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RNA polymerase I transcription on nucleosomal templates: the transcription termination factor TTF-I induces chromatin remodeling and relieves transcriptional repression

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Eukaryotic ribosomal gene promoters are preceded by a terminator element which is recognized by the transcription termination factor TTF-I. We have studied the function of this promoter-proximal terminator and show that binding of TTF-I is the key event which leads to ATP-dependent nucleosome remodeling and transcriptional activation of mouse rDNA preassembled into chromatin. We have analyzed TTF-I mutants for their ability to bind to free or nucleosomal DNA, and show that the DNA binding domain of TTF-I on its own is not sufficient for interaction with chromatin, indicating that specific protein features exist that endow a transcription factor with chromatin binding and remodeling properties. This first analysis of RNA polymerase I transcription in chromatin provides a clue for the function of the upstream terminator and establishes a dual role for TTF-I both as a termination factor and a chromatin-specific transcription activator.

Keywords: chromatin remodeling/RNA polymerase I/ transcription/transcription termination factor TTF-1

Introduction

The genes that code for eukaryotic ribosomal RNA (rDNA) are arranged in large clusters of tandem repeats in which the pre-rRNA coding region alternates with an intergenic spacer region. The intergenic spacer contains all of the elements that regulate transcription by RNA polymerase I (pol I). Even though the sequence and location of regulatory elements that govern efficient transcription vary considerably between species (for review, see Paule, 1994), the overall structural organization of the rDNA repeats is very similar. Several types of regulatory elements have been identified in the intergenic spacer, including (i) the gene promoter at the 5′ end of the pre-rRNA coding region, (ii) a transcription terminator immediately upstream of the gene promoter, (iii) enhancer elements that stimulate transcription, (iv) one or more spacer promoters and (v) terminator elements at the 3′ end of the pre-rRNA coding region. Specific transcription factors bind directly or via protein–protein interactions to these regions and thus promote the synthesis of faithfully initiated and terminated ribosomal precursor RNA.

Spacer transcripts are co-directional with rRNA and are terminated at the promoter-proximal terminator, termed T_0 in mammals (Grummt *et al.*, 1986a). In mouse, the sequence of the T_0 element is almost identical to the 18 bp downstream terminator motif, pragmatically called 'Sal box', which is repeated several times (T_1–T_{10}) downstream of the 3′ end of the pre-rRNA coding region and has been shown to mediate transcription termination *in vivo* and *in vitro* (Grummt *et al.*, 1985; Bartsch *et al.*, 1988; Kuhn and Grummt, 1990). The 'Sal box' terminator elements are recognized by a nucleolar protein, the transcription termination factor TTF-I. Alterations in the 'Sal box' that reduce TTF-I binding also impair transcription termination (Grummt *et al.*, 1986b).

The functional relevance of the promoter-proximal terminator has been elusive. In mouse, rat, human, *Xenopus laevis* and *X.borealis*, this terminator is located 150–200 bp upstream of the transcription initiation site. The conservation of a terminator-related element at a precise position just upstream of the gene promoter suggests that this element serves an important function in rDNA transcription initiation. Indeed, T_0 has been shown to stimulate transcription initiation *in vivo* (Grummt *et al.*, 1986a; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986). The positive effect on transcription has been interpreted to result from shielding the promoter from polymerases that read through from spacer promoters, thereby inactivating or 'occluding' assembled initiation complexes (Henderson and Sollner-Webb, 1986; Bateman and Paule, 1988). On the other hand, the presence of the upstream terminator increases the competition strength of an adjacent gene promoter, and the terminator must be in its natural position to exert a stimulatory effect on transcription initiation. Changing the spacing between the upstream terminator and the promoter severely impaired transcriptional activity (McStay and Reeder, 1986).

The positive effect of the upstream terminator is difficult to reveal in *in vitro* assays. In nuclear extracts or purified reconstituted transcription systems, binding of TTF-I to T_0 has no effect on the efficiency of transcription. Since chromatin is the physiological template *in vivo*, we wondered whether TTF-I bound to T_0 may stimulate transcription on chromatin templates. Little is known about the implications of chromatin structure for ribosomal gene transcription by pol I. In yeast, where the chromatin structure of the flanking spacer was analyzed in some detail, about half of the ribosomal genes are transcriptionally active and devoid of nucleosomes (Dammann *et al.*, 1993). On the other hand, the inactive genes as well as their 5′-flanking spacers are characterized by a regular chromatin structure. Significantly, the chromatin structure characterisic for active and inactive transcription units is not inherited directly by the newly synthesized daughter strands during chromosome duplication, but the replication machinery entering upstream of a transcrip-

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tionally active rRNA gene generates two newly replicated coding regions regularly packaged into nucleosomal arrays (Lucchini and Sogo, 1995). Apparently, post-replicative processes regenerate an exposed chromatin conformation on newly replicated rRNA gene promoters shortly after the passage of the replication fork.

To study pol I transcription on chromatin templates, we reconstituted chromatin on rDNA templates *in vitro* using extracts from early *Drosophila* embryos, which contain large amounts of chromatin precursors (Becker and Wu, 1992). Chromatin reconstituted in this system is complex and contains activities that are responsible for dynamic properties of chromatin which allow DNA binding proteins to gain access to their sites of action (Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995). Here we show that binding of TTF-I to the upstream terminator activates transcription from chromatin templates. This transcriptional activation is accompanied by ATP-dependent nucleosome repositioning. Significantly, stable interaction of TTF-I with chromatin requires, in addition to the DNA binding domain, the part of TTF-I which is essential for transcription termination. This finding demonstrates that DNA binding of TTF-I is separable from chromatin remodeling and termination functions. We conclude that in addition to its well-documented role as a transcription termination factor, TTF-I also plays an important role as a chromatin-specific transcription activator.

Results

TTF-I relieves chromatin-mediated repression of rDNA transcription

In order to assess the effect of chromatin on pol I transcription, we used a cell-free chromatin reconstitution system derived from *Drosophila* embryos (Becker and Wu, 1992; Sandaltzopoulos *et al.*, 1994). A linear plasmid containing the murine ribosomal gene promoter (pMrWT) was immobilized on paramagnetic beads and reconstituted into nucleosomes in the absence or presence of different amounts of TTF-I. The chromatin was purified in the magnetic field, and used as template in a transcription assay containing a fractionated mouse nuclear extract (DEAE-280 fraction). This transcription system contains all proteins required for rDNA transcription initiation (Schnapp and Grummt, 1991) and promotes several rounds of transcription during a standard 50 min incubation. On naked DNA templates, the amounts of transcripts synthesized were not affected by the presence or absence of TTF-I (Figure 1, lanes 1 and 2). On pre-assembled chromatin templates, on the other hand, TTF-I had a pronounced effect on transcriptional activity. In the absence of TTF-I, no transcripts were synthesized, indicating that the promoter was repressed (lanes 3 and 4). However, if increasing amounts of recombinant TTF-I were added to the chromatin assembly reaction, a dose-dependent activation of transcription was observed (lanes 5–12). The amount of transcripts from the chromatin templates was ~30% of that observed with naked DNA, which indicates that a major fraction of the chromatin templates was transcribed. The strong transcriptional activation brought about by TTF-I suggests that TTF-I interacts with chromatin and that this interaction counteracts nucleosomal repression from pre-assembled chromatin templates *in vitro*.

