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TTF-I determines the chromatin architecture of the active rDNA promoter

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Transcription of ribosomal genes assembled into chromatin requires binding of the transcription termination factor TTF-I to the promoter-proximal terminator T_0. To analyze the mechanism of TTF-I-mediated transcriptional activation, we have used mutant templates with altered sequence, polarity and distance of T_0 with respect to the transcription start site. Transcription activation by TTF-I is chromatin specific and requires the precise positioning of the terminator relative to the promoter. Whereas termination by TTF-I depends on the correct orientation of a terminator, TTF-I-mediated transcriptional activation is orientation independent. TTF-I can bind to nucleosomal DNA in the absence of enzymatic activities that destabilize nucleosome structure. Chromatin-bound TTF-I synergizes with ATP-dependent cofactors present in extracts of *Drosophila* embryos and mouse cells to position a nucleosome over the rDNA promoter and the transcription start site. Nucleosome positioning correlates tightly with the activation of rDNA transcription. We suggest that transcriptional activation by TTF-I is a stepwise process involving the creation of a defined promoter architecture and that the positioning of a nucleosome is compatible with, if not a prerequisite for, transcription initiation from rDNA chromatin.

Keywords: chromatin/nucleosome remodeling/RNA polymerase I/transcription activation/TTF-I

Introduction

It is well established that chromatin structure is an active participant in the regulation of eukaryotic gene expression. The assembly of nucleosomes onto DNA efficiently represses transcription by RNA polymerases II and III (Knezetic and Luse, 1986; Lorch *et al.*, 1987; Almouzni *et al.*, 1990; Felts *et al.*, 1990; Clark and Wolffe, 1991) by restricting access of the transcriptional machinery to DNA (for a review see Owen-Hughes and Workman, 1994). As a result, the chromatin structure at enhancer and promoter regions is often reconfigured prior to, or concurrent with, the induction of gene transcription. It has become apparent that this initial remodeling of chromatin is a key step in transcriptional regulation. Changes in chromatin structure can occur independently of DNA replication or may result from transcription factors binding to their target sites prior to chromatin assembly during DNA replication (Barton and Emerson, 1994). Activation of chromatin templates is due mainly to anti-repression of nucleosomal inhibition of transcription, possibly through contacts with the general transcription machinery and in synergy with chromatin remodeling activities that directly destabilize chromatin structure (for reviews see Becker, 1994; Felsenfeld, 1996; Tsukiyama and Wu, 1997). However, there are also indications that precisely positioned nucleosomes may be integral parts of active promoter structure. The bending of DNA around a histone octamer brings distant sequence elements into proximity, that may facilitate the functional interaction between transcription regulators which may otherwise be unable to contact each other (Wallrath *et al.*, 1994; Wolffe, 1994). In a few instances, the interaction of transcription factors with DNA wrapped over precisely positioned nucleosomal particles has been suggested to be essential for optimal regulation of transcription (McPherson *et al.*, 1993; Truss *et al.*, 1995).

In contrast to genes transcribed by RNA polymerases II and III, very little is known about transcription by RNA polymerase I (Pol I) on chromatin templates. Previously we described that transcriptional activation on nucleosomal rDNA templates *in vitro* requires the presence of the transcription termination factor TTF-I (Längst *et al.*, 1997) and suggested that TTF-I serves an important role in transcription initiation on chromatin besides its established function as a transcription termination factor (Grummt *et al.*, 1986a; Evers *et al.*, 1995) and replication fork barrier (Gerber *et al.*, 1997). As initially discovered in *Xenopus* (Moss, 1983), a transcription terminator precedes all vertebrate rDNA promoters studied so far. In mouse, this promoter-proximal terminator element, known as T_0, is positioned 170 bp upstream of the transcription initiation site (Grummt *et al.*, 1986b). Similarly to the repeated terminator elements (T_1–T_{10}) located downstream of the pre-rRNA coding region (Grummt *et al.*, 1985), the T_0 element is recognized specifically by TTF-I. Binding of TTF-I to its cognate site can stop elongating Pol I *in vitro* (Bartsch *et al.*, 1988; Smid *et al.*, 1992; Evers *et al.*, 1995). The functional relevance of the promoter-proximal terminator has been elusive. Nuclease S1 mapping and nucleolar run-on experiments have revealed that transcripts initiated at the spacer promoter are terminated at this site. Moreover, the upstream terminator has been shown to augment transcription from an adjacent promoter *in vivo* and *in vitro* (Grummt *et al.*, 1986a; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986). The same mutations within T_0 that impair termination also reduce the amount of transcripts from the adjacent rDNA promoter in the absence of transcription from upstream (Grummt *et al.*, 1986b; McStay and Reeder, 1986).

The mechanism by which the upstream terminator stimulates transcription is not yet understood. It has been suggested that the terminator could serve a role in preventing transcriptional interference between duplicated gene promoters, i.e. inhibition of a downstream promoter by transcription from an upstream gene. In this 'promoter occlusion' model, TTF-I bound to T_0 shields the rDNA promoter from polymerases that read through from spacer promoters, thereby inactivating initiation complexes assembled at the rDNA promoter (Bateman and Paule, 1988; Henderson *et al.*, 1989). However, other models are conceivable to explain how this transcriptional stimulation may occur. For instance, the terminator could 'hand-off' polymerase to the adjacent promoter efficiently in a concerted reaction, called 'readthrough enhancement' (Moss 1983). Alternatively, it can be envisaged that the terminator is, or overlaps with, a promoter element. In this scenario, T_0 would be part of the upstream control elements that stimulate transcription from the rDNA core promoter. In agreement with this hypothesis, the upstream terminator in *Xenopus* rDNA augments transcription initiation by a mechanism that depends on the helical alignment between the upstream terminator and the core promoter (McStay and Reeder, 1990). These observations, together with the conservation of the position of T_0 with respect to essential promoter elements, suggest that the promoter and terminator activities are intimately connected.

