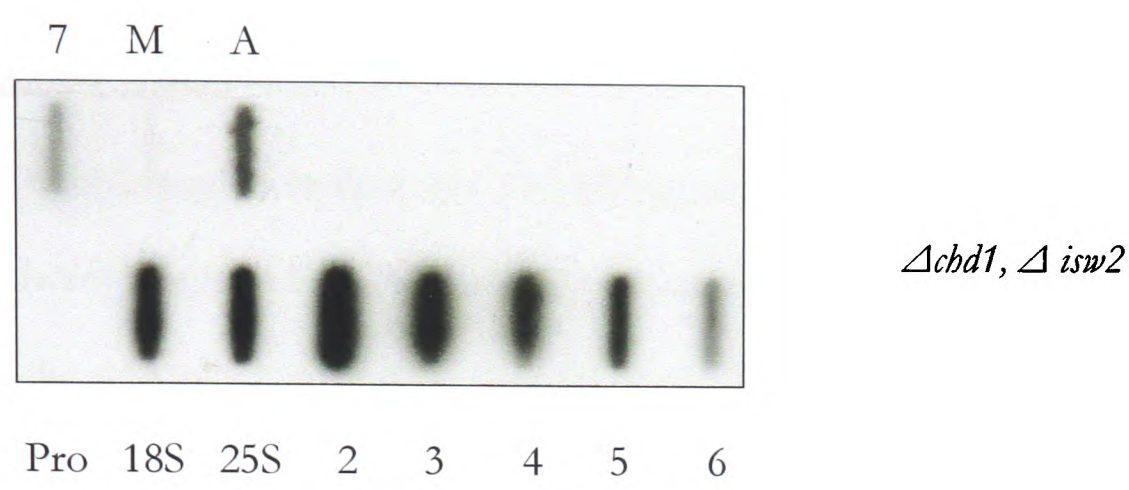
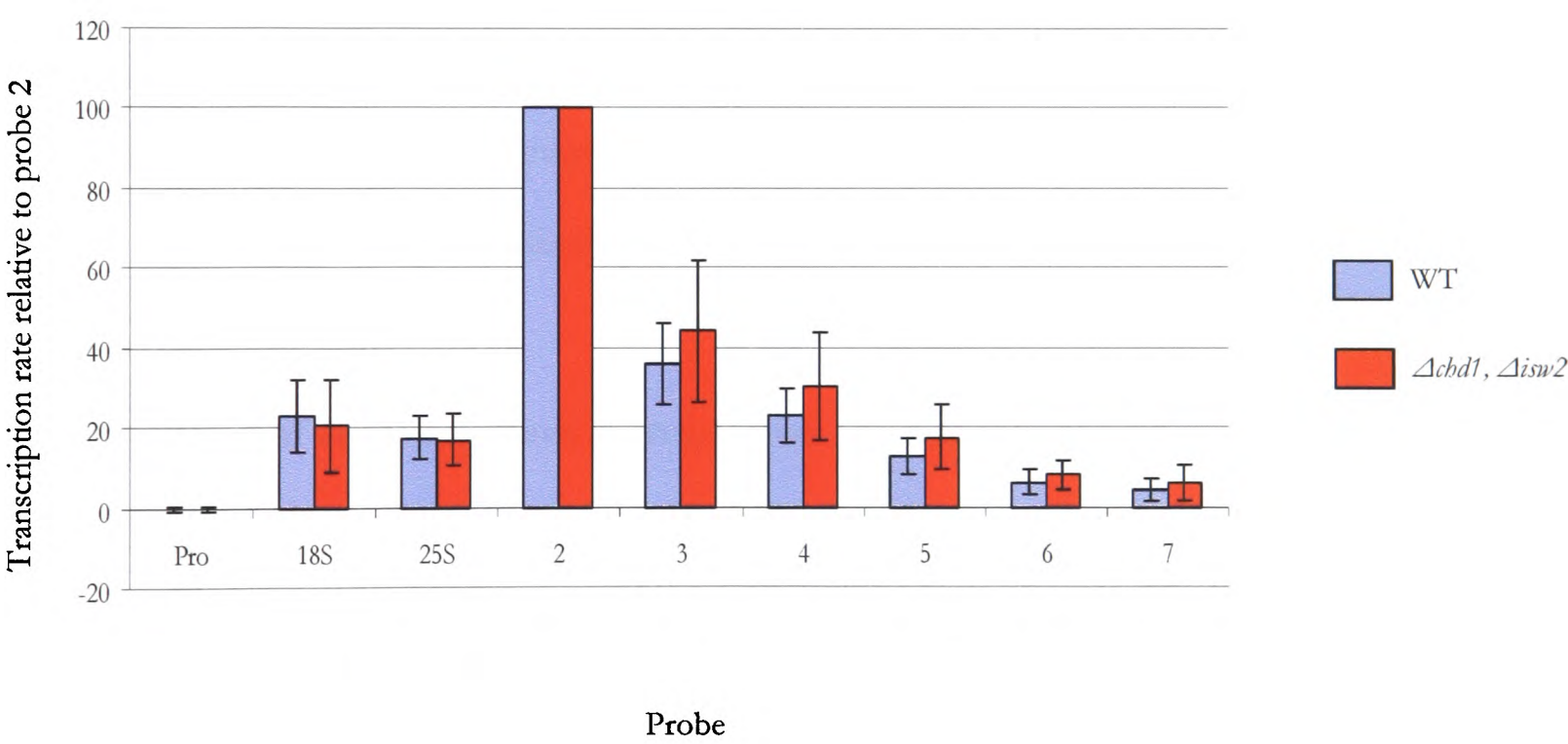


Figure 5.5.2: Transcription profile across the rDNA repeat in a YTT225 $\Delta chd1$, $\Delta isw2$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. (B) Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B



the $\Delta chd1 \Delta isw1$ double mutant (Figure 5.2.1), it is clear that loss of Chd1p and Isw2p does not cause a read-through phenotype or distort the transcription profile.

5.5.3 $\Delta isw1, \Delta isw2$

The final double mutant studied was YTT199, in which both Isw1p and Isw2p are deleted. A raw data set (Figure 5.5.3A) and average quantitated data (Figure 5.5.3B) are shown. As seen for the previous two double mutants, loss of Isw1p and Isw2p does not alter the transcription profile.

5.6 Summary of double mutant transcription run-on analysis

Figure 5.6 shows a composite graph of all double mutant profiles compared to that of the wild-type. It can be seen that all double mutants give a profile very similar to that seen for the wild-type. All show strong pausing over probe 2 indicating that Pol I in all strains continues to recognise the Reb1p pause protein and the T-rich pause element. In addition, elongation does not appear to be affected.

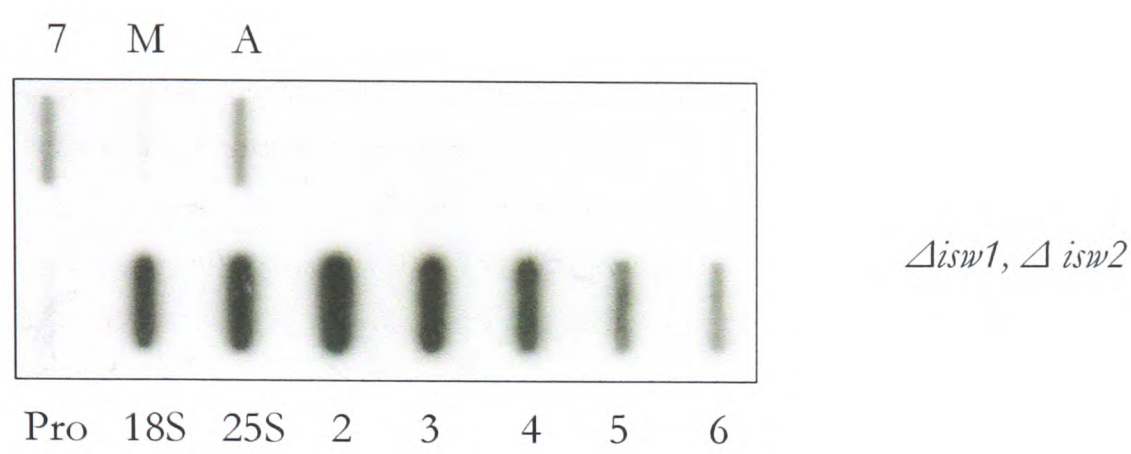
5.7 Analysis of transcription termination in $\Delta chd1, \Delta isw1, \Delta isw2$