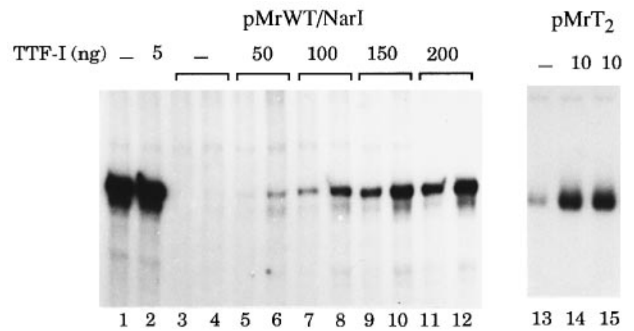

Fig. 1. Transcriptional activation of chromatin templates by TTF-I. Transcription from the linear template pMrWT/NarI (lanes 1–12) or the circular template pMrT_2 (lanes 13–15). Reactions contained 20 ng of free DNA (lanes 1 and 2), 60 ng (lanes 3, 5, 7, 9 and 11) or 140 ng (lanes 4, 6, 8, 10 and 12) of linear or 20 ng of circular nucleosomal template (lanes 13–15). Increasing amounts of TTF-I were added at the onset (lanes 3–12 and 14) or after completion of nucleosome assembly (lane 15).

On the linear template used, TTF-I had to be added at the onset of the chromatin assembly reaction to activate transcription. On circular templates, however, transcription stimulation was also observed when TTF-I was added after chromatin reconstitution was complete (lanes 13–15). For this experiment, we chose the template pMrT_2 which contains a pol I terminator (T_2) derived from the 3′ end of the rDNA transcription unit inserted downstream of the coding region of pMrWT. Termination at the downstream terminator results in transcripts of defined size, and therefore allows monitoring of specific transcription on circular templates (Grummt *et al.*, 1986b). The DNA was assembled into soluble chromatin either in the absence of TTF-I (lane 13) or in the presence of TTF-I which was added either at the onset (lane 14) or after completion (lane 15) of chromatin assembly. An aliquot of the assembly reaction was then used as template in the murine transcription system. In this soluble system, transcriptional repression by chromatin was overcome whether TTF-I was added before or after chromatin assembly. This result demonstrates that TTF-I interacts with chromatin and can activate transcription from pre-assembled chromatin templates *in vitro*.

TTF-I interacts with chromatin and remodels nucleosome structures in an energy-dependent manner

To assess whether transcriptional activation by TTF-I involves chromatin rearrangements, we analyzed the nucleosome positions on the promoter with micrococcal nuclease (MNase), a nuclease known for its preference for nucleosomal linker DNA. Plasmid DNA was assembled into chromatin as before, partially digested with MNase, and the cleavage sites were mapped by indirect end labeling (Wu, 1980). Digestion of protein-free plasmid DNA revealed the sequence preference of the nuclease (Figure 2A, lanes 1 and 2). The MNase digestion pattern of chromatin assembled in the absence of TTF-I resembled that of naked DNA (lanes 3 and 4). Clearly, the interaction of TTF-I with chromatin led to a repositioning of nucleosomes, from more or less random positions to defined

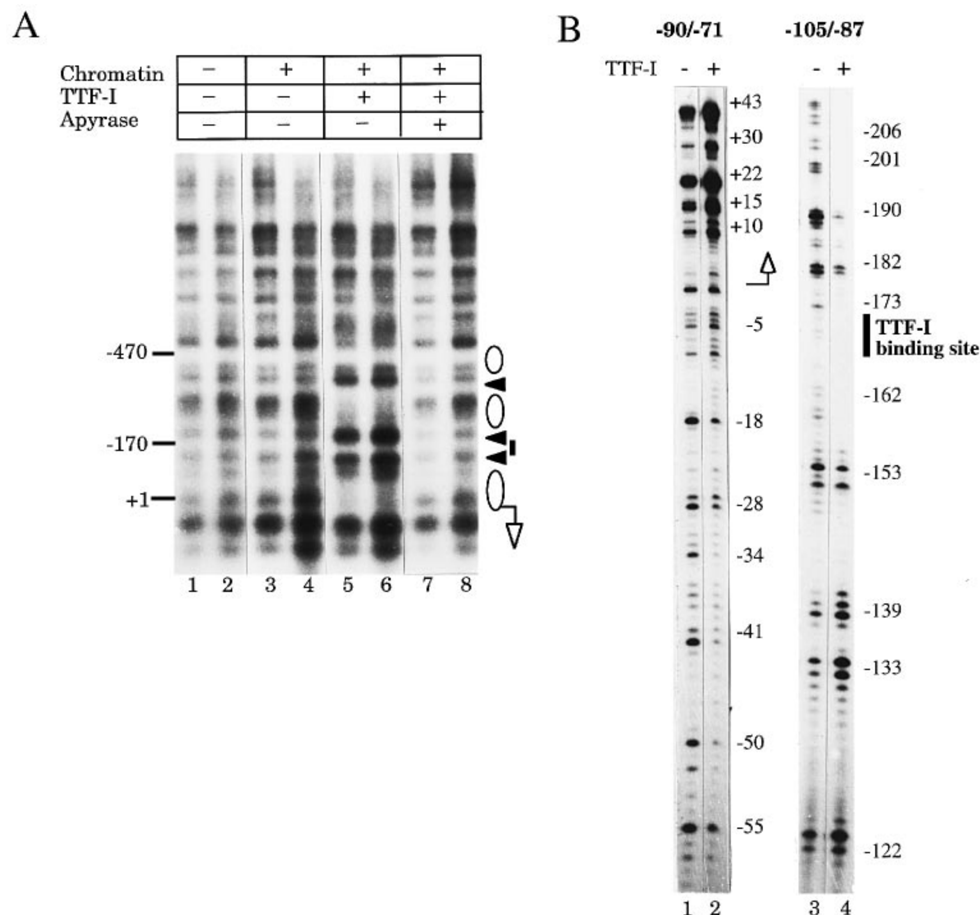

Fig. 2. Energy-dependent nucleosome remodeling by TTF-I. (**A**) Mapping of MNase cleavage sites on naked pMrWT DNA (lanes 1 and 2) or on reconstituted chromatin templates (lanes 3–8) by indirect end labeling. Each reaction was digested with 10 U of MNase for 20 or 60 s. Lanes 3 and 4 show the MNase pattern of chromatin assembled in the absence of TTF-I. In the reactions represented in lanes 7 and 8, the reconstituted chromatin was treated with apyrase before TTF-I addition. Predominant nucleosome positions at the rDNA promoter region are indicated by open ellipses; hypersensitive sites due to TTF-I binding are marked by filled triangles. The TTF-I binding site is indicated by a bar. (**B**) High resolution mapping of nucleosome boundaries. Chromatin assembled on pMrWT in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of TTF-I was digested with MNase. Mononucleosome-sized fragments were gel purified and the ends mapped by extension of internal oligonucleotide primers. Probe –90/–71 anneals to the lower strand of rDNA and maps the 3′ boundaries. Oligonucleotide –105/–87 anneals to the upper strand and maps the 5′ boundaries. The borders of the nucleosomes are marked by numbers which correspond to nucleotide positions upstream and downstream of the transcription start site. The TTF-I binding site is indicated by a bar; the arrow marks the transcription start site.