Since access of protein factors to their recognition sites is influenced by the assembly of DNA into chromatin, an understanding of the upstream terminator function must take into account the chromatin structure. In this study, we investigated the correlation of TTF-I binding to the promoter-proximal terminator assembled into chromatin, nucleosome remodeling and transcriptional activation. The data suggest that activation of Pol I transcription on chromatin templates by TTF-I can be dissected into several steps. First, TTF-I is able to interact with its target site on nucleosomes in the absence of ATP-dependent cofactors. Second, a remodeling activity which is present both in *Drosophila* embryo extracts and in the partially purified murine transcription system aligns nucleosomes upstream and downstream of the TTF-I-binding site in an ATP-dependent fashion, thus placing a nucleosome over the rDNA promoter including the transcription start site. Remarkably, the positioning of a nucleosome on the rDNA promoter correlates tightly with transcriptional activation. TTF-I-induced transcription on chromatin templates has tight spatial constraints, i.e. it requires the natural distance of the terminator relative to the promoter. The important function of TTF-I as a chromatin-specific activator provides a molecular explanation for the evolutionary conservation of terminator elements within eukaryotic ribosomal gene promoters.

Results

TTF-I counteracts transcriptional repression on reconstituted chromatin templates

Transcription on naked DNA and chromatin, respectively, was assayed on the template $pMrT_2$, an artificial ribosomal minigene which represents a fusion between a 5′- and 3′-terminal murine rDNA fragment and therefore contains both Pol I promoter and terminator sequences. A murine protein fraction (DEAE-280) was used as a source of RNA polymerase I and all other factors required for transcription initiation on mouse rDNA. As this fraction lacks the termination factor TTF-I, long heterogeneous readthrough transcripts are synthesized on circular, non-nucleosomal $pMrT_2$ (Figure 1, lane 1). Addition of TTF-I yields 172 nucleotide RNA molecules which correspond to specifically terminated transcripts (lane 2). To study Pol I transcription on chromatin, $pMrT_2$ was assembled into chromatin with extracts from early *Drosophila* embryos and used as template in the murine transcription system. Consistent with previous results (Längst *et al.*, 1997), TTF-I did not affect the overall transcriptional activity on naked DNA, but counteracted transcriptional repression on chromatin templates. In the absence of TTF-I, transcription on reconstituted chromatin templates was repressed (Figure 1, lane 3), and this repression was overcome by increasing amounts of recombinant TTF-I (lanes 4–6). In the presence of saturating levels of TTF-I, the amount of transcripts synthesized from the nucleosomal template $pMrT_2$ was about one-third of that observed with naked DNA. Thus, on chromatin templates, TTF-I exerts two functions, i.e. it facilitates initiation and mediates termination of transcription.

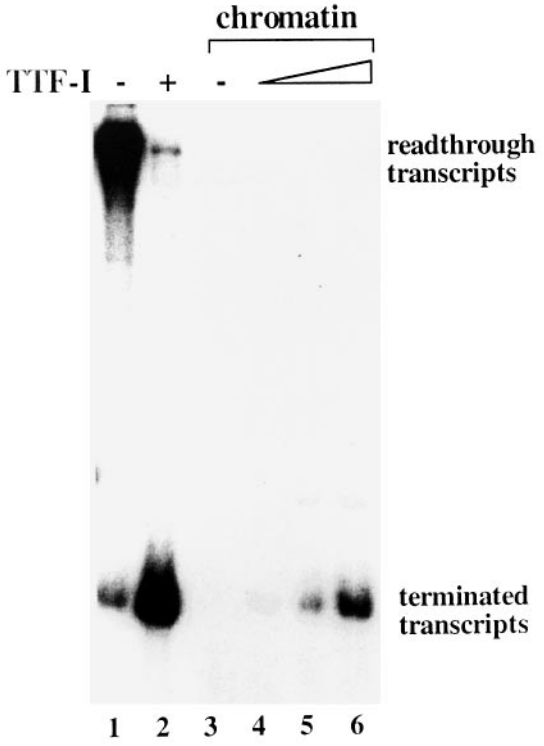

Fig. 1. TTF-I activates transcription on chromatin templates. The transcription reactions contained 15 ng of circular $pMrT_2$ (lanes 1 and 2) or 15 ng of $pMrT_2$ that has been pre-assembled into chromatin (lanes 3–6) and incubated with increasing amounts of TTF-I as indicated. The positions of the non-terminated readthrough transcripts and the terminated transcripts are indicated.

Chromatin remodeling and transcriptional activation requires TTF-I binding to the upstream terminator T_0

To determine the specificity of transcriptional activation, we tested the ability of TTF-I to induce chromatin