triple mutant

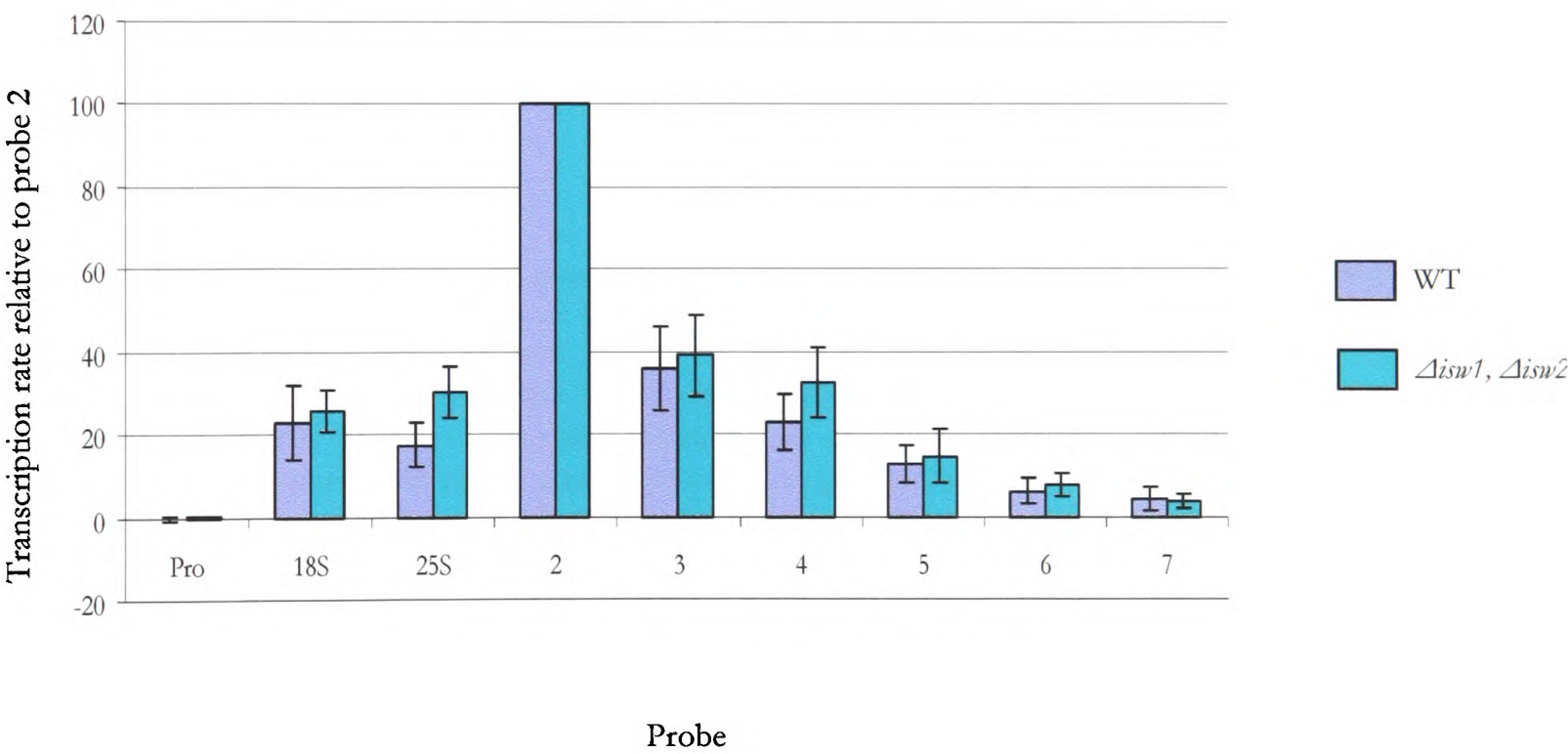
As shown above, loss of one or two chromatin remodeling factors does not lead to significant defects in transcription termination. This may indicate that one of the remodeling factors studied is able to compensate for loss of one or two of the other factors studied. This idea of redundancy amongst the three remodeling factors was investigated by carrying out TRO analysis on YTT227, which lacks Chd1p, Isw1p and Isw2p. A raw data set (Figure 5.7A) and average quantitated data (Figure 5.7B) are shown. If the raw data set is first considered, it can be seen that, in contrast to the single and double mutants, the triple mutant shows decreased pausing over probe 2. This probe spans the +93 primary

Figure 5.5.3: Transcription profile across the rDNA repeat in a YTT199 $\Delta isw1$, $\Delta isw2$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. (B) Average quantitation of multiple data sets (n=5) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B



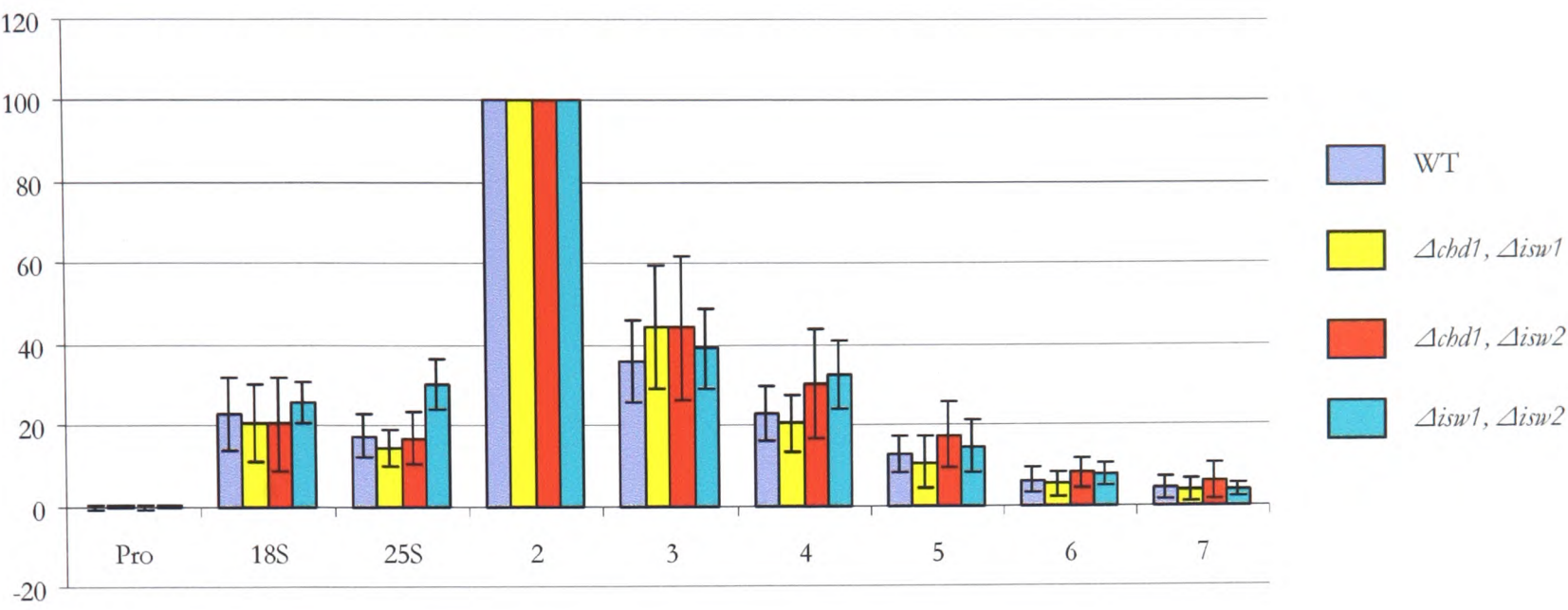
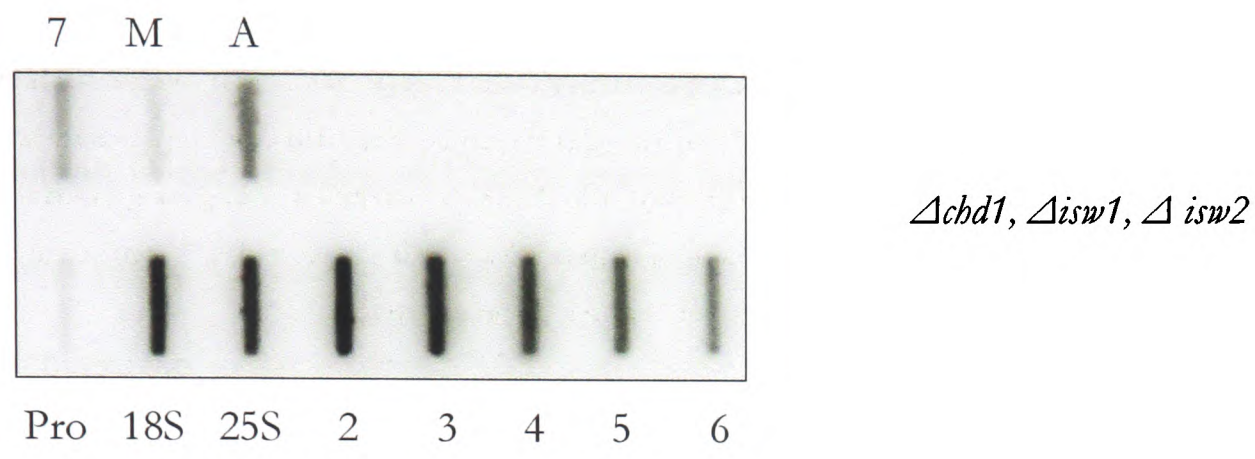