positions flanking the TTF-I recognition site. Two hypersensitive sites flanking the T_0 sequence were induced and strongly preferred MNase cleavage sites were protected in adjacent regions (lanes 5 and 6). The protected region extended from position –146 to +10. An identical cleavage pattern was observed whether TTF-I was added during or after chromatin assembly. Importantly, this nucleosome remodeling required energy. Addition of apyrase which depletes the assembly reaction of ATP prevented TTF-induced remodeling and, therefore, yielded an MNase digestion pattern that is indistinguishable from naked DNA or regularly spaced chromatin (lanes 7 and 8). Thus, TTF-I mediates ATP-dependent nucleosome remodeling at the rDNA promoter.

To map the 3′ and 5′ borders of nucleosomes at the nucleotide level, chromatin was assembled in the presence or absence of TTF-I and digested with MNase to yield predominantly mononucleosomes. The DNA was purified, separated on an agarose gel and nucleosome-sized fragments (146 bp) were isolated. The ends of these fragments, which define the nucleosome positions, were visualized by multiple extensions of internal primers. In the absence of TTF-I, the nucleosomes gave rise to a series of fragment endpoints distributed over the entire promoter area on both strands (Figure 2B, lanes 1 and 3). Fragment endpoints on either site tentatively can be matched to yield fragments of nucleosomal size. Significantly, if TTF-I was included in the assembly reaction, the nucleosomes that occupied the T_0 sequence (bar in Figure 2B) were shifted towards the transcription initiation site (lanes 2 and 4). This result demonstrates that TTF-I induces the positioning of a nucleosome in the vicinity of the transcription start site.

Chromatin remodeling and DNA binding functions of TTF-I are separable

Next we examined whether the DNA binding domain of TTF-I is sufficient to counteract nucleosomal repression or whether additional regions of TTF-I are involved in transcription stimulation from chromatin templates. For this, we tested the ability of several N-terminally truncated TTF-I derivatives (TTFΔN185, TTFΔN323 and TTFΔN445) to rearrange the chromatin structure and to

Fig. 3. TTF-I domains involved in nucleosome rearrangement and chromatin-specific transcriptional activation. (**A**) Indirect end labeling of MNase cleavage sites of chromatin assembled on pMrWT in the absence of TTF-I (lanes 5 and 6) or in the presence of different N-terminal TTF-I deletion mutants indicated above the lanes. The nucleosome positions flanking T_0-bound TTF-I are indicated by open ellipses; hypersensitive sites induced by TTF-I binding are marked by filled triangles. The TTF-I binding site is indicated by a bar, the transcription start site is marked by an open arrow. (**B**) Transcriptional analysis of TTF-I deletion mutants on chromatin templates. Chromatin that was assembled on the immobilized linear template pMrWT/NarI (lanes 1–4) or on circular $pMrT_2$ (lanes 5–8) either in the absence (lanes 1 and 5) or presence of different TTF-I mutants (lanes 2–4 and 6–8) was assayed for transcriptional activity. The arrows mark the positions of specific run-off and terminated transcripts, respectively.

activate transcription. These TTF-I mutants differ in their DNA binding and transcription termination properties (Evers *et al.*, 1995). Briefly, deletion of the N-terminal part of TTF-I (TTFΔN185, TTFΔN323) affects neither DNA binding nor the termination function of TTF-I. Mutant TTFΔN445 still binds specifically and efficiently to its target site on naked DNA but its termination activity is strongly impaired.

As shown in Figure 3A, the two termination-competent mutants TTFΔN185 and TTFΔN323 efficiently repositioned nucleosomes (lanes 7–10). By contrast, TTFΔN445, whose DNA binding affinity compares with that of TTFΔN185 and TTFΔN323, was unable to induce changes in chromatin structure (lanes 11 and 12). Thus, the ability of TTF-I to reorganize nucleosomal structures does not only depend on its DNA binding function, but requires protein features located between amino acids 323 and 445. These sequences also harbor the termination function of TTF-I (Evers *et al.*, 1995).

Figure 3B shows the activity of the TTF-I mutants in the reconstituted transcription system on a linear and circular template, respectively. Consistent with the notion that chromatin remodeling is necessary for transcriptional activation, both TTFΔN185 and TTFΔN323 were capable of directing transcription on the chromatin template, whereas TTFΔN445 failed to do so. However, significant differences in the activation efficiency of TTFΔN323 were observed depending on whether a linear or a circular chromatin template was used. On the linear chromatin template, TTFΔN323 activated transcription much less efficiently than TTFΔN185 (lanes 2 and 3). On the circular template, however, the activation properties of TTFΔN323 and TTFΔN185 were similar (lanes 6 and 7).

TTF-I domains required for stable interaction with chromatin

The failure of the mutant TTFΔN445 to remodel chromatin may be due to its inability either to stably bind chromatin or to influence the structure or position of nucleosomes. To decide between these two possibilities, TTF-I binding to chromatin was monitored by DNase I footprinting. Naked DNA (pMr600) or DNA assembled into chromatin in the absence or presence of TTF-I was partially digested with DNase I, and the purified DNA was analyzed by primer extension. As shown in Figure 4, TTF-I strongly binds to the upstream terminator to yield a footprint which extends from nucleotides –175 to –148, and is flanked by a marked hypersensitive site at –147. Both TTFΔN185 (panel 2) and TTFΔN445 (panel 3) bound with the same affinity to naked DNA and protected both the upstream terminator and adjacent nucleotides. A different result was obtained on chromatin templates. When either of the two proteins were included in a chromatin assembly reaction, only TTFΔN185 efficiently bound to chromatin and protected the same sequences as those observed on naked DNA (lanes 5). The DNase I digestion pattern of TTFΔN445, on the other hand, was indistinguishable from that of chromatin assembled in the absence of TTF-I (panel 4), indicating that this TTF-I derivative was not able to stably bind to chromatin (panel 6). Thus, consistent with the low resolution nucleosome mapping (see Figure 3A), TTFΔN185, but not TTFΔN445, interacts with its target site in chromatin. We conclude that the DNA binding region of TTF-I on its own is not sufficient for binding to its target sequence in chromatin. Stable interactions with chromatin which lead to nucleosome remodeling and subsequent chromatin-specific transactivation appear to require additional sequences located between amino acids 323 and 445.