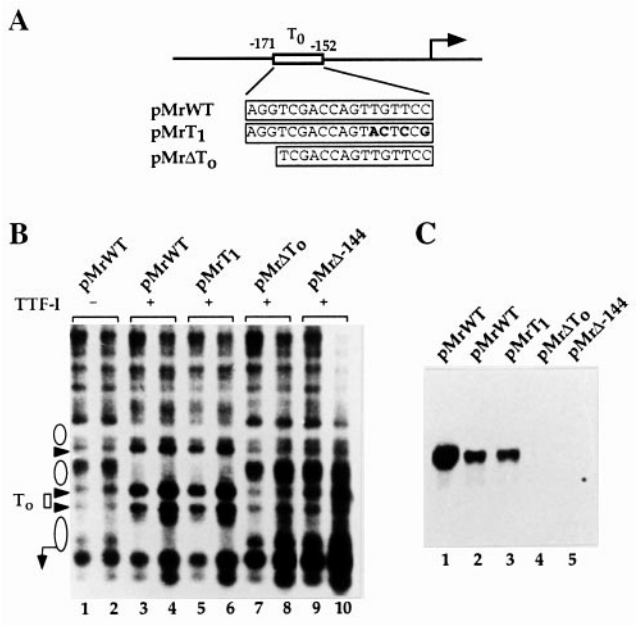

Fig. 2. Nucleosome rearrangement and transcription activation require binding of TTF-I to the promoter-proximal terminator. (**A**) Nucleotide sequence from –171 to –152 that varies between the constructs. The T_0 sequence is boxed. Nucleotide exchanges between the upstream terminator T_0 and the downstream terminator T_1 are indicated by bold letters. (**B**) Nucleosome remodeling by TTF-I on mutant templates. The individual templates were assembled into chromatin in the absence or presence of TTF-I as indicated, and nucleosome positions were assayed by partial MNase digestion (10 U of MNase, 10 and 30 s) and indirect end-labeling. The predominant nucleosome positions at the promoter are indicated by open circles; filled triangles indicate new boundaries after factor binding. The TTF-I-binding site is marked by a box and the transcription initiation site by an arrow. (**C**) TTF-I-mediated transcriptional activation of mutant chromatin templates. Transcriptions were performed on naked pMrWT/*Nar*I (lane 1) or on the different chromatin templates in the presence of TTF-I (lanes 2–5).

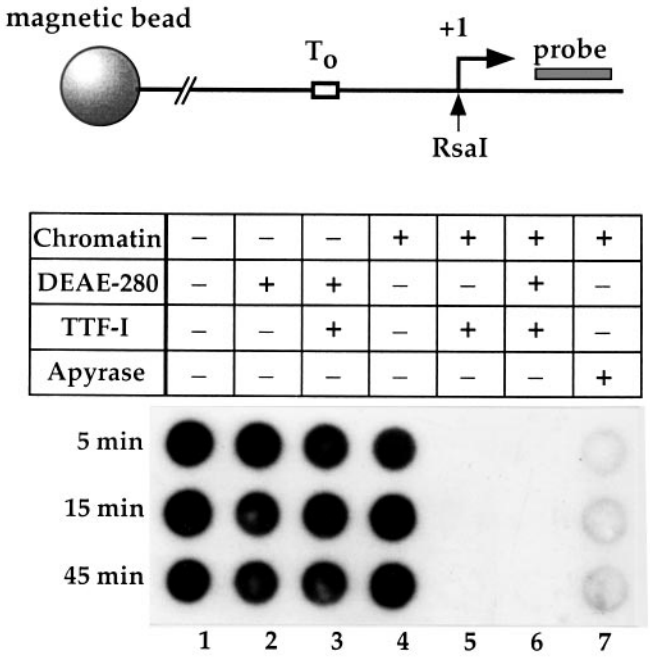

Fig. 3. Restriction site protection assay. pMrWT was immobilized on paramagnetic beads, assembled into chromatin and incubated with TTF-I and/or the DEAE-280 fraction as indicated. Aliquots of the individual reactions were incubated under standard transcription conditions in the presence of *Rsa*I for the indicated times. The accessibility of the *Rsa*I site (between –1 and +1) was monitored by dot hybridization of DNA released into the supernatant.

remodeling and promote transcription from mutant rDNA templates containing alterations in the sequence of the upstream terminator. T_0 was either mutated ($pMrΔT_{0)}$, deleted (pMrΔ-144) or replaced by T_1, the first of the 10 downstream Sal box terminator elements ($pMrT_1$). The templates were assembled into chromatin in the absence or presence of TTF-I and analyzed by partial micrococcal nuclease (MNase) digestion, followed by Southern blotting and indirect end-labeling (Längst *et al.*, 1997). In the absence of TTF-I, a regular pattern of MNase-sensitive sites was observed (Figure 2B, lanes 1 and 2) which corresponds to that of naked DNA (data not shown). Significantly, in the presence of TTF-I, the pattern of hypersensitive sites changed. The most remarkable features observed in the presence of TTF-I were the appearance of hypersensitive sites flanking the terminator T_0 and protection of MNase cleavage sites in adjacent regions (Figure 2B, lanes 3–6). The size of the protected regions and the pattern of hypersensitivity of sequences flanking T_0 have been interpreted as re-alignment of nucleosomes with respect to bound TTF-I (Längst *et al.*, 1997). Replacement of T_0 by the downstream terminator T_1 ($pMrT_1$) which has a higher affinity for TTF-I exhibited the same MNase cleavage pattern (Figure 2B, lanes 5 and 6) and a transcriptional activity comparable with that of the natural construct pMrWT (Figure 2C, lanes 2 and 3). On the other hand, when T_0 was either mutated or deleted ($pMrΔT_0$ and pMrΔ-144), TTF-I was unable to rearrange nucleosomal positions (Figure 2B, lanes 7–10) and to activate transcription (Figure 2C, lanes 4 and 5). It is noteworthy that none of the mutants had any effect on transcription from naked rDNA templates (data not shown). The finding that TTF-I-mediated chromatin re-alignment correlates with activation of rDNA transcription on chromatin templates suggests that these two processes are intimately related.