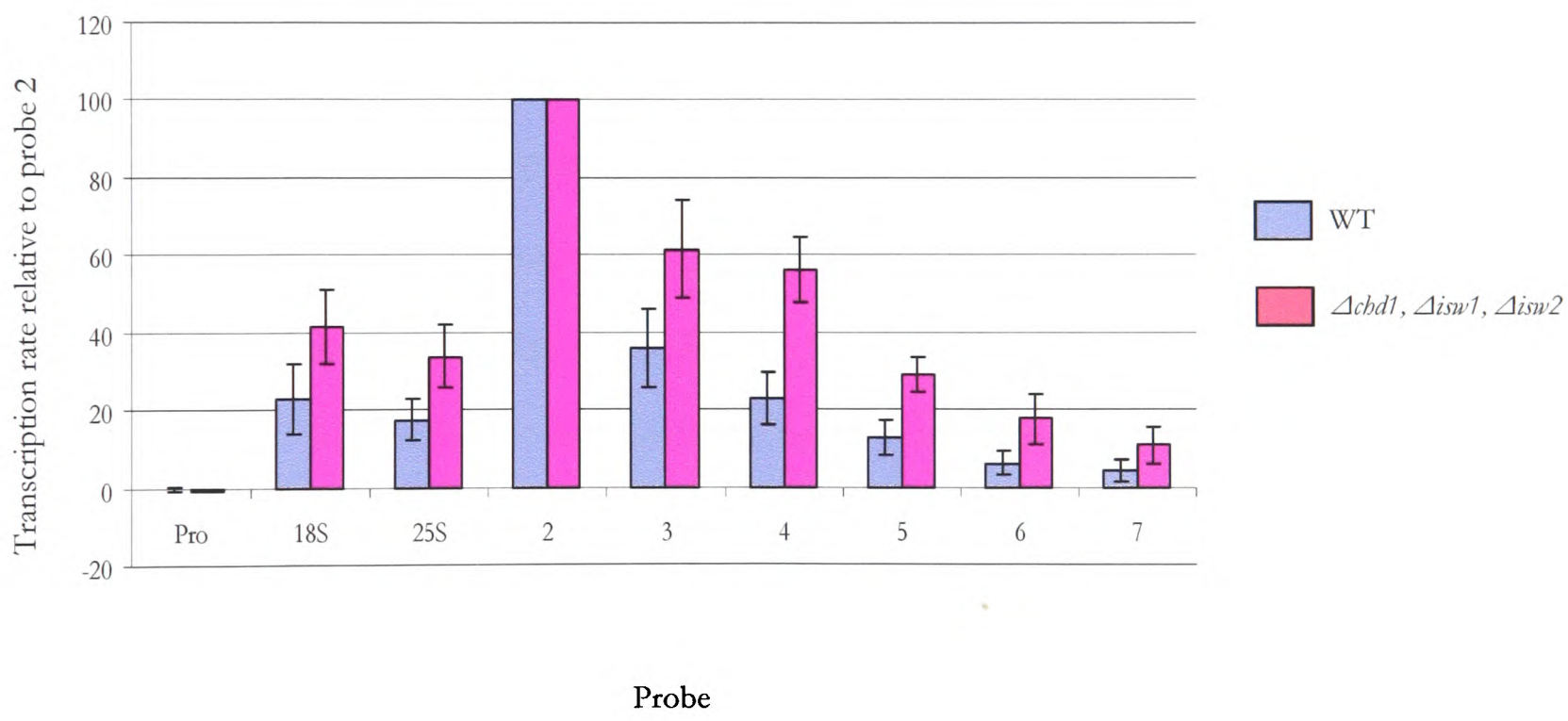
Figure 5.6: Composite graph of quantitated transcription run-on data from wild-type and double mutant strains. Transcription run-on profiles obtained from double mutant strains are not significantly different to the profile obtained from an isogenic wild-type strain, indicating that loss of two chromatin remodeling factors does not affect the transcription profile of RNA Polymerase I. Average quantitation of multiple data sets is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

Figure 5.7: Transcription profile across the rDNA repeat in a YTT227 $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ strain. (A) A representative data set showing hybridisation of radiolabeled rDNA transcripts to the membrane containing single stranded DNA probes. Probe “M” contains a fragment of *Schizosaccharomyces pombe* *URA4* gene and acts as a negative control. Probe “A” contains a fragment of *S. cerevisiae* *ACT1* gene and is used to monitor transcription levels. **(B)** Average quantitation of multiple data sets (n=4) is presented. Standard deviations are shown as black bars. Filters were exposed to a Phosphor-Imager screen and signals quantified using Image Quant software. Values obtained were corrected for the “U” content of each probe. Signals are plotted as a percentage of probe 2 (set to 100%) to allow for direct comparison between different experiments.

A



B



termination site and this decrease in signal indicates that the polymerase is reading through this site. If the quantitated data are next considered, where probe 2 is normalised to 100%, it can be seen that the $\Delta chd1$, $\Delta isw1$, $\Delta isw2$ triple mutant strain shows a significant (2-3 fold) increase in signal over the spacer (4-7). This confirms that Pol I reads past the normal site of termination in the absence of Chd1p, Isw1p and Isw2p. The greatest increase in signal is seen over probe 4, which spans the +250 failsafe termination site. Furthermore, increases in signal over the genic probes “18” and “25” suggest defects in elongation as well as termination.

5.8 Discussion

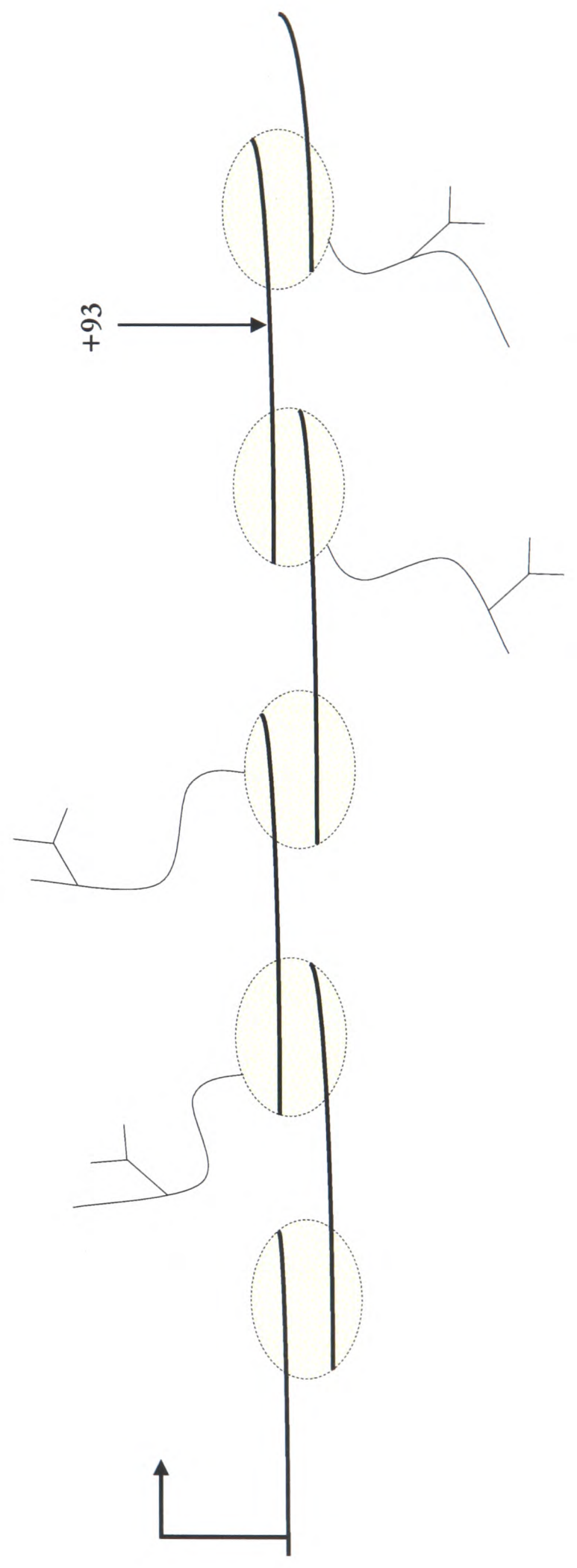
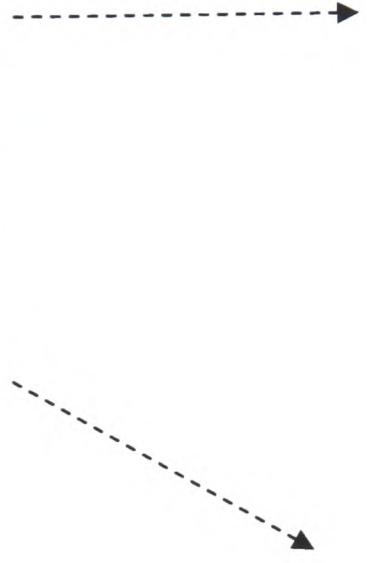
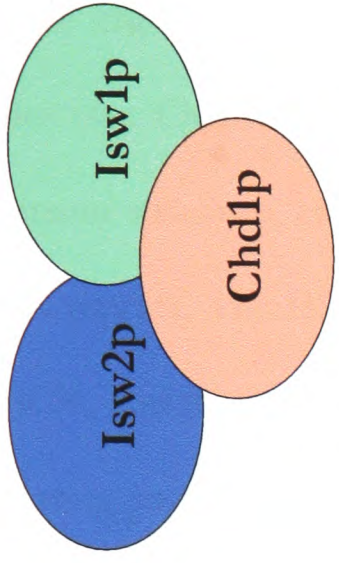
The aim of the first half of this chapter was to investigate the roles played by chromatin remodeling factors in Pol I transcription termination. This was achieved by employing TRO analysis, which gives a read-out of polymerase density across the region probed. Comparison of wild-type and mutant strains thus allows defects in transcription to be diagnosed. Here, TRO analysis was performed on strains lacking one, two or three of the chromatin remodeling factors studied (Chd1p, Isw1p, Isw2p). First, single mutant strains were analysed (Figures 5.3.1-.3). It was found that loss of a single remodeling factor did not lead to termination defects, with all strains showing strong pausing upstream of the Reb1p site (probe 2), little read-through downstream (probes 4-7) and no real distortion in elongation (probes 18S and 25S). This would initially suggest that these factors are not involved in Pol I transcription. However, the presence of the remaining two remodeling factors could compensate for the loss of the third. To investigate this, TRO analysis was repeated in double mutant strains (Figures 5.5.1-.3). Here, it was again found that loss of two remodeling factors does not lead to defects in termination, nor in elongation, with Pol I terminating as well as, or slightly better than wild-type. This suggested that redundancy may occur between all three remodeling factors. Thus the TRO analysis was repeated in a triple

mutant strain (Figure 5.7). In contrast to loss of single or double remodeling factors, it was found that loss of Chd1p, Isw1p and Isw2p leads to distortions in Pol I transcription, during both the termination and elongation phases. Increased signals are seen over the genic region, suggesting that Pol I is pausing, or is less able to transcribe processively. Over the termination region, Pol I pausing is decreased and the polymerase reads through past the normal +93 site of termination. The greatest increase in signal is seen over probe 4. This probe spans the +250 failsafe termination site which, in the wild-type case, is reached by only ~20% of polymerases. Loss of Chd1p, Isw1p and Isw2p however results in ~60% of all polymerases reaching the +250 failsafe terminator (probe 4). Furthermore, half of the polymerases that reach the +250 site read through even further down stream (compare probes 4 and 5). This indicates that whilst termination does still occur, it is occurring much further downstream than is seen in the wild-type.