Chromatin perturbations occur on transcriptionally active rDNA templates

To find out whether TTF-I binding affects the regularity of the nucleosomal array in the promoter region, we

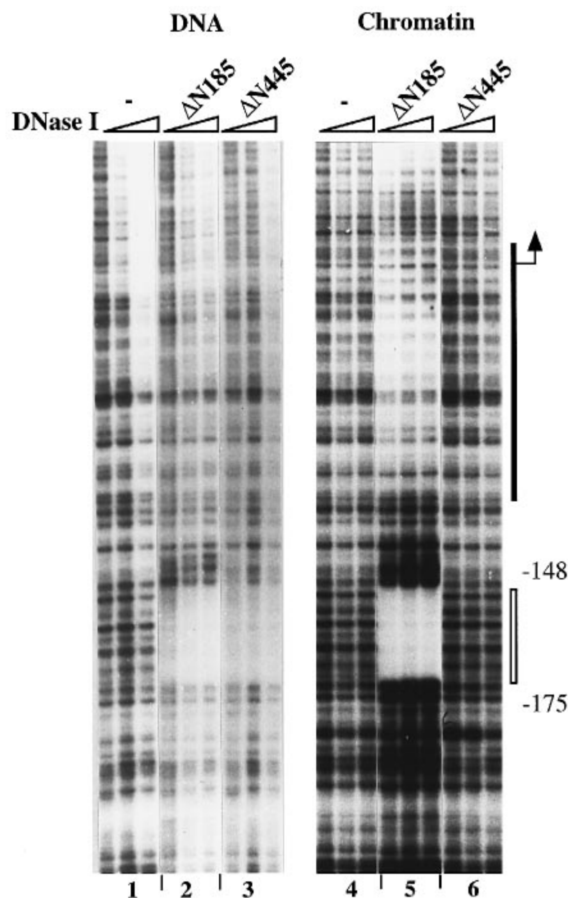

Fig. 4. The DNA binding domain of TTF-I is not sufficient for binding to chromatin. pMr600 was assembled into chromatin in the absence of TTF-I (lanes 4), or in the presence of TTFΔN185 (lanes 5) and TTFΔN445 (lanes 6), partially digested with DNase I and DNA was analyzed by primer extension with the labeled oligonucleotide –232/–214. Lanes 1–3 show the DNase I footprint on free DNA. The numbers refer to nucleotides which are protected by TTF-I. The transcription start site is marked by an arrow. The bar marks the positioned nucleosome at the promoter.

employed an assay that has been used previously to define nucleosome positions on *Drosophila* heat shock promoters (Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995). Chromatin was first assembled on circular $pMrT_2$, then combinations of TTF-I and the DEAE-280 fraction were added and the assays were incubated for 15 min at 30°C in the presence of nucleotides to allow transcription to occur. The chromatin was partially digested with MNase and the resulting oligonucleosomal DNA ladders were visualized by sequential hybridization to probes that correspond either to sequences upstream of the transcription start site (Figure 5A), to transcribed sequences (Figure 5B) or non-transcribed vector DNA (Figure 5C). In the absence of specific transcription factors, periodic nucleosomal arrays were observed in the rDNA promoter region (probe –90/–71; lanes 1–4). Also the presence of pol I transcription factors *per se* (DEAE-280) did not affect the regularity of the nucleosomal array (lanes 9–12). In the presence of TTF-I, a new fragment appeared between the mono- and the dinucleosome (arrow, lanes 5–8) which results from binding of TTF-I right next to a nucleosome. Significantly, under transcription conditions, i.e. when both TTF-I and the DEAE-280 fraction were present, the chromatin became much more sensitive towards MNase digestion. At intermediate stages of MNase digestion, the oligonucleosomal DNA ladder was lost, indicating that the nucleosomal array around the promoter was perturbed (lanes 13–16). This increased accessibility in the presence of both TTF-I and DEAE-280 was also observed in the transcribed region (probe +111/+130), but not in vector sequences (Figure 5C). This region of the template is not transcribed because the downstream terminator in $pMrT_2$ stops elongating pol I. Consistent with the idea that the perturbation of nucleosomes is dependent on transcription, the chromatin perturbation spread into the vector sequences if a plasmid was used that lacks the terminator (data not shown). The presence of regularly spaced nucleosomal arrays in the non-transcribed region, on one hand, and the increased MNase sensitivity of the promoter and transcribed regions in the presence of both TTF-I and DEAE-280, on the other hand, strongly suggest that perturbations in chromatin structure are correlated directly with ongoing transcription. Moreover, the fact that these perturbations were observed with bulk chromatin confirms our earlier notion that a major fraction of the templates is transcribed.

Nucleosome remodeling is a prerequisite for efficient initiation

The experiments shown above suggest that TTF-I-directed transcriptional activation involves movement of a nucleosome over the gene promoter. This was an unexpected result which raises the question of whether a nucleosomal configuration is compatible with the assembly of initiation complexes or whether additional remodeling steps occur under transcription conditions. To assess whether the nucleosomal state of the promoter would be altered by assembly of the initiation complex and transcription, we mapped MNase cleavages by indirect end labeling as before, but in the presence of TTF-I, the DEAE-280 fraction and nucleotides to allow transcription to occur. As shown in Figure 6, the DEAE-280 fraction did not alter the pattern of MNase-sensitive sites of chromatin (compare lanes 1 and 2 with 5 and 6). Also the TTF-I-mediated protection over the promoter region remained essentially unchanged both in the absence and presence of the DEAE-280 fraction. However, the relative intensity of the hypersensitive site at the transcription start site markedly decreased under transcription conditions (lanes 7 and 8). The extent of protection of this hypersensitive site correlates with the estimated fraction of active templates (25–30%). On the basis of this finding, we postulate that the primary nucleosome rearrangement by TTF-I is followed by an additional remodeling step which presumably acts in concert with transcription complex assembly and transcription initiation.

Discussion

Reconstitution of faithful initiation, elongation and termination of pol I transcription on chromatin templates

Activation of gene transcription is accompanied by perturbations of the regular nucleosome structure in promoters and other regulatory elements (for reviews, see Elgin, 1988; Becker, 1994; Wallrath *et al.*, 1994; Wolffe, 1994).