A nucleosome covers the transcription start site

Single nucleotide resolution mapping resolved the positioned nucleosome into a heterogeneous family of nucleosomes with different translational positions (Längst *et al.*, 1997). In order to estimate the degree of accessibility of the transcription start site on the promoter under various conditions, we made use of the fact that nucleosomal DNA usually is not cleaved by restriction enzymes (Almer and Hörz, 1986; Archer *et al.*, 1991) and, therefore, protection of a restriction site can yield information as to whether or not a DNA region is covered by a nucleosome. To monitor positioned nucleosomes at the rDNA promoter, the accessibility of immobilized chromatin templates towards the restriction endonuclease *Rsa*I was assayed. Since *Rsa*I cleaves mouse rDNA between nucleotides –1 and +1, a nucleosome at the transcription initiation site should protect the template from digestion. In the experiment shown in Figure 3, chromatin was assembled on the immobilized rDNA template in the presence or absence of TTF-I and/or the DEAE-280 fraction, respectively, incubated for 5, 15 or 45 min under transcription conditions with *Rsa*I, and cleavage was assessed by dot-

blot hybridization of DNA released from the beads. In the presence of the DEAE-280 fraction, TTF-I or both, *Rsa*I cleavage occurred as efficiently as on protein-free DNA (Figure 3B, lanes 1–3). Significantly, the *Rsa*I site was also accessible in chromatin which had been assembled in the absence of TTF-I (lane 4). This accessibility of chromatin to restriction enzymes most likely relies on ATP-dependent cofactors which promote nucleosome movement (Varga-Weisz *et al.*, 1997). These movements come to a stop in the absence of ATP (presence of Apyrase, lane 7). Under those conditions, cleavage is ~10% of the free DNA, which presumably reflects the fraction of templates in which the *Rsa*I site is accessible in a linker. Remarkably, if TTF-I was added to chromatin under conditions where nucleosome re-positioning and efficient transcription occur, cleavage at the start site was inhibited quantitatively both in the absence and presence of the DEAE-280 fraction (lanes 5 and 6). Apparently the binding of TTF-I to chromatin restricted the mobility of nucleosomes inherent in the system (Varga-Weisz *et al.*, 1995), such that a nucleosome protected the promoter from *Rsa*I cleavage. Under those conditions, at least 30% of the templates are active, which can be visualized by a transcription-dependent chromatin perturbation (Längst *et al.*, 1997). Together with the results of the indirect end-labeling analysis, this suggests that a nucleosomal configuration of the transcription start site is compatible with transcription initiation.

Transcription termination, but not TTF-I mediated transcriptional activation on chromatin templates, is polar

A unique property common to all Pol I transcription terminators is the strict polarity of the termination reaction, i.e. the terminator element has to be in its natural orientation to arrest an elongating RNA polymerase I (Grummt *et al.*, 1985, 1986a). Also, the replication fork barrier (RFB) function of TTF-I is polar, requiring the TTF-I binding site to be in the natural orientation to arrest replication forks coming from downstream and thus prevent collision with the transcription machinery (Gerber *et al.*, 1997). In view of this orientation dependence, we studied whether transcription activation by TTF-I on chromatin templates would require the correct orientation of the TTF-I-binding site with respect to the rDNA promoter. We used two minigene templates in which T_0 was replaced by a synthetic oligonucleotide corresponding to the terminator T_1 in either orientation. The two templates (T_1 and T_1*, see Figure 4A) also contained the rDNA terminators T_1–T_{10} at the end of the transcription unit. The two plasmids were assembled into chromatin, TTF-I was added (or not) to induce nucleosome repositioning and the templates were then analyzed by the standard transcription reaction which monitors discrete transcripts terminating at T_1–T_{10} (Figure 4A). In parallel, the chromatin structure was analyzed by partial MNase digestion and indirect end-labeling (Figure 4B). Remarkably, TTF-I stimulated transcription and induced nucleosome remodeling, irrespective of whether the TTF-I-binding site was inserted in the natural or opposite orientation. Thus, in contrast to transcription termination and RFB activity, TTF-I-mediated chromatin remodeling and transcriptional activation do not require the native orientation of the binding site.

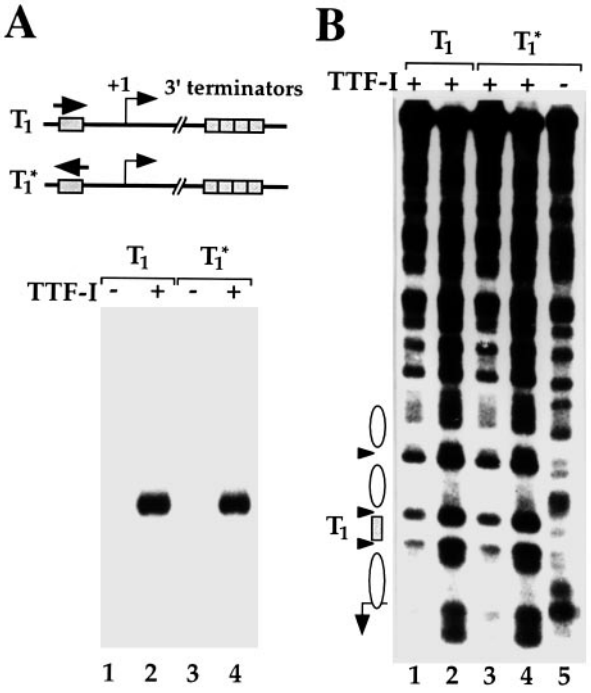

Fig. 4. TTF-I mediated chromatin remodeling and transcriptional activation is independent of the orientation of the TTF-I target site. (**A**) Transcription on reconstituted chromatin templates. Twenty ng of circular $pMrT_1$–$T_{1–10}$ (abbreviated here as T_1) and $pMrT_1$*-$T_{1–10}$ (abbreviated here as T_1*) were assembled into chromatin and assayed for transcriptional activity in the absence or presence of 0.1 pmol of TTF-I. The arrows show the orientation of the terminator. (**B**) MNase digestion pattern. Chromatin was assembled on $pMrT_1$ (lanes 1 and 2) and $pMrT_1$* in the absence or presence of TTF-I (lanes 3–6), digested with 10 U of MNase for 30 and 90 s and the cleavage pattern was analyzed by indirect end-labeling. The nucleosome positions flanking DNA-bound TTF-I are indicated by open circles; hypersensitive sites induced by TTF-I binding are marked by filled triangles. The TTF-I binding site is indicated by a bar and the transcription start site is marked by an arrow.