All of the above data indicate that Chd1p, Isw1p and Isw2p act redundantly at the rDNA locus to facilitate efficient transcription, at both the level of elongation and termination. Redundancy amongst these three remodeling factors has been seen before at the Pol II-transcribed *GAL10-7* locus (Alen et al., 2002). Thus this result indicates a further similarity between Pol I and Pol II transcription. The role of Chd1p, Isw1p and Isw2p during elongation and termination is also consistent with the presence of these factors across the entire rDNA repeat.

The results of this chapter are summarised in Figure 5.8. The chromatin remodeling factors Chd1p, Isw1p and Isw2p act redundantly at the rDNA locus. They act during both elongation and termination to ensure efficient transcription.

Figure 5.8: Updated model of the role of chromatin remodeling factors in Pol I transcription. An active rDNA repeat is shown. The black lines represent actively transcribed rDNA organised into nucleosome-like structures composed of, at least, histones H2B and H3 (circles surrounded by dotted lines). The start site of transcription is indicated by the black arrow and the +93 primary termination site is indicated by the “+93” black arrow. The black wavy lines extending from the dotted circles represent the N-terminal histone tails. Black forked structures represent dimethylation of histone tails. Chd1p, Isw1p and Isw2p act redundantly, during both elongation and termination, to ensure efficient transcription by RNA Polymerase I.



Chapter 6: Concluding remarks

6.1 Conclusions

The aims of this thesis were to investigate the chromatin environment of rRNA genes during active transcription. Furthermore, the role of chromatin remodeling factors at this locus was investigated. This study found that modified histones are present across all-active repeats. The relative ratios of the histones studied, H3 and H2B, were unequal, suggesting that the chromatin structure of active repeats is dynamic. In light of previous disparate studies (see Chapter 1: Introduction), the results seen here would suggest that a form of chromatin structure, consisting of non-canonical, dynamic nucleosomes is present on active rDNA repeats. The H3 histones found on active rDNA repeats are methylated at both K4 and K36, and the patterns of methylation seen are compatible with those seen at Pol II loci, suggesting parallels between Pol II and Pol I transcription. The presence of histones across the rDNA locus and tail modifications known to recruit chromatin remodeling factors suggested both a need for these factors, and a means of recruiting them. Consistent with this, Chd1p, Isw1p and Isw2p are found at the rDNA locus. Their association is seen across the entire repeat and would suggest the involvement of these factors in both transcription elongation and termination. The role of these chromatin remodeling factors was assayed using transcription run-on analysis in strains deleted for the factors. The resulting nascent profiles of transcription indicated that these factors act redundantly to influence both elongation and termination phases. It is therefore possible to imagine a number of different scenarios to explain their role in efficient transcription. The density of polymerases on rRNA genes may require the temporary eviction of histones from the rDNA template. Thus the remodeling factors may act to chaperone the histones as Pol I elongates through the rRNA gene. The build-up of polymerase over the genic region in the absence of Chd1p, Isw1p and Isw2p is not as large as that seen over the termination region. Therefore it could alternatively be imagined that the remodeling factors function primarily during transcription

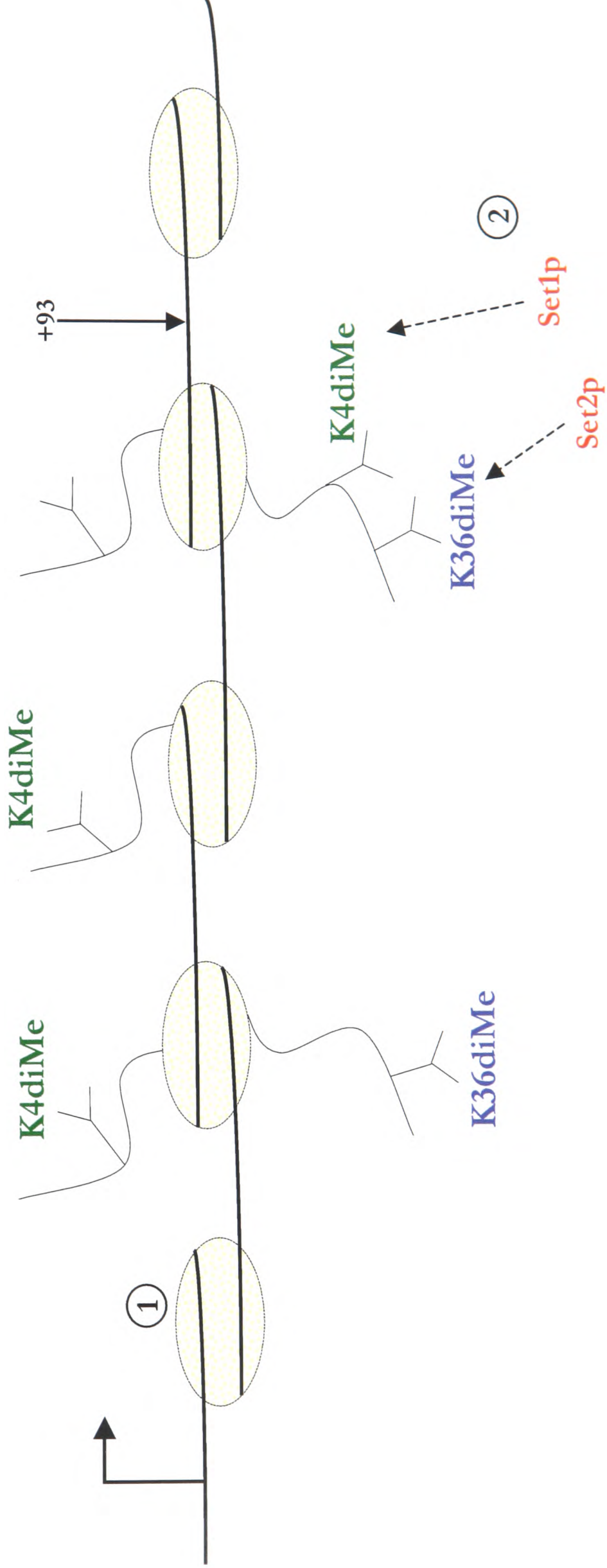
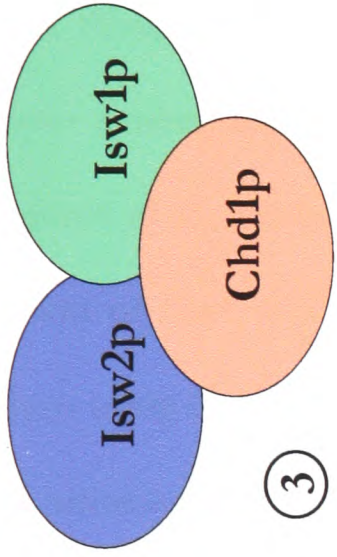
termination. They may be required to form a chromatin structure over the termination region that is refractory to the passage of Pol I downstream. These two hypotheses are not mutually exclusive and the remodeling factors may function during both elongation and termination. It should also be noted that whilst the high ChIP signals seen for histones H2B and H3 in the all-active strain indicate that these nucleosome components are present on active rDNA repeats, the ChIP analysis does not allow one to determine whether all 42 repeats in the all-active strain possess histones. Thus there may be a sub-fraction of active rDNA repeats that possess a chromatin structure and chromatin remodeling factors are required at these repeats to ensure high levels of rDNA transcription.

The work presented in this thesis allows an updated model of rDNA transcription to be presented (Figure 6):

- 1) Histones are present across actively transcribed rDNA repeats.
- 2) These histones show modifications that parallel those seen on actively transcribed Pol II genes. Furthermore, the modifications are compatible with the recruitment of chromatin remodeling factors.
- 3) Chromatin remodeling factors Chd1p, Isw1p and Isw2p are recruited to rDNA repeats.
- 4) These remodeling factors act redundantly, possibly during both elongation and termination, to ensure efficient Pol I transcription.

Figure 6: Updated model of the role of chromatin and chromatin remodeling factors in rDNA transcription.

1. Histones H2B and H3 are present across actively transcribed rDNA repeats.
2. The N-terminal tails of these histones undergo dimethylation at residues lysine 4 and lysine 36, catalysed by Set1p and Set2p respectively. These modifications parallel those seen on actively transcribed Pol II genes. Furthermore, the modifications are compatible with the recruitment of chromatin remodeling factors.
3. Chromatin remodeling factors Chd1p, Isw1p and Isw2p are recruited to rDNA repeats via interactions involving lysine 4 dimethylated histone tails and rRNA.
4. These remodeling factors act redundantly, possibly during both elongation and termination, to ensure efficient Pol I transcription.



6.2 Future prospects

The work presented in this thesis raises a number of questions which could be explored further:

6.2.1 Further analysis of chromatin structure of active rDNA repeats

ChIP analysis using core histone antibodies (Chapter 2) indicates that actively transcribed repeats possess both histones H2B and H3. The amounts of these histones found at active repeats, compared to inactive repeats, is unequal suggesting that the chromatin structure may be dynamic and non-canonical. The presence of histones H2B and H3 would lead one to expect though that histones H2A and H4 would also be present, especially in light of *Xenopus* studies indicating that H2A and H4 are present on actively transcribed repeats (Dimitrov et al., 1990; Dimitrov et al., 1992) and the requirement of histone H4 tails for the function of Isw1 and Isw2 complexes (Clapier et al., 2001; Fazzio et al., 2005). As ChIP analysis using commercial antibodies against these histones was unsuccessful, it would be of great interest to raise antibodies against histones H2A and H4 and to use these antibodies in further ChIP analysis. The presence and relative amounts of these histones on actively transcribed rDNA repeats would give insight into the nature of rDNA nucleosomes and the extent to which they are canonical. In addition, the analysis could be furthered to include the study of variant histones such as Htz1 and may give insights into the mechanism by which Pol I transcribes through its nucleosomal template.