Up to now, all chromatin remodeling studies have been performed on genes transcribed by RNA polymerase II and RNA polymerase III. Therefore, very little is known about the mechanisms of transcriptional activation of class I genes in chromatin. Studies in yeast suggest that chromatin remodeling precedes the activation of ribosomal gene promoters (Lucchini and Sogo, 1995). The active state of a ribosomal gene is transiently erased at replication, when the two newly synthesized daughter strands are assembled rapidly into regular nucleosomal arrays. The staggered appearance of open promoters and coding regions following the passage of the replication fork suggested that a local disruption of chromatin over the rRNA gene promoter precedes the assembly of a functional transcription initiation complex. Lucchini and Sogo (1995) suggested that the perturbation of chromatin at the promoter might be mediated by pReb1p, the yeast homolog of TTF-I, which has a strong binding site located 200 bp upstream of the transcription initiation site (Fedor *et al.*, 1988; Morrow *et al.*, 1989).

Our results strongly support this hypothesis. We have shown that binding of TTF-I to the terminator element upstream of the mouse rDNA transcription unit mediates nucleosome rearrangements in pre-assembled chromatin. This remodeling correlates with transcriptional activation on otherwise repressed nucleosomal rDNA templates. Since TTF-I does not stimulate transcription on naked DNA templates, this factor appears to counteract repressive chromatin structures. Thus, TTF-I is a multifunctional protein that, in addition to its established role as a pol I-specific termination factor (Bartsch *et al.*, 1988; Smid *et al.*, 1992; Evers *et al.*, 1995), is also able to trigger nucleosome remodeling and to antagonize repression of ribosomal gene transcription on chromatin templates. Once initiated, pol I is able to elongate through nucleosomes and to terminate faithfully. This finding opens up a new experimental avenue for studying the fate of nucleosomes during transcription by pol I in chromatin.

DNA binding and nucleosome remodeling are separable functions

When added to rDNA templates covered in regular arrays of randomly positioned nucleosomes, TTF-I interacts with its binding site in chromatin and directs nucleosomes to adjacent, more defined positions. Both the ATP dependence of this process and the alteration of MNase digestion patterns are reminiscent of 'nucleosome remodeling' by GAGA factor (GAF) and heat shock factor (HSF) on *Drosophila* heat shock promoters (Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995). Nucleosome remodeling by these transcription factors requires the presence of a co-factor, termed nucleosome remodeling factor (NURF), a complex of four polypeptides, which is abundant in the extracts used for chromatin reconstitution (Tsukiyama and Wu, 1995). Although we are still ignorant of the protein(s) and the mechanisms that govern TTF-I-directed nucleo-

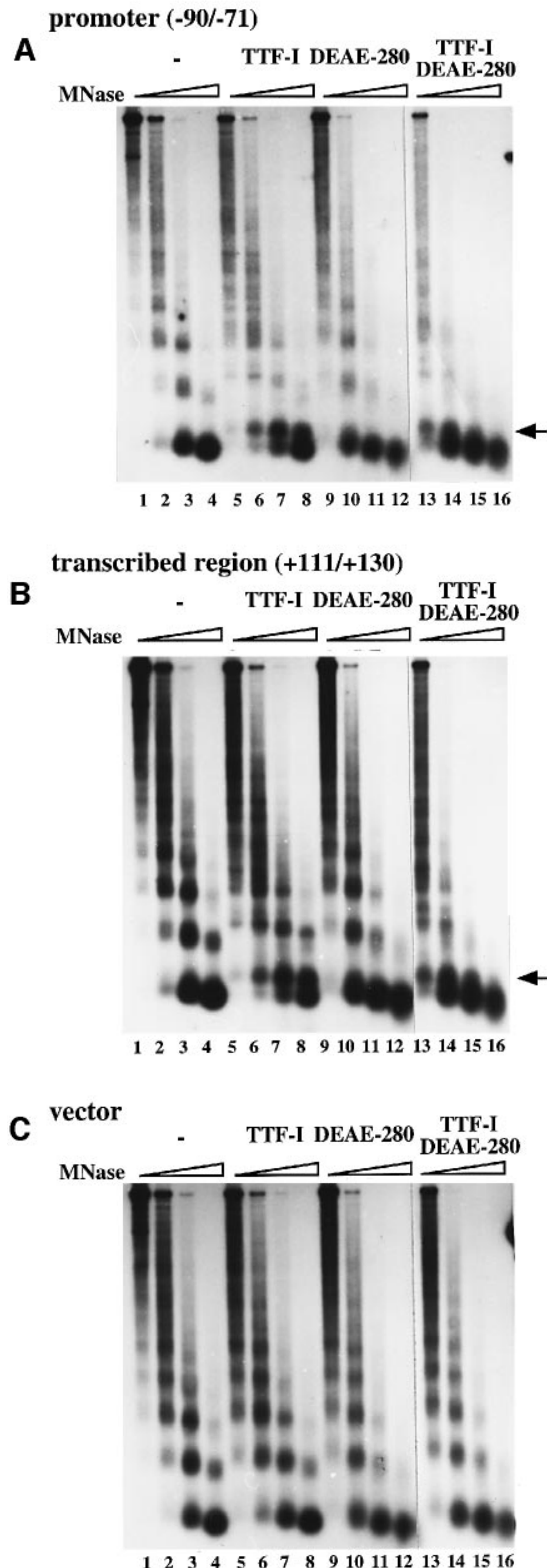

Fig. 5. Changes in chromatin structure under transcription conditions. Visualization of the nucleosome pattern at the promoter (**A**), in the transcribed region (**B**) and on vector DNA (**C**). 40 ng of pMrT$_2$ and 160 ng of phage λ DNA were reconstituted into chromatin in the absence of additional proteins (lanes 1–4), in the presence of TTF-I (lanes 5–8), in the presence of fractionated mouse cell extract (DEAE-280, lanes 9–12) and in the presence of both DEAE-280 and TTF-I (lanes 13–16). The chromatin was digested with MNase and DNA fragments were resolved by electrophoresis, blotted and hybridized sequencially to probes from the rDNA promoter region (–90/–71, A), the transcribed region (+111/+130, B) or to a vector fragment (pUC9 sequences from 1582 to 2274, C) to visualize the respective nucleosomal pattern.

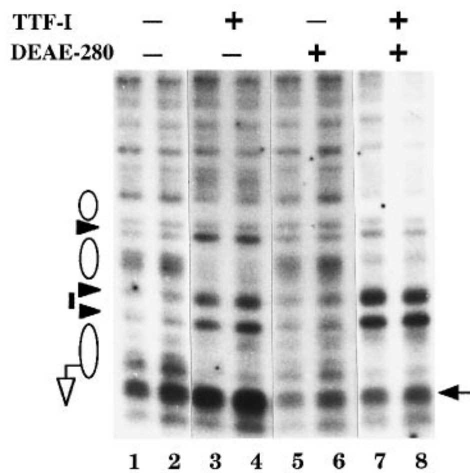

Fig. 6. Changes in chromatin structure of transcriptionally active rDNA templates. MNase cleavage sites were mapped by indirect end labeling as in Figure 2. Chromatin (lanes 1 and 2) was incubated under transcription conditions with TTF-I (lanes 3 and 4), with DEAE-280 (lanes 5 and 6) and with TTF-I plus DEAE-280 (lanes 7 and 8). The nucleosome positions flanking T_0-bound TTF-I are indicated by open ellipses; hypersensitive sites induced by TTF-I binding are marked by filled triangles. The TTF-I binding site is indicated by a bar, the transcription start site is marked by an open arrow. The hypersensitive site downstream of the initiation site which is specifically protected by the mouse factors is marked by an arrow.

some rearrangement, the ATP requirement suggests that NURF or a related activity present in the fly extracts plays a central role in perturbation of the nucleosomal structure at the rDNA promoter.