Transcriptional activation requires a precise spacing between T_0 and the promoter

To examine whether TTF-I-mediated transcriptional activation requires the correct positioning of the terminator with respect to the transcription initiation site, we constructed a series of templates in which the distance of the T_0 element from the promoter was changed by –4 to +90 nucleotides with respect to its normal position. Consistent with TTF-I not affecting transcription of naked DNA, all mutants had the same transcriptional activity when used as templates in transcription assays, irrespective of whether TTF-I was present or not (Figure 5A, lanes 1–6).

A strikingly different result was obtained if the same series of mutants was assembled into chromatin. Only the wild-type construct $pMrPA_0$ and the deletion mutant $pMrPA_{–4}$ were activated efficiently in the presence of TTF-I (Figure 5A, lanes 7–10), whereas increasing the distance between T_0 and the promoter by 4, 10, 15 and 30 nucleotides was deleterious (lanes 11–18). This result indicates that the defined distance of the TTF-I binding site relative to the promoter is a prerequisite for factor-mediated transcriptional activation on chromatin tem-

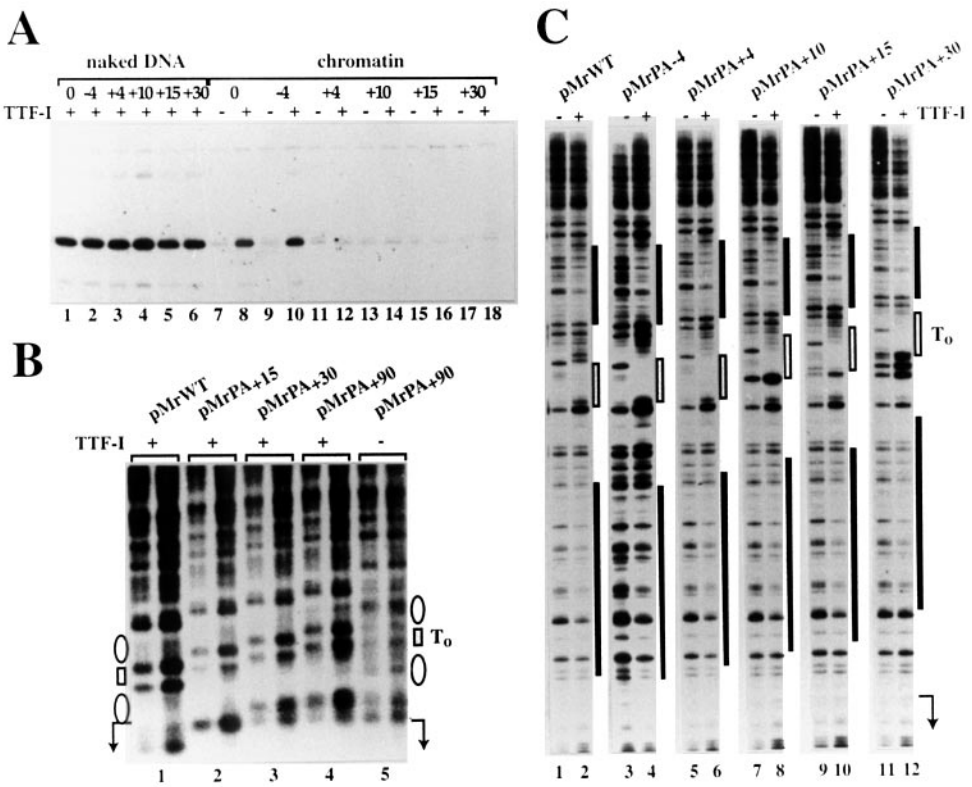

Fig. 5. Effect of spacing changes between T_0 and the promoter. (**A**) Transcriptional analysis. The push-apart constructs, changing the distance between T_0 and the promoter, were used as circular, naked DNA (lanes 1–6) or as chromatin templates (lanes 7–18) in the standard transcription assay in the absence or presence of 0.1 pmol of TTF-I as indicated. Transcripts were analyzed by primer extension. (**B**) Indirect end-labeling of MNase cleavage sites. The templates were assembled into chromatin in the presence (lanes 1–4) or absence (lanes 5) of TTF-I and analyzed by partial MNase digestion and indirect end-labeling. The nucleosome positions flanking DNA-bound TTF-I are indicated by open circles. The TTF-I binding site is indicated by a bar, the transcription start site is marked by an arrow. (**C**) MNase footprint. The templates were assembled into chromatin in the presence or absence of TTF-I, digested with MNase and analyzed by primer extension. The black bars mark the regions protected by nucleosomes. The gray box indicates the position of the upstream terminator T_0.

plates. Contrary to previous microinjection data in *X.laevis* showing that the stimulatory effect of the upstream terminator could be partially rescued when the distance was changed in units of 10 bp (McStay and Reeder, 1990), TTF-I failed to relieve transcriptional repression of all push-apart mutants tested (i.e. $pMrPA_{+4/+10/+15/+30}$). Thus, the correct helical alignment of T_0 could not compensate for incorrect spacing. To see whether the transcriptional activity of the templates affected the chromatin remodeling, we analyzed the chromatin structure of the change-of-distance mutants in the presence and absence of TTF-I by MNase digestion and indirect end-labeling as before (Figure 5B). In contrast to transcription activation, TTF-I-mediated nucleosome repositioning did not require the correct spacing between the terminator and the promoter. In all mutants tested, two hypersensitive sites at T_0 and nucleosome-sized protections upstream and downstream of the TTF-I binding site were observed, indicating that nucleosomes flank DNA-bound TTF-I, irrespective of the length and nucleotide composition of the inserted sequence.