Secondly, the analysis of histone tail modifications could be extended. Histone H2B is known to be ubiquitylated at K123 and at Pol II loci, this modification is a prerequisite for H3 K4 dimethylation (Dover et al., 2002; Sun and Allis, 2002). It would therefore be of great interest to investigate whether this modification is present at actively transcribed rDNA repeats, and if so, whether the presence of H3 K4diMe shows the same dependency on H2B ubiquitylation as seen for Pol II loci.

There are more than 30 residues known to undergo histone tail modification. In addition to the methylation studies carried out here, a much more comprehensive analysis could be carried out on active rRNA genes, with acetylation, arginine methylation and phosphorylation investigated. Thus the pattern of histone modifications could be analysed and comparisons made between the patterns seen for Pol I and Pol II.

For all of these additional analyses, it would be desirable to alter the sonication conditions to produce both smaller chromatin fragments and a smaller range of fragments. The sonication conditions used in this study produced fragments ranging in size from 100bp to 1kb, with an average size of 450bp. The production of smaller chromatin fragments would increase the resolution of the analysis and would decrease the likelihood of false positive signals being seen. This is particularly important over the termination region where the short distance between the end of the 25S gene and the 5S gene results in primer pairs 2+3, 5 and 7 being located close together.

6.2.2 Interactions between, and recruitment of, chromatin remodeling factors

The results presented here indicate that the chromatin remodeling factors Chd1p, Isw1p and Isw2p act to facilitate efficient transcription by Pol I. The redundant function of these factors at the rDNA locus is consistent with their physical interaction (Gavin et al., 2002). It is known that Chd1p travels with the elongating Pol II through interactions with the Paf1 complex and the elongation factors Spt4-5 and Spt16-Pob3 (Krogan et al., 2002a; Simic et al., 2003). As such elongation factors have not been identified for Pol I transcription, it would be of interest to investigate whether any of the remodeling factors interact with Pol I by carrying out co-immunoprecipitation experiments with tagged strains.

The recruitment of remodeling factors was also addressed in this study. As discussed in Chapter 3, Chd1p is recruited to the rDNA locus by a mechanism involving both H3 K4 dimethylation and rRNA. To study the role of rRNA further, binding assays could be

performed to investigate whether Chd1p can bind directly to rRNA. It would also be of interest to investigate whether Chd1p interacts with histone H3 tails dimethylated at K36, as has been proposed (Hampsey and Reinberg, 2003). If K36diMe tails are found to bind Chd1p, the question of recruitment could then be furthered to investigate whether K4diMe or K36diMe tails (or both) are responsible for recruiting Chd1p. To do this, the ChIP analysis could be repeated in a $\Delta set2$ background, as was done in Chapter 3 for the $\Delta set1$ background. Moreover, the impact of Chd1p deletion on K4 and K36 dimethylation could be investigated. By carrying out ChIP analysis for K4 and K36 in a $\Delta chd1$ strain, the temporal order of modifications could be probed. For instance, Set1p and K4 methylation may be required for recruitment of Chd1p whilst the presence of Chd1p may be required for further methylation at K36. Finally, further Isw1p and Isw2p studies could be carried out. It would be of interest to know whether Isw1p functions at the rDNA locus with any of its Ioc partners. This would give insight into the relative contributions of Isw1a and Isw1b complexes. In addition, ChIP analysis could be carried out using an antibody against the Itc1p, Dpb4p and Dls1p components of the Isw2 complex.

6.2.3 Recruitment of Set1p and Set2p

At Pol II loci, the function of Set1p is known to be dependent on the presence of a serine 5 phosphorylated CTD (Ng et al., 2002). Furthermore, the recruitment of Set2p is dependent on a serine 2 phosphorylated CTD (Krogan et al., 2003c; Li et al., 2003; Li et al., 2002; Ng et al., 2002; Schaft et al., 2003; Xiao et al., 2003). Thus the Pol II CTD plays a crucial role in both the recruitment and regulation of these methyltransferase activities. The A43 and A14 subunits of Pol I form a stalk structure that has been suggested to fulfil a similar role to the Pol II CTD (Bischler et al., 2002). Thus interaction studies with Set1p, Set2p and the A43/A14 subunits of Pol I may shed light on the recruitment of these methyltransferases to

the rDNA locus. Such a study could be particularly interesting for Set2p as the Pol II interaction domain has been identified (Kizer et al., 2005).

Chapter 7: Materials

Reagents

Most chemicals and reagents were purchased from BDH and were of ‘AnalaR’ grade.

Chemical reagents obtained from other sources are listed below:

Agar, technical	Difco
Agarose	Melford
Ampicillin	Sigma
Bactopeptone	BD
β-mercaptoethanol	Sigma
Bovine Serum Albumin (BSA)	Sigma
Bromophenol Blue	Sigma
Chloroform	Sigma
Complete, EDTA-free tablets	Roche
Deoxyribonucleotide triphosphates (dNTPs)	Invitrogen
Dithiothreitol (DTT)	Roche
Ethanol	BDH
Ethidium Bromide	Sigma
Formaldehyde	BDH
Glass beads, acid washed, 425-600μm	Sigma
Glucose	BDH
Hybond NX	Amersham-Pharmacia
Lambda DNA	Q BIOgene
PEG 3500, 8000	Sigma
Phenol	Sigma
Protein A-Sepharose CL-4B beads	Amersham

Quantitect Sybr Green mix	Qiagen
Ribonucleoside triphosphates	Roche
Salmon sperm DNA	Sigma
Sodium N-Lauryl sarcosine sulphate	BDH
Sorbitol	Sigma
Spermidine	Sigma
Tris	MP Biomedicals
Xylene Cyanol	Sigma
Yeast Extract	Oxoid

Enzymes

Restriction endonucleases were obtained from New England Biolabs. Other enzymes were obtained from the following suppliers:

Micrococcal nuclease	USB/Pharmacia
Proteinase K	Roche
RNase A/T1 cocktail	Ambion
<i>Taq</i> DNA Polymerase	Promega
Yeast lytic enzyme	MP Biomedicals

Antibodies

FLAG (F 3165)	Sigma
HA (clone F7)	Santa-Cruz Biotech.
c-Myc (clone 9E10)	Sigma
H2B	D. Bentley
H3 (ab1791)	Abcam

H3K36diMe (ab9049)	Abcam
H3K36diMe (07-369)	Upstate
H3K4diMe (07-030)	Upstate
Rna15	D. Barilla

Radioisotopes

[$\alpha^{32}\text{P}$] dATP	3000 Ci/mmol; 10 mCi/ml
[$\alpha^{32}\text{P}$] rUTP	3000 Ci/mmol; 10 mCi/ml

All radioisotopes were obtained from Amersham.

Kits

Qiagen DyeEX Kit	Qiagen
Qiaquick Gel Purification Kit	Qiagen
Qiagen PCR Purification Kit	Qiagen
Prime-It II Random primer labelling kit	Stratagene

Organic Reagents

Phenol pH 8, DNA work	Sigma
Phenol pH4.3, RNA work	Sigma

Molecular Weight Markers

1kb Plus DNA ladder	Invitrogen
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Centrifuges

50ml cultures were spun at 4°C in a Heraeus Multifuge 3s. 1ml and 1.5ml tubes were spun at room temperature in a Heraeus Biofuge Pico and at 4°C in a Sorvall Fresco.

***S. cerevisiae* media**

YP-medium (per litre)

Bactopeptone	20 g
Yeast extract	10 g
Glucose added to	2%

YP-plates (per litre)

Bactopeptone	20 g
Yeast extract	10 g
Agar	20 g
Glucose added to	2%

Solutions and Buffers

Gel Electrophoresis Buffers

50x TAE-Buffer

Sodium acetate	250 mM
EDTA	50 mM
Tris	2 M
Glacial acetic acid	91 ml/litre

10x Orange G dye

Ficoll	5% w/v
Orange G	2.5 mg/ml
EDTA	10 mM

5x TBE

Tris	50 mM
EDTA	10 mM
Boric acid	50 mM

Hybridisation solutions**50x Denhardt's**

Ficoll	1%
BSA	1%
Polyvinylpyrrolidone	1%

20x SSC

NaCl	3 M
Sodium citrate	300 mM
Adjusted to pH 7 with HCl	

20x SSPE

NaCl	3 M
NaH ₂ PO ₄	200 mM
EDTA	20 mM
Adjusted to pH 7.4 with NaOH	

Chromatin immunoprecipitation reagents

SDS buffer		
SDS		1%
EDTA		10mM
Tris-HCL pH 8		50mM
PMSF-EtOH		0.5mM
Complete, EDTA-free		1 tablet/5ml
IP buffer		
SDS		0.01%
Triton-X-100		1.1%
EDTA		1.2mM
Tris-HCl pH 7.5		16.7mM
NaCl		167mM
PMSF-EtOH		0.5mM
Complete, EDTA-free		1 tablet/5ml
TSE150 wash		
Triton-X-100		1%
SDS		0.1%
EDTA		2mM
Tris-HCl pH 8		20mM
NaCl		150mM