A molecular dissection of TTF-I revealed that the DNA binding domain on its own was unable to interact with chromatin and to promote nucleosome remodeling. Thus, additional protein features located between amino acids 323 and 445 are required for chromatin-specific functions. This is an important result because, by separating the ability of TTF-I to interact productively with chromatin from its DNA binding function, we provide the first example of a distinct domain of a transcription factor in facilitating binding to chromatin. On the other hand, we are aware of the possibility that the inability of TTFΔN445 to bind to chromatin may be due to an artificial property of truncation of the protein, and therefore would not necessarily reflect the function of this region of TTF-I *in vivo.*

Previous studies have shown that the DNA binding domain of the GAL4 protein can interact with its binding sites and disrupts the nucleosome to which it binds (Taylor *et al.*, 1991; Axelrod *et al.*, 1993; Morse 1993; Pazin *et al.*, 1994), while its activation domains lead to disruption of an adjacent nucleosome on the *GAL1* promoter (Adams and Workman, 1995). Similarly, activation of the *PHO5* promoter requires disruption of adjacent nucleosomes. For disruption to occur, the activation domain of PHO4 or a heterologous activation domain is required (Svaren *et al.*, 1994). Our finding that TTFΔ445, the deletion mutant which binds efficiently to its target site on naked DNA and is almost inactive in transcription termination, does not bind to nucleosomal DNA points to an active role for other parts of TTF-I, in addition to the DNA binding domain, in the interaction with chromatin templates. It is tempting to speculate that the part of TTF-I required for chromatin-specific functions may provide a surface for interactions with dedicated 'nucleosome remodeling machines'.

Moreover, the tight correlation between transcription termination, chromatin remodeling and transcription activation, which all reside between amino acids 323 and 445, suggests that these different activities are functionally interrelated. It will be interesting to find out whether the diverse functions of TTF-I rely on overlapping or separable structural features. Similarly, a recent analysis of NFκB activity in reconstituted chromatin demonstrated that nucleosome remodeling and chromatin-specific transcriptional activation can be separated (Pazin *et al.*, 1996). The p50 subunit, which lacks a transactivation domain, was able to bind chromatin and to remodel nucleosomes around the promoter even more efficiently than the active p65 subunit.

Chromatin remodeling and activation of ribosomal gene transcription

Previous studies have demonstrated that gene induction is often accompanied by alterations in chromatin structure. Our results suggest that this is also true for class I gene transcription. However, in contrast to pol II promoters where transcription is strongly repressed when the transcription start site and the binding sites for general transcription factors are occluded by a nucleosome (for review, see Owen-Hughes and Workman, 1994), the interaction of TTF-I with T_0 results in a relocation of nucleosomes such that the promoter and the transcription start site are in a nucleosomal configuration. The obvious question that arises is whether the DNA molecules with the promoter-bound nucleosome are indeed the transcriptionally active templates. Quantitative S1 nuclease mapping revealed that several transcripts were synthesized per DNA template in a 5 min reaction (data not shown). On the nucleosomal template, TTF-I-activated transcription reached 20–30% of the levels obtained with nucleosome-free DNA, which suggests that a major fraction of the chromatin templates was transcribed. Consistent with this interpretation, we found a specific perturbation of the regular nucleosome pattern both in the rDNA promoter region and in transcribed sequences. This result indicates that the initial nucleosome rearrangement is not only compatible with, but rather is a prerequisite for, transcriptional activation. In this scenario, the presence of the promoter-bound nucleosome would facilitate assembly of the transcription initiation complex. We postulate that a second, perhaps transient, remodeling step perturbs the nucleosome at the promoter by either loosening histone–DNA contacts or displacing histone dimers or tetramers. This second remodeling step could be the mechanism which facilitates binding of the transcription machinery to the promoter.

Previous experiments in yeast strongly support the *in vivo* relevance of our results. The yeast homolog of TTF-I, Reb1p, is supposed to play a much more general role in the cell than solely in termination of yeast rDNA transcription. Reb1p is is an essential protein for growth of yeast and is postulated to have a function in both the expression and organization of the DNA in the nucleus and nucleolus. Interestingly, Reb1p exerts its effect on transcription by influencing the chromatin structure and

creating a nucleosome-free region surrounding its binding site (Fedor *et al.*, 1988). Moreover, by analysis of tagged integrated rDNA transcription units in which either one or both of the Reb1p binding sites have been inactivated, Planta and collegues found that mutation of the promoter-proximal Reb1p binding site diminished transcription by ~50% (Kulkens *et al.*, 1992). When both binding sites were removed, the effects were even more drastic. Thus, Reb1p is essential for rDNA transcription in the chromosomal context, and for efficient transcription both binding sites need to be intact.

Recent work has uncovered a surprising and provocative connection between chromatin structure and transcriptional activation. A pol II holoenzyme has been described which contains stoichiometric amounts of the SWI–SNF complex which in turn endows the holoenzyme with the ability to disrupt nucleosomes (Wilson *et al.*, 1996). The presence of SWI–SNF proteins in the pol II holoenzyme could answer the question of how nucleosomes covering a transcription start site adjacent to bound activators are displaced. Whether or not an analogous pol I holoenzyme complex exists is still a matter for speculation. Further studies with the biochemical approach described here should help to clarify the role of TTF-I in transcriptional activation from chromatin templates and to find out whether nucleosome-destabilizing activities are also associated with RNA polymerase I.

Materials and methods

Plasmids and probes

pMrWT contains mouse rDNA sequences from –170 to +155 including the upstream terminator T_0 at position –170. pMr600 contains a 600 bp *Pvu*II fragment covering nucleotides from –326 to +292 cloned into the *Sma*I site of pUC9. pMrT_2 is a minigene construct containing an 83 bp 3′-terminal rDNA fragment (from +603 to +686 with respect to the 3′ end of 28S RNA) downstream of the coding region of pMrWT. For run-off transcription, pMrWT was linearized with *Nde*I, 5′-protruding ends were filled in with biotin-16-dUTP, then cleaved with *Nar*I and bound to Dynabeads–strepatavidin M 280 (Dynal). In the immobilized template, the rDNA promoter is spaced ~2.6 kb from the beads and produces a 332 bp run-off transcript.