To map the position of the nucleosomes at higher resolution, chromatin was assembled in the presence of TTF-I, partially digested with MNase and analyzed by primer extension (Figure 5C). Consistent with the results of the indirect end-labeling experiments, TTF-I binding to the upstream terminator induced MNase-hypersensitive sites in the flanking sequences. Since MNase not only cuts within the linker region, but also produces single strand cuts in nucleosomal DNA (Cockell *et al.*, 1983), the two positioned nucleosomes upstream and downstream of the TTF-I-binding site were less obvious in this assay. Nevertheless, in all spacing mutants, the position of the downstream nucleosome was altered depending on the number of nucleotides that were deleted or inserted between T_0 and the transcription initiation site.

TTF-I binds to nucleosomal DNA

The results presented so far did not reveal whether TTF-I binding to chromatin and nucleosome re-positioning are independent processes, or whether nucleosome remodeling by energy-consuming cofactors present in embryo extracts (Tsukiyama and Wu, 1995; Varga- Ito *et al.*, 1997; Weisz *et al.*, 1997) is a prerequisite for TTF-I binding. To examine whether the two processes can be separated, we first analyzed the interaction of TTF-I with nucleosome core particles. Mononucleosomes were assembled *in vitro* on a 176 bp 5′-terminal rDNA fragment (from –232 to –56) using purified core histones and polyglutamic acid. Electrophoretic mobility shift analysis (EMSA) revealed that ~50% of the DNA fragment was reconstituted into nucleosomal cores (Figure 6A, lanes 1 and 2). To select for DNA fragments which were assembled into nucleosomes, the reactions were incubated with *Eco*RII. *Eco*RII

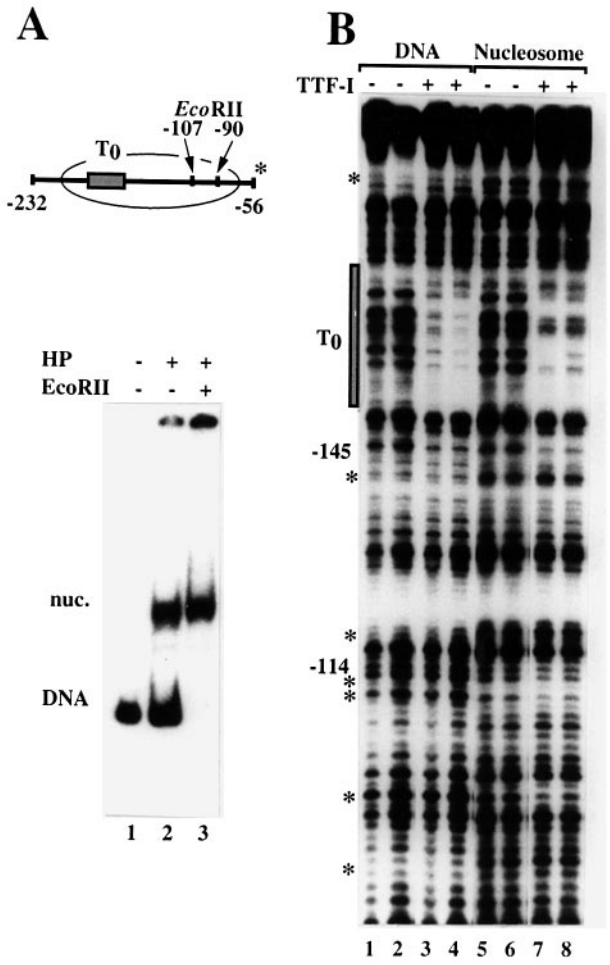

Fig. 6. TTF-I binds to *in vitro* reconstituted nucleosomes. (**A**) EMSA of nucleosome core particles assembled *in vitro*. A labeled DNA fragment spanning rDNA sequences fom –232 to –56 was reconstituted into mononucleosomes. Free DNA was digested with *Eco*RII (lane 3). Lanes 1 and 2 are free DNA and undigested nucleosomes respectively. The positions of free DNA and nucleosome cores (nuc.) are marked. The scheme above shows the rDNA fragment used and indicates the *Eco*RII cleaveage sites as well as the site of labeling (*). HP indicates nucleosome reconstitution by histones and polyglutamic acid. (**B**) DNase I footprinting of TTF-I bound to mononucleosomes. Free DNA (lanes 1–4) and nucleosomes (lanes 5–8) were incubated in the absence or presence of 50 ng of TTF-I, digested with DNase I (0.02 and 0.1 U for free DNA and 0.1 and 0.5 U for chromatin, respectively) and analyzed on a 6% sequencing gel. The TTF-I-binding site T_0 is indicated by a gray box on the left. Differences between the naked DNA and the nucleosomal DNA are indicated by asterisks.

cleaves the free rDNA fragment, but not if it is assembled into a mononucleosome (lane 3). Therefore, digestion with *Eco*RII was used as a tool to separate nucleosomal from naked DNA which would interfere in protein interaction assays.