TSE500 wash

Triton-X-100	1%
SDS	0.1%
EDTA	2mM
Tris-HCl pH 8	20mM
NaCl	500mM

LiCl wash

LiCl	0.25M
NP-40	1%
Dioxycholate	1%
EDTA	1mM
Tris-HCl pH 8	10mM

Elution buffer

SDS	1%
NaHCO ₃	0.1M

CL-4B buffer

Tris-HCl pH 8	10mM
EDTA	1mM
BSA	10µg/ml

In vivo chromatin analysis reagents

Yeast Lytic Enzyme buffer

YLE	10mg/ml
Sorbitol	1M
β-mercaptoethanol	5mM

Sphaeroplast digestion buffer

Sorbitol	1M
NaCl	50mM
Tris-HCl pH 7.5	10mM
MgCl ₂	5mM
CaCl ₂	1mM
β-mercaptoethanol	1mM
Spermidine	0.5mM
NP40	0.075% v/v

Micrococcal nuclease dissolved at 15units/μl in

10mM Tris-HCl pH 7.5
10mM NaCl
100μg.ml BSA

Stop solution

SDS	5%
EDTA	250mM

Denaturing solution

NaCl	1.5 M
NaOH	0.5 M

Neutralising solution

NaCl	1.5 M
Tris-HCl, pH 7	0.5 M
EDTA	1 mM

Transcription run-on analysis reagents

AE Buffer

NaOAc	50mM
EDTA	10mM
Adjust to pH 5 with acetic acid	

TES

Tris-HCl, pH 7.5	10 mM
EDTA	10 mM
SDS	0.5%

2.5x Transcription Buffer

Tris-HCl, pH 7.4	50 mM
MgCl ₂	80 mM
KCl	500 mM

Hybridisation solution

SSPE	5x
Formamide	50%
Denhardt's Solution	10x
tRNA	100 µg/ml
SDS	0.2%

Denaturing solution (see above)

Neutralising solution (see above)

Yeast genomic DNA prep reagents

Breaking buffer

Triton-X-100	2%
SDS	1%
NaCl	100mM
Tris-HCl pH 8	10mM
EDTA	1mM

TE

Tris-HCl pH 8	10mM
EDTA	1mM

S. cerevisiae strains

Strain name	Genotype	Source	Use*
YTT166	MATa <i>ade2-1 can1-100 his3-11,15</i> <i>leu2-3,112 trp1-1 ura3-1 RAD5+</i>	T. Tsukiyama	CMT
YTT400	YTT166 with <i>cbd1::TRP1</i>	" "	MT
YTT186	YTT166 with <i>isw1::ADE2</i>	" "	MT
YTT196	YTT166 with <i>isw2::LEU2</i>	" "	MT
YTT223	YTT166 with <i>isw1::ADE2</i> <i>cbd1::TRP1</i>	" "	MT
YTT225	YTT166 with <i>cbd1::TRP1</i> <i>isw2::LEU2</i>	" "	MT
YTT199	YTT166 with <i>isw1::ADE2</i> <i>isw2::LEU2</i>	" "	T
YTT227	YTT166 with <i>cbd1::TRP1</i> <i>isw1::ADE2 isw2::LEU2</i>	" "	MT
YTT166 <i>set2</i>	YTT166 with <i>set2::KanMx</i>	J. Mellor	C

YHT117	MAT α <i>ura3-52 lys2-801 leu2-Δ1</i> <i>his3-Δ200 Δtrp1 pep4Δ::HIS3</i> <i>prb1-Δ1.6R</i>	A. Johnson	C
YHT149	YHT117 with <i>6MYC::6HIS::</i> <i>CHD1::TRP1</i>	A. Johnson	C
NOY1051	NOY886 but copy number $\sim$ 140	M. Nomura	C
NOY886	MAT α <i>rpa135Δ::LEU2 ade2-1 ura3-1</i> <i>his3-11 trp1-1 leu2-3,112 can1-100</i> <i>fob1Δ::HIS3 pNOY117 [CEN</i> <i>RPA135 TRP1]</i>	M. Nomura	C
AS14	MAT α <i>ade2 ura3-52 trp1Δ63 TRP1</i> <i>his3Δ200 leu2Δ1 lys2-801 RPA14-</i> <i>3HA</i>	M. Riva	C
UCC1001	MAT α <i>ade2-101 his3Δ-200 leu2Δ1</i> <i>trp1Δ1 lys2-801 TELadhb4::URA3</i>	J. Mellor	C
UCC1001 set1	UCC1001 with <i>set1::URA3</i>	J. Mellor	C
GHY773 CHD1-HA	MAT α <i>HA1₃-CHD1 his3Δ200</i> <i>lys2-128δ leu2Δ1 ura3-52</i>	G. Hartzog	C

GHY773 CHD1-HA	GHY773 with <i>set1::KanMX</i>	G. Hartzog	C
<i>Δset1</i>			
CEN.PK2-1D	MATα <i>ura3-52 trp1-289 leu2-3,112</i>	J. Mellor	C
	<i>his3Δ1 MAL2-8^c SUC2</i>		
Isw1-Myc	CEN.PK2-1D with <i>ISW1::13Myc::</i>	J. Mellor	C
	<i>KanMX6</i>		
Isw2-Myc	CEN.PK.2-1D with <i>ISW2::13Myc::</i>	" "	C
	<i>KanMX6</i>		
YTT1401	YTT166 with <i>Mata ste2::HygB</i>	T Tsukiyama	C
	<i>ste3::KanMX</i>		
YTT2164	YTT166 with <i>Mata ste2::HPH</i>	" "	C
	<i>ste3::NAT isw2-K215R-3FLAG-</i>		
	<i>KanMX</i>		

* C = used in ChIP experiments, M = used in MNase experiments, T = used in TRO experiments

Chapter 8: Methods

Chromatin immunoprecipitation analysis

The analysis was carried out according to (Morillon et al., 2003b) with a number of modifications and is summarised below.

Chromatin isolation

50ml cultures were grown to an OD₅₉₅ of 0.7-1.0 (density of 1.5-4.0 x 10⁷ cells/ml). 1.35ml formaldehyde was added to each culture and incubated with agitation at room temperature for 6 minutes. Cells were then rapidly harvested at 2,500 rpm for 2.5 minutes. Cells were washed in 30ml cold PBS and reharvested. A second wash in 10ml PBS and reharvesting followed. The PBS was poured off to leave ~2ml, which was used to resuspend the cell pellet. Cells were then transferred to round-bottomed Eppendorf tubes.

Cells were centrifuged at 13,000 rpm at 4°C, PBS was removed and cells were resuspended in 250µl SDS buffer on ice. 500µl glass beads were added and cells were vortexed 8x 30 seconds, with 1 minute rests on ice. A further 250µl SDS buffer was then added. Eppendorf tubes were needle pierced and placed in 15ml Falcon tubes (containing second Eppendorf tube to catch flow-through). Falcon tubes were spun at 2,000 rpm for 30 seconds. 25µl “pre-sonication” sample was removed for sonication analysis.

Sonication

Sonication was performed using a Diagenode bioruptor. Sonication was carried out at 4°C, with 20 cycles of 10 seconds ON/30 seconds OFF. Sonication conditions were optimised to produce sonicated fragments with a size range 100bp-1kb and an average size of ~450bp.

Antibody addition and RNase A treatment

Sonicated samples were centrifuged for 10 minutes at 13,000 rpm and the supernatant transferred to 15ml Falcon tubes. 25µl “post-sonication” sample was removed for sonication analysis.

5ml cold IP buffer was added to the supernatant, followed by 15 minutes incubation on ice. Samples were then centrifuged at 3,500 rpm for 10 minutes and the supernatant transferred to fresh Falcon tubes. The supernatant was aliquoted into 4 Eppendorf tubes: 300µl TOT (Total input DNA) and genomic DNA, and 1ml IP (Immunoprecipitated sample) and NO (No antibody control). TOT and genomic DNA samples were stored at -20°C. Antibody was added to IP sample only. IP and NO samples were then incubated on a rotating wheel at 4°C overnight.

The RNase A protocol is adapted from Abruzzi et al. (2004) and is performed prior to antibody addition. For RNase A treatment, 2 sets of samples were aliquoted. To one set of TOT, IP and NO, RNase A was added at the following volumes; TOT 6µl, IP and NO 19µl. Both sets of samples were incubated at room temperature for 30 minutes. Antibody was then added as described above.

Volumes of antibody added

FLAG	10µl
HA	20µl
c-Myc	5µl
H2B	2µl
H3	5µl
H3K36diMe	5µl
H3K4diMe	5µl
Rna15	5µl

Isolation of chromatin:antibody complexes

To all samples was added: 6µl 0.4mg/ml sonicated λ DNA and 40µl ProteinA-sepharose beads in CL-4B buffer. Samples were then incubated on rotating wheel at room temperature for 90 minutes.

Beads were then washed with 950µl of the following buffers for 3 minutes (incubation on the wheel) followed by centrifugation at 13,000 rpm for 1 minute: TSE-150, TSE-500, LiCl. 950µl dH₂O was added and beads were transferred to new Eppendorfs followed by centrifugation. 250µl elution was buffer was added to the beads followed by incubation on the wheel for 15 minutes. Beads were centrifuged and supernatant was transferred to new Eppendorf tubes. Beads were then incubated with further 250µl elution buffer followed by centrifugation and removal of supernatant as before.

Sonication of λ DNA and preparation of Protein A-Sepharose CL-4B beads

λ DNA was sonicated at an amplitude of -20 with 4 cycles of 10 seconds ON/30 seconds OFF using a Diagenode Bioruptor.

500µl Protein A-Sepharose CL-4B beads were dissolved in 10ml PBS then centrifuged for 5 minutes at 3,000 rpm. PBS was removed and beads resuspended in 1ml CL-4B buffer. Beads were centrifuged for 1 minute at 3,000 rpm and buffer removed. Beads were resuspended in 500µl CL-4B buffer and stored at 4°C for less than a month.

Proteinase K treatment and reversal of cross-links

Genomic DNA and TOT samples were thawed out. To genomic DNA, TOT, IP and NO samples was added 0.5µl proteinase K, 0.2M NaCl and 4mM Tris-HCl pH7.5. Samples were incubated at 42°C for 1 hour to achieve protein degradation, followed by overnight incubation at 65°C to reverse cross-links.