The designation of oligonucleotides used in the various assays (+111/+130, –90/–71, –105/–87, –232/–214) indicates the position of rDNA sequences relative to the transcription start site. The probe used for indirect end labeling of pMrWT assembled into chromatin was an *Eco*RI–*Nde*I fragment derived from pUC9. The vector-specific probe used for hybridization of oligonucleosomes contained pUC9 sequences from 1582 to 2274.

Expression and purification of recombinant TTF-I

cDNAs encoding histidine-tagged TTF-I mutants were inserted into the baculovirus expression vector pBacPAK9 (Clontech) and were expressed in Sf9 cells. Forty eight hours after infection, extracts were prepared by sonicating the cells for 10 s in lysis buffer [300 mM KCl, 20 mM Tris–HCl (pH 8.0), 1% NP-40, 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Extracts were centrifuged at 10 000 *g* and incubated with Ni^{2+}-NTA–agarose (Qiagen) at 4°C for 30 min. The resin was washed with lysis buffer containing 0.5% NP-40 and 1 mM imidazole and lysis buffer containing 0.5% NP-40 and 10 mM imidazole. TTF-I was released from the Ni^{2+}-NTA–agarose resin by elution with lysis buffer containing 200 mM imidazole. The eluted proteins were dialyzed against buffer BC-100 [100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.9), 0.2 mM EDTA and 20% glycerol]. TTF-I activity was measured by electrophoretic mobility shift assays (Smid *et al.*, 1992). Unless otherwise stated, all experiments were performed with mutant TTFΔN185 because full-length recombinant TTF-I is hard to express in sufficient amounts and exhibits low DNA binding activity (Sander *et al.*, 1996).

Chromatin assembly

Preparation of assembly extracts and chromatin reconstitution were performed as described (Becker and Wu, 1992; Becker *et al.*, 1994). 40 μl reactions which contained 200 ng of DNA and 11 μl of *Drosophila* extract were complemented with TTF-I either at the onset of chromatin assembly (0 h) or after 5.5 h. To remove ATP, 1 U of apyrase was added after 5.5 h of assembly and incubated for 20 min. Then TTF-I was added and incubated for a further 30 min. Immobilized chromatin was washed with 100 mM salt before being used for *in vitro* transcription.

Analysis of chromatin structure

For indirect end labeling, chromatin was digested with 10 U of micrococcal nuclease for 20 and 60 s in a total volume of 100 μl in the presence of 3 mM $CaCl_2$. The reactions were stopped by the addition of 0.2 volumes of 4% SDS–0.1 M EDTA. Proteins were digested with 10 μg of proteinase K for 1 h at 50°C. Isolated DNA was cleaved with *Nde*I, separated on 1.3% agarose gels, blotted and hybridized with a 207 bp *Eco*RI–*Nde*I fragment from pUC9.

To map the nucleosome boundaries by linear PCR, chromatin was digested for 3 min with 150 U of MNase to yield mainly mononucleosomal DNA. DNA was purified by treatment with RNase A and proteinase K. DNA fragments of 146 bp were isolated and linearly amplified by extension of ^{32}P-labeled oligonucleotide primers. Each reaction contained, in a volume of 50 μl, 0.2 pmol of primer, 2 ng of *in vitro* assembled core DNA, 2.5 mM dNTPs and 2.5 U of *Taq* DNA polymerase (Boehringer). Cycling involved 4 min at 95°C, then 30 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C. Fragments were resolved on 8% sequencing gels and mapped with respect to labeled size markers.

For mapping the nucleosomal structure, 40 ng of pMrT_2 and 160 ng of phage λ DNA were assembled into chromatin. Transcription conditions were established by adding 660 μM nucleotides, 40 μl of DEAE-280 fraction and 10 ng of TTF-I in a volume of 120 μl. After 15 and 50 min incubation at 30°C, 10 μl aliquots of the reactions were used to estimate the overall transcription rate by quantitative S1 mapping. MNase digestions were performed in a volume of 100 μl in the presence of 3 mM $CaCl_2$. Reactions without the DEAE-280 fractions were incubated for 10, 40, 160 and 600 s with 45 U of MNase. Reactions containing DEAE-280 were incubated for the same times with 500 U of MNase. DNA was isolated and blotted as described above. The Southern blot was hybridized sequentially with the ^{32}P-labeled rDNA oligonucleotides –90/–71 and +111/+130, and then with the vector probe. For rehybridization, membranes were stripped by boiling with 0.5% SDS for 10 min.

DNase I footprinting

Circular pMr600 (100 ng) was reconstituted into chromatin in the absence or presence of TTF-I, and 50 ng aliquots were digested at room temperature in a total volume of 55 μl with 5 U of DNase I (Worthington) for 10, 30 and 90 s. Protein-free DNA was digested with 0.002 U of DNase I. The reaction was stopped by addition of 0.2 volumes of 4% SDS–0.1 M EDTA. Purified DNA was denatured in 8 μl of 0.125 M NaOH for 5 min at 68°C. After addition of 2 μl of 560 mM TES, 240 mM HCl and 100 mM $MgCl_2$, 0.2 pmol (7×10^5 c.p.m.) of labeled primer (–232/–214) were annealed and primer extension was performed for 10 min at 70°C with 1 U of *Taq* DNA polymerase (Boehringer) and 0.2 mM dNTPs. DNA was purified and analyzed on a 6% sequencing gel.

In vitro transcription assays

The 25 μl assays contain 12 mM Tris–HCl (pH 7.9), 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 80 mM KCl, 10 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP, CTP and GTP, 0.01 mM UTP and 1–2 μCi of [α-^{32}P]UTP, 20–200 ng of either naked template DNA or reconstituted chromatin, and 5 μl of murine nuclear extract proteins that have been partially purified by chromatography on DEAE–Sepharose CL-6B (DEAE-280 fraction). Chromatin assembled on immobilized templates was washed with buffer BC-100 before assaying in the transcription system. Reactions were incubated for 1 h at 30°C and stopped by the addition of 25 μl of 0.4 M ammonium acetate pH 5.5, 0.4% SDS and 0.2 mg/ml yeast tRNA, followed by organic extraction and ethanol precipitation. Run-off transcripts were analyzed on 4.5% polyacrylamide gels.

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References

Adams,C.C. and Workman,J.L. (1995) Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol. Cell. Biol.*, **15**, 1405–1421.

Axelrod,J., Reagan,M.S. and Majors,J. (1993) GAL4 disrupts a repressing nucleosome during activation of GAL1 transcription *in vivo*. *Genes Dev.*, **8**, 3891–3897.

Bartsch,I., Schoneberg,C. and Grummt,I. (1988) Purification and characterization of TTFI, a factor that mediates termination of mouse ribosomal DNA transcription. *Mol. Cell. Biol.*, **8**, 3891–3897.