To assess whether TTF-I was capable of binding its cognate site in the nucleosome, TTF-I was added to reactions containing either naked DNA or *Eco*RII-selected nucleosomes respectively, and binding was assayed by DNase I footprinting (Figure 6B). The pattern of DNase I cleavage sites was modulated by the wrapping of the DNA around the nucleosome (asterisk in Figure 6B). The lack of a clear 10 bp 'ladder' of digestion products is consistent with previous results showing multiple positions of nucleosomes at the rDNA promoter (Längst *et al.*, 1997). Significantly, TTF-I bound to T_0 both in free DNA and on nucleosomes, as revealed by protection of the upstream terminator in the presence of TTF-I (lanes 3, 4, 7 and 8). The DNase I cleavage pattern diagnostic for the nucleosome was unaltered upon TTF-I binding, indicating the formation of a ternary complex. Remarkably, binding occurred in the absence of ATP and cofactors, demonstrating that TTF-I on its own is capable of interacting with its target site packaged into a nucleosome.

Nucleosome re-alignment requires ATP-dependent cofactors present in both the Drosophila extract and the murine transcription system

To separate TTF-I binding to chromatin from nucleosome re-alignment, plasmid DNA (pMrWT) was first assembled into chromatin with *Drosophila* embryo extract in the absence of TTF-I and then incubated for 5 min with 0.08% Sarkosyl. This detergent inactivates the main nucleosome remodeling factors known in the extract (Tsukiyama and Wu, 1995; Varga-Weisz *et al.*, 1997). The chromatin was purified on a spin column, TTF-I and ATP were added and the chromatin structure was analyzed by MNase and DNase I footprinting (Figure 7A and B). Consistent with the data above, the MNase cleavage pattern of untreated chromatin revealed protection of a diagnostic MNase-sensitive site (asterisk in Figure 7A) within the upstream terminator T_0 by TTF-I, the appearance of flanking hypersensitive sites and nucleosome-sized protected regions adjacent to the TTF-I binding site (Figure 7A, lanes 1 and 2). In the absence of TTF-I, the digestion pattern of Sarkosyl-treated chromatin was similar to that of the untreated sample (lanes 1 and 3). Remarkably, TTF-I was able to interact with chromatin in Sarkosyl-washed chromatin (lane 4), even in the absence of ATP (data not shown). Apparently, TTF-I binding to chromatin did not require Sarkosyl-sensitive nucleosome remodeling factor(s) present in the *Drosophila* embryo extract, in accord with its ability to interact with the nucleosome (Figure 6).

However, there were marked differences in the MNase pattern of flanking sequences in untreated and Sarkosyl-treated chromatin. Although TTF-I bound to both samples with similar efficiency, no re-positioning of nucleosomes to align with bound TTF-I occurred in detergent-treated chromatin. Neither the hypersensitive sites flanking T_0 nor the protected regions upstream and downstream of the TTF-I binding site were observed (Figure 7A, lane 4). However, consistent with Sarkosyl treatment inactivating or eliminating cofactors in the *Drosophila* embryo extract that facilitate nucleosomal remodeling, addition of fresh *Drosophila* extract partially restored TTF-I-dependent alterations of chromatin structure (lane 5).

Similar results were obtained by DNase I footprinting. As shown in Figure 7B, TTF-I binding protected the upstream terminator from DNase I digestion, and binding was observed both in untreated and detergent-treated chromatin (lanes 2, 4 and 6). Again, the hypersensitive sites flanking T_0 were not observed in Sarkosyl-treated chromatin (lanes 3 and 4), but were generated after addition of *Drosophila* extract (lanes 5 and 6). Thus, Sarkosyl-sensitive activities present in the *Drosophila*