DNA isolation

DNA was isolated using a Qiaquick PCR purification kit (Qiagen). Samples were eluted in 50µl dH₂O.

Analysis of sonication

25µl dH₂O was added to “pre-sonication” and “post-sonication” samples. Samples were treated with proteinase K and cross-links were reversed as described above. Samples were then run on a 1% agarose gel (see below for details of Gel Electrophoresis). Comparison between pre- and post-sonication samples ensured sonication had occurred efficiently and that the resultant fragments were of a size range 100bp – 1kb, with an average size of ~450bp.

Real-time PCR analysis

Real-time PCR analysis was carried out on the Corbett Rotorgene 3000 system. TOT DNAs were diluted 1/20, IPs and NOs were diluted 1/5. All samples were run in triplicate. Standards were made by serial dilution of genomic DNA sample. All primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are detailed below. Primers were tested by both normal PCR and real-time PCR analysis. A melt-curve analysis was performed for each analysis to ensure the integrity of the PCR products.

Reaction mix

	Stock	Volume used (μl)
Sybr Green mix	2x	7.5
Forward primer	5μM	0.75
Reverse primer	5μM	0.75
Template		2
dH ₂ O		4
		15

Cycling parameters

95°C	15 minutes	
95°C	30 seconds	45 cycles
57.5°C	30 seconds	
72°C	30 seconds	

Quantitation

Relative fluorescent intensities were calculated from:

$$[(\Sigma \text{ IP signals} / \Sigma \text{ IP samples}) - (\Sigma \text{ NO signals} / \Sigma \text{ NO samples})] / [(\Sigma \text{ input signals} / \Sigma \text{ input samples}) - (\Sigma \text{ NO signals} / \Sigma \text{ NO samples})].$$

To calculate the percentage of ChIP signal contributed by an all-active strain relative to a wild-type strain, the relative fluorescent intensity obtained for the all-active strain over a given region was divided by that obtained for the wild-type strain and multiplied by 100 as shown: [(AA/WT)*100].

Primer pairs for chromatin immunoprecipitation analysis

Primer	Sequence	Location*	Size (bp)
<i>rDNA locus</i>			
3' of 5S	FP 5' TGGTGCAAAAGACAAATGGA 3'	-954/-702	251
	RP 5' CCGTCCCTCCAAATGTAAAA 3'		
Pro	FP 5' GCACCTGTCACTTTGGAAAAA 3'	-203/45	249
	RP 5' TTCGTTTCCAAACTCTTTTCG 3'		
18,1	FP 5' AAACGGCTACCACATCCAAG 3'	1098/1344	247
	RP 5' GGCCCAAAGTTCAACTACGA 3'		
18,2	FP 5' GATGCCCTTAGACGTTCTGG 3'	2134/2374	241
	RP 5' GGCCTCACTAAGCCATTCAA 3'		
5.8	FP 5' CCCAGAGGTAACAAACACAAAC 3'	2770/3020	251
	RP 5' GGAAATGACGCTCAAACAGG 3'		
25,1	FP 5' AAAAGAAACCAACCGGGATT 3'	3310/3551	242
	RP 5' CCCACTTAGAGCTGCATTCC 3'		
25,2	FP 5' CCGAATGAACTAGCCCTGAA 3'	4533/4778	246
	RP 5' CGACTAACCCACGTCCAAC 3'		

25,3	FP 5' GCC CCC TTGATTTTGATTTT 3' RP 5' ACC CAG CTCACGTTCCCTAT 3'	5956/6203	248
2+3	FP 5' CAACGGGGTATTGTAAGCAG 3' RP 5' CTCTTACACACTATCATCCT 3'	6571/6819	248
5	FP 5' AGGGCTTTCACAAAGCTTCC 3' RP 5' TGTCCCCACTGTTCACTGTT 3'	6918/7163	246
7	FP 5' GATGGCAAGTTCCAGAGAGG 3' RP 5' CTTATTCCTTCCCGCTTTCC 3'	7214/7457	244
<i>Pol II loci</i>			
CYC1 5'	FP 5' TTAGGCTCGAGAACAATAGGG 3' RP 5' GATGTCGTCTCACACGGAAA 3'	-689/-449	241
CYC1 3'	FP 5' CATCCGCTCTAACCGAAAAG 3' RP 5' GATGATGAGAGCGACGATGA 3'	400/644	245
ISY1	FP 5' CCACTGCCATCTCTTCCATT 3' RP 5' TTTCAAGAGCAGCAAGCAGA 3'	42/281	240
INO1	FP 5' TCACATGGAGCAGAGAAAGC 3' RP 5' CACGTAGTCTTGAACAGTGG 3'	-179/164	343

INO1 G FP 5' AACCGGATTTCATTCAAA 3' 1282/1531 250
RP 5' CTAAGGCGGTTCTTTGCTTG 3'

* All coordinates are given with respect to the start of the 35S transcript

Note. Primers were designed to cover similar regions to those covered by the transcription run-on probes (see below). Therefore the following ChIP and TRO probes span comparable regions of the rDNA locus:

ChIP primer pair	TRO probe
Pro	Pro
18,2	18S
25,3	25S
2+3	2, 3
5	5, 6 and part of 7

ChIP primer pair 7 is therefore downstream of the region covered by TRO probe 7.

Gel electrophoresis (agarose)

Samples in 1x Orange G loading dye were run on 1% agarose/TAE buffer gels. Gels contained 1µg/ml ethidium bromide. Gels were run at a maximum of 200mA and visualised and photographed using a Syngene Gene Genius Bioimaging system.

For isolation of fragments from gels, a 254nm UV light box was used and the gel fragment excised using a razor blade. DNA was isolated using a Qiaquick Gel Extraction kit (Qiagen).

Genomic DNA prep

1ml cultures were grown until stationary phase, then harvested at 2,500 rpm for 3 minutes. Cells were resuspended in 200µl breaking buffer and transferred to an Eppendorf. 200µl glass beads and 200µl phenol:chloroform (pH 8) were added. Cells were vortexed at full speed for 3 minutes. 200µl TE was added and samples were mixed. Samples were then centrifuged at 13,000 rpm for 5 minutes. The upper phase was removed and transferred to a new Eppendorf tube. DNA was precipitated with two volumes cold ethanol and 0.3M sodium acetate at -20°C. The pellet was then resuspended in an appropriate amount of H₂O.

Growth of *S. cerevisiae*

S. cerevisiae were grown in YPD at 30°C with shaking.

In vivo chromatin analysis

The analysis was carried out according to Kent et al. (1993) and Wu and Winston (1997), using the rapid sphaeroplasting technique of Kent and Mellor (1995). Indirect-end labelling was carried out as described in Kent et al. (1994).

Cell harvest and chromatin digestion

100ml cultures were grown to mid-log phase ($1.0-4.0 \times 10^7$ cells/ml) and counted using a haemocytometer. 1.2×10^9 cells were harvested at 2,500 rpm for 3 minutes. The media was decanted, leaving ~1ml in which the pellet was resuspended. Cells were transferred to a round-bottomed Eppendorfs and centrifuged to remove remaining media. Cell pellet was resuspended in 950µl Yeast Lytic Enzyme buffer. Sphaeroplasting was carried out by gently inverting the tube for a predetermined length of time (in the range of 20-60 seconds). Cells were pelleted by centrifuging for 5 seconds at 13,000 rpm followed by removal of the YLE buffer. Cells were washed twice with 950µl 1M sorbitol then resuspended in 1.2ml

Sphaeroplast Digestion Buffer. 200µl aliquots (containing 2×10^8 cells) were transferred to Eppendorf tubes containing either Micrococcal nuclease or Stop solution as shown below.

Tube	Addition of	Concentration units/µl	Use
1	MNase	150	Sample 1
2	MNase	300	Sample 2
3	Stop	-	Naked DNA
4	Stop	-	Markers

All tubes were incubated at 37°C for 4 minutes. Digestion in tubes 1 and 2 was halted by addition of 20µl Stop solution.

DNA purification

200µl phenol:chloroform (pH 8) was added to each tube. Tubes were centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a new Eppendorf containing 5µl 10mg/ml RNase A. Samples were mixed well and incubated at 37°C for 30 minutes. A further 200µl phenol:chloroform (pH 8) was added and centrifugation performed to separate the phases.

The aqueous phase was transferred to a new Eppendorf and the DNA precipitated with 1.5M ammonium acetate and 1 volume isopropanol. DNA was pelleted by centrifuging at 13,000 rpm for 10 minutes. DNA was then washed with 190µl 80% ethanol followed by centrifugation and aspiration of the ethanol. DNA pellets were dried at room temperature for 5 minutes and the DNA resuspended in 18µl TE.

Naked DNA controls

300µl SDB was added to an 18µl sample of undigested, purified DNA (tube 3 above). 2µl of 1:20 dilution of MNase was added and incubated for a predetermined length of time (in range of 20-40 seconds). Digestion was terminated by the addition of 300µl phenol:chloroform (pH 8). DNA was then purified and washed as described above and resuspended in 18µl TE.

Markers

3x 5µl aliquots were taken from an 18µl sample of undigested, purified DNA (tube 4 above). Each aliquot was digested with an appropriate restriction enzyme (as described below). DNA was purified by phenol:chloroform (pH 8) as described above. Aqueous phases were pooled and the DNA precipitated as described above. DNA was finally resuspended in 18µl TE.

End-digest and gel

All samples (MNase digested/Naked DNA controls/markers) were digested overnight with appropriate end-digest restriction enzyme. DNA loading buffer (containing bromophenol blue only). MNase digested and naked samples were loaded onto a 1.5% TBE-agarose gel with 4µl of the markers. The gel was run at a constant current of no more than 6mA/cm for approximately 4 hours, until the bromophenol blue was 1cm from the bottom of the gel.