Bateman,E. and Paule,M.R. (1988) Promoter occlusion during ribosomal RNA transcription. *Cell*, **54**, 985–992.

Becker,P.B. (1994) The establishment of active promoters in chromatin. *BioEssays*, **16**, 541–547.

Becker,P.B. and Wu,C. (1992) Cell-free system for assembly of transcriptionally repressed chromatin from *Drosophila* embryos. *Mol. Cell. Biol.*, **12**, 2241–2249.

Becker,P.B., Tsukiyama,T. and Wu,C. (1994) Chromatin assembly extracts from *Drosophila* embryos. *Methods Cell Biol.*, **44**, 207–223.

Dammann,R., Lucchini,R., Koller,T. and Sogo,J.M. (1993) Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 2331–2338.

Elgin,S.C.R. (1988) The formation and function of DNase I hypersensitive sites in the process of gene activation. *J. Biol. Chem.*, **263**, 19259–19262.

Evers,R., Smid,A., Rudloff,U., Lottspeich,F. and Grummt,I. (1995) Different domains of the murine RNA polymerase I-specific termination factor mTTF-I serve distinct functions in transcription termination. *EMBO J.*, **14**, 1248–1256.

Fedor,M.J., Lue,N.F. and Kornberg,R.D. (1988) Statistical positioning of nucleosomes by specific protein-binding to an upstream activating sequence in yeast. *J. Mol. Biol.*, **204**, 109–127.

Grummt,I., Maier,U., Öhrlein,A., Hassouna,N. and Bachellerie,J.P. (1985) Transcription of mouse rDNA terminates downstream of the 3′ end of 28S RNA and involves interaction of factors with repeated sequences in the 3′ spacer. *Cell*, **43**, 801–810.

Grummt,I., Kuhn,A., Bartsch,I. and Rosenbauer,H. (1986a) A transcription terminator located upstream of the mouse rDNA initiation site affects rRNA synthesis. *Cell*, **47**, 901–911.

Grummt,I., Rosenbauer,H., Niedermeyer,I., Maier,U. and Öhrlein,A. (1986b) A repeated 18 bp sequence motif in the mouse rDNA spacer mediates binding of a nuclear factor and transcription termination. *Cell*, **45**, 837–846.

Henderson,S. and Sollner-Webb,B. (1986) A transcriptional terminator is a novel element of the promoter of the mouse ribosomal RNA gene. *Cell*, **47**, 891–900.

Kuhn,A. and Grummt,I. (1990) Specific interactions of the murine termination factor TTF-I with class-I RNA polymerases. *Nature*, **344**, 559–562.

Kulkens,T., van de Sante,C., Dekker,A.F., van Heerikhuizen,H. and Planta,R.J. (1992) A system to study transcription by yeast RNA polymerase I within the chromosomal context: functional analysis of the ribosomal DNA enhancer and the RBP1/REB1 binding sites. *EMBO J.*, **11**, 4665–4674.

Lucchini,R. and Sogo,J.M. (1995) Replication of transcriptionally active chromatin. *Nature*, **374**, 276–280.

McStay,B. and Reeder,R.H. (1986) A termination site for *Xenopus* RNA polymerase I also acts as an element of an adjacent promoter. *Cell*, **47**, 913–920.

Morrow,B.E., Johnson,S.P. and Warner,J.R. (1989) Proteins that bind to the yeast rDNA enhancer. *J. Biol. Chem.*, **264**, 9061–9068.

Morse,R.H. (1993) Nucleosome disruption by transcription factor binding in yeast. *Science*, **262**, 1563–1566.

Owen-Hughes,T. and Workman,J.L. (1994) Experimental analysis of chromatin function in transcriptional control. *Crit. Rev. Eukaryotic Gene Expr.*, **8**, 1–39.

Paule,M.R. (1994) Transcription of ribosomal RNA by eukaryotic RNA polymerase I. In Conaway,R.C. and Conaway,J.W. (eds), *Transcription: Mechanisms and Regulation.* Raven Press, New York, pp. 83–106.

Pazin,M.J., Kamakaka,R. and Kadonaga,J.T. (1994) ATP-dependent nucleosome reconfiguration and transcriptional activation from preassembled chromatin templates. *Science*, **266**, 2007–2011.

Pazin,M.J., Sheridan,P.L., Cannon,K., Cao,Z., Keck,J.G., Kadonaga,J.T. and Jones,K.A. (1996) NF-kappaB-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer *in vitro*. *Genes Dev.*, **10**, 37–49.

Sandaltzopoulos,R., Blank,T. and Becker,P.B. (1994) Transcriptional repression by nucleosomes but not H1 in reconstituted preblastoderm *Drosophila* chromatin. *EMBO J.*, **13**, 373–379.

Sander,E.E., Mason,S.W., Munz,C. and Grummt,I. (1996) The amino-terminal domain of the transcription termination factor TTF-I causes protein oligomerization and inhibition of DNA binding. *Nucleic Acids Res.*, **24**, 3677–3684.

Schnapp,A. and Grummt,I. (1991) Transcription complex formation at the mouse rDNA promoter involves the stepwise association of four transcription factors and RNA polymerase. *J. Biol. Chem.*, **266**, 24588–24595.

Smid,A., Finsterer,M. and Grummt,I. (1992) Limited proteolysis unmasks specific DNA-binding of the murine RNA polymerase I-specific transcription termination factor TTF-I. *J. Mol. Biol.*, **227**, 635–647.

Svaren,J. Schmitz,J. and Hörz,W. (1994) The transactivation domain of Pho4 is required for nucleosome disruption at the PHO5 promoter. *EMBO J.*, **13**, 4856–4862.

Taylor,C.A., Workman,J.L., Schuetz,T.J. and Kingston,R.E. (1991) Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: differential function of DNA binding domains. *Genes Dev.*, **5**, 1285–1298.

Tsukiyama,T., Becker,P.B. and Wu,C. (1994) ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. *Nature*, **367**, 525–532.

Tsukiyama,T. and Wu,C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell*, **83**, 1011–1020.

Varga-Weisz,P.D., Blank,T.A. and Becker,P.B. (1995) Energy-dependent chromatin accessibility and nucleosome mobility in a cell-free system. *EMBO J.*, **14**, 2209–2216.

Wallrath,L.L., Quinn,L., Granok,H. and Elgin,S.C.R. (1994) Architectural variations of inducible eukaryotic promoters: preset and remodeling chromatin structures. *BioEssays*, **16**, 165–170.

Wilson,C.J., Chao,D.M., Imbalzano,A.N., Schnitzler,G.R., Kingston,R.E. and Young,R.A. (1996) RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell*, **84**, 235–244.

Wolffe,A.P. (1994) Transcription: in tune with the histones. *Cell*, **77**, 1–4.

Wu,C. (1980) The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. *Nature*, **286**, 854–860.

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