Southern blot

The gel was denatured with 2x 15 minute treatments with denaturing solution. The gel was then rinsed with water and neutralised with 2x 15 minutes treatments with neutralising solution. The gel was then blotted overnight in 20xSSC to MAGNA nylon hybridisation membrane. The membrane was then baked at 80°C for 2 hours.

Production and random priming of end-label

DNA fragment containing chosen restriction enzyme site (see below) was amplified up by PCR and purified using the Qiaquick Gel Extraction kit (Qiagen). DNA was then digested by the appropriate restriction enzyme as described and purified as described above.

The end-label was random primed using the Prime-It II kit (Stratagene) and [$\alpha^{32}\text{P}$] dATP and purified using the DyeEX kit (Qiagen).

Hybridisation of end-label and washing

The membrane was pre-hybridised at 64°C for 60 minutes in 1.5x SSC, 5x Denhardt's and 0.1% SDS. The end-label was boiled in 250-500 μl 2.5mg/ml salmon sperm DNA for 5 minutes and quenched on ice for 2 minutes. The end-label/salmon sperm DNA mix was then added to the pre-hybridisation solution and incubated at 64°C overnight. The membrane was washed 1x in 100ml 2xSSC and 0.1% SDS at 60°C for 20 minutes and 2x in 100ml 2x SSC and 0.1% SDS at 64°C for 15 minutes. The membrane was then sealed in Saranwrap and exposed to Fuji RX film at -80°C for an appropriate length of time.

Experimental design

S. cerevisiae rDNA sequence was scrutinised to locate restriction enzyme sites for use as end-digest and marker enzymes. Maximum resolution achieved with this technique is ~2kb from the location of the end-digest restriction enzyme. Within this distance, 3 further restriction enzymes are chosen. These enzymes individually digest purified DNA which is then pooled and used as markers. There should be only a single occurrence of cutting by each of the enzymes within 2kb, up- and downstream of the end-digest enzyme.

Analysis of rDNA termination region:

End-digest enzyme = *Ava*II (+5814bp)

Marker enzyme I = *Afl*III (+6765bp)

Marker enzyme II = *Ava*I (+6937bp)

Marker enzyme III = *Hpa*I (+7881bp)

(Coordinates denote the position of enzyme cleavage and are given relative to the start site of transcription).

To generate the end-label, PCR was performed to generate a DNA fragment of ~1kb. The PCR mix was run on a 1% TAE-agarose gel and purified by Qiaquick Gel Extraction (Qiagen) and then digested overnight with *Ava*II. The digestion mix was run on a gel and the ~400bp digestion product purified as before.

End-label to probe rDNA termination region:

Forward primer 5' CAG GTC GTA CTG ATA ACC 3'

Reverse primer 5' CTC ACG ACG GTC TAA ACC 3'

Size of product = 1141bp

Size of products after *Ava*II digest = 405bp, 736bp

Purified product for use as end-label fragment = 405bp

Maxipreps**M13 maxiprep**

After 6 hours growth, 2ml of XL1 blue culture was added to 50µl of phage stock and incubated at room temperature for 5 minutes. The mix was then added to 200ml 2x TY and

grown with shaking at 37°C for 6 hours. The culture was then centrifuged at 10,000 rpm for 15 minutes in a Beckman ultra-centrifuge. The supernatant was decanted and 50ml 20% PEG 8000/2.5M NaCl was added, followed by overnight incubation at 4°C. The mixture was next spun down at 13,000 rpm for 30 minutes. The supernatant was discarded and the pellet resuspended in 600µl TE. The sample was then centrifuged at 13,000 rpm for 1 minute and the supernatant transferred to a new Eppendorf tube. ss DNA was extracted with 1x phenol, then 1x phenol:chloroform and finally with 1x chloroform (all phenol pH 8). The ssDNA was ethanol precipitated and the pellet was resuspended in 150µl TE. The concentration of ssDNA was determined by spectrophotometer at OD₂₈₀.

Phenol:chloroform extraction/ethanol precipitation

Equal volumes of phenol (pH 4.3 for RNA work, pH 8 for DNA work) and chloroform was mixed. An equal volume of this phenol:chloroform solution was added to the sample, vortexed vigorously and centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a new Eppendorf tube and the process repeated until a clean interface was seen between aqueous and organic phases. The DNA was then precipitated with 0.3M sodium acetate and 2.5 volumes 100% ethanol at -20°C for 30 minutes. The DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed in 70% ethanol, followed by 2 rounds of centrifugation at 13,000 rpm to ensure all ethanol was removed. The pellet was then dried at room temperature for 5 minutes and resuspended in an appropriate amount of dH₂O.

PCR

Conventional PCR

PCR reactions were carried out in a final volume of 100µl containing 2µl DNA template (variable concentration), 0.4mM dNTP, 100 pmol forward and reverse primer, 2mM MgCl₂,

1x Mg Free Thermophilic DNA Polymerase Buffer (Promega), 5 U *Taq* DNA Polymerase and the remainder of the volume with dH₂O.

PCR reactions were performed on a Biometra TRIO-Thermoblock PCR machine with a heated lid. Cycle parameters are shown below:

Initial denature	95°C	5 minutes	
Denature	95°C	45 seconds	30 cycles
Anneal	X°C	1minute	
Elongation	72°C	30 seconds/ 500bp amplicon	
Final elongation	72°C	3 minutes	

The annealing temperature was calculated using the formula [4GC + 2xAT]-5. The final elongation step of 3 minutes allowed elongation to go to completion.

Real-time PCR

See “Real-time PCR analysis” in Chromatin immunoprecipiation section.

Transcription run-on analysis

Transcription Run-on analysis was performed as described in Birse et al. (1997) and is summarised below.

Harvesting cells

Cultures were grown to an OD₅₉₅ of 0.05-0.15 and harvested by centrifugation at 2,500 rpm for 3 minutes. Pellets were washed with ice cold dH₂O and resuspended in 950µl dH₂O.

50µl 10% sodium N-Lauryl sarcosine sulphate was added, the tube was gently inverted six times to mix and incubated for 20 minutes (all done in 4°C cold room). Cells were pelleted by centrifugation at 5,000 rpm for 1 minute.

***In vitro* transcription**

Cells were resuspended in 60µl 2.5x transcription buffer, 8µl of an ACG rNTP mix (10mM each) and 3µl 100mM DTT. Upon addition of 4µl [$\alpha^{32}\text{P}$]-rUTP, the mixture was incubated at 30°C for 5 minutes. Addition of 900µl AE buffer stopped the transcription reaction. Cells were the pelleted by centrifugation at 5,000 rpm for 2 minutes.

RNA extraction

Cells were resuspended in 400µl TES buffer, transferred to a tube containing 400µl phenol pH4.3 at 65°C. The mixture was incubated at 65°C for 90 minutes with vortexing every 15 minutes. Tubes were transferred to ice for 5 minutes before centrifugation at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a new Eppendorf tube containing 400µl phenol: chloroform pH 4.3. After centrifugation at full speed as above, the aqueous phase was removed and transferred to a new Eppendorf tube, with further extractions carried out as necessary to ensure a clean interface between aqueous and organic phases. After the final extraction, the RNA was precipitated by a 30 minute incubation at -20°C with 1/10 volume 5M LiCl and 2.5 volumes ice cold ethanol.

RNA hydrolysis

The pellet was pelleted by centrifugation at 13,000 rpm for 15 minutes. After resuspension in 40µl dH₂O, partial hydrolysis of the RNA was achieved by addition of 10µl 1M NaOH with incubation on ice for 5 minutes. The mixture was then neutralised by addition of 20µl 0.5M Tris/0.5M HCl.

Hybridisation

To each sample was added 10µg M13 with an *S. pombe* *URA4* insert, followed by 650µl hybridisation solution. The tubes were incubated at 42°C for 90 minutes with shaking. After incubation, the sample was added to the pre-hybridised filters (see below) and incubated overnight at 42°C in a mini-Hybaid oven.

Filter Preparation

Filters were made with a GIBCO-BRL hybrislot manifold apparatus and Hybond-NX membrane. The membrane was prewet in 5x SSPE and assembled in the manifold. Wells were rinsed with 400µl 5x SSPE.

Single-stranded M13 probes (Prescott et al., 2004) were diluted to 5µg/well in 200µl 5x SSPE. Probes were applied to wells and washed with 400µl 5x SSPE. The manifold apparatus was disassembled with the suction still on and the membrane removed.

The DNA was denatured by immersion in denaturing solution for 5 minutes followed by neutralisation with neutralising solution for 2 minutes. The membrane was air dried and the DNA fixed to the membrane by UV cross-linking using a Stratagene UV Stratalinker 1800. The filters were cut from the membrane and added to tubes containing hybridisation solution and 1µl 10mg/ml tRNA. Filters were incubated at 42°C in a mini-Hybaid oven for at least 3 hours prior to addition of hot RNA.

Filter washing

Filters were washed 4x 10 minutes in 2x SSC and 0.1% SDS at room temperature. The filters were then removed, wrapped in Saranwrap and exposed to a Molecular Dynamics Phosphor storage cassette for a minimum of 24 hours. The Phosphor screen was analysed by a PhosphorImager and data quantified using ImageQuant software. Filters were also exposed to BioMax MR film at -80°C overnight.

M13 probes for transcription run-on analysis

Probe	Location*	Size (bp)	U content
Pro	-85/-310	225	61
18S	2070/2284	214	54
25S	5953/6152	199	62
2	6592/6730	138	29
3	6701/6847	146	47
4	6804/6972	168	40
5	6902/7075	173	49
6	6948/7128	180	57
7	6999/7198	199	66

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