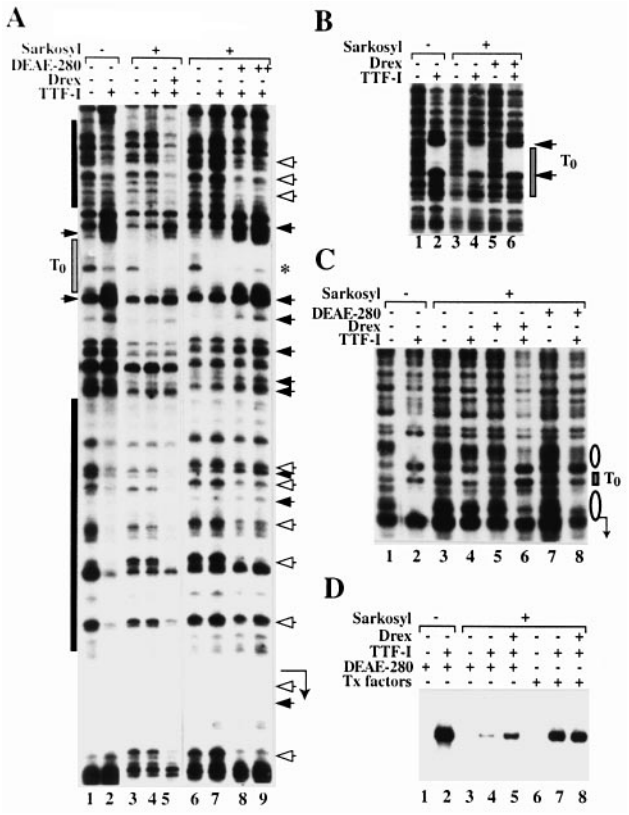

Fig. 7. Cellular cofactors are required for TTF-I-mediated nucleosome remodeling. (**A**) MNase footprint. Chromatin was assembled on plasmid pMrWT, incubated for 5 min in the absence (lanes 1 and 2) or presence of 0.08% Sarkosyl (lanes 3–9) and purified on a Sephacryl 300 HR spin column. Purified chromatin was incubated for 30 min with TTF-I, *Drosophila* extract (Drex) or increasing amounts of DEAE-280 as indicated. Chromatin was partially digested with MNase and analyzed by primer extension. Protected regions due to a positioned nucleosome are marked by a black box. The TTF-I-binding site T_0 is indicated by a gray box. Increased sensitivity of sites after the addition of DEAE-280 are indicated by filled arrows; protected sites are marked by open arrows. (**B**) DNase I footprint. Chromatin and Sarkosyl-treated chromatin were incubated in the absence or presence of TTF-I and *Drosophila* extract (Drex) as indicated. Chromatin was partially digested with DNase I and analyzed by primer extension. The footprint shows the TTF-I-binding region, indicated by a gray box. Hypersensitive sites are marked by filled arrows. (**C**) MNase digestion pattern of reconstituted chromatin. Chromatin was incubated in the absence (lanes 1 and 2) or presence of Sarkosyl (lanes 3–8), purified and incubated for 30 min with TTF-I, DEAE-280 and *Drosophila* extract (Drex) as indicated. After digestion for 30 s with either 1 U (lanes 3 and 4) or 10–30 U of MNase (lanes 1, 2 and 5–8), the chromatin structure was analyzed by Southern blotting and indirect end-labeling. The TTF-I binding site is indicated by a bar; the protected regions due to nucleosome remodeling are marked by an open circle; the transcription start site is marked by an arrow. (**D**) Transcription on Sarkosyl-treated chromatin. Chromatin was assembled on plasmid $pMrT_{1-10}$ and incubated in the absence (lanes 1 and 2) or presence of 0.08% Sarkosyl (lanes 3–8) for 5 min. After gel filtration on a spin column, purified chromatin was analyzed for transcriptional activity in the DEAE-280 fraction (lanes 1–5), or in a reconstituted system containing partially purified Pol I, TIF-IA, TIF-IB, TIF-IC and recombinant UBF (Tx factors; lanes 6–8) in the absence or presence of TTF-I and *Drosophila* embryo extract (Drex) as indicated.

extract were required to facilitate re-alignment of nucleosomes with respect to bound TTF-I.

To test whether similar activities are also contained in the mouse protein fraction used for transcription, the Sarkosyl-treated chromatin was incubated with ATP and increasing amounts of the DEAE-280 fraction. The analysis by MNase footprinting (Figure 7A, lanes 8 and 9) and indirect end-labeling (Figure 7C) demonstrated that the DEAE-280 fraction contained activities that could substitute functionally for Sarkosyl-sensitive *Drosophila* factors. The hypersensitive sites flanking the TTF-I footprint were restored after addition of either the *Drosophila* embryo extract or the murine DEAE-280 fraction (Figure 7A, lanes 5, 8 and 9). The protection of the promoter region was less obvious with the murine protein fraction than with the *Drosophila* extract, which may be due to specific binding of mouse transcription factors to rDNA control elements. The presence of remodeling activities in the mouse protein fraction which can replace the *Drosophila* enzyme(s) was also revealed by indirect end-labeling (Figure 7C). Both the *Drosophila* extract (lane 6) and the murine DEAE-280 fraction (lane 8) induced TTF-I-dependent nucleosome re-alignment.

The presence of chromatin remodeling activities in the DEAE-280 fraction suggests that TTF-I should be capable of activating transcription from Sarkosyl-treated chromatin. To test this, untreated and detergent-treated chromatin was analyzed for transcriptional activation by TTF-I (Figure 7D). Chromatin templates without Sarkosyl wash were activated by TTF-I (lanes 1 and 2). Significantly, transcriptional activation by TTF-I also occurred on Sarkosyl-treated chromatin in the absence of *Drosophila* extract, irrespective of whether the reactions contained the DEAE-280 fraction (lanes 3 and 4) or a reconstituted system containing recombinant upstream binding factor (UBF) and partially purified transcription factors (lanes 6 and 7). In the latter case, TTF-I activated transcription on detergent-treated chromatin templates almost as efficiently as on the untreated control (compare lanes 2 and 7).

Discussion

Previously we have shown that activation of murine rDNA transcription on chromatin templates required binding of the termination factor TTF-I to the upstream terminator T_0 which triggered an energy-dependent re-positioning (remodeling) of nucleosomes (Längst *et al.*, 1997). The action of TTF-I in chromatin resulted in a specific activation of the chromatin template, whereas binding of TTF-I to chromatin-free templates did not affect transcription. The tight correlation between TTF-I binding, ATP-dependent nucleosome re-positioning and transcriptional activation suggested that these processes are intimately connected.

We now report that the process of transcriptional activation by TTF-I can be dissected into three distinct steps: the initial binding of TTF-I to nucleosomal DNA, the re-positioning (remodeling) of nucleosomes, requiring energy-consuming cofactors and, finally, the activation of transcription. This result is reminiscent of the events that lead to activation of Pol II transcription by the thyroid receptor (TR), where the process of transcriptional activation involves the local chromatin disruption upon TR–RXR binding, extensive, hormone-dependent chromatin rearrangements and, finally, activation domain-dependent activation of transcription (Wong *et al.*, 1997a).

Efficient transcription of rDNA depended on narrow spatial constraints: changing the distance between the

upstream terminator and the transcription start site by as little as four nucleotides abolished the chromatin-specific transcriptional activation by TTF-I. The same deleterious effect was observed in mutants where the distance between the terminator and the promoter was increased by one or three integral helical turns, indicating that the correct helical alignment of T_0 could not compensate for incorrect spacing. While active transcription requires a precise distance between the terminator and the transcription start site, the position of TTF-I also defines the nucleosome positions on both sides of T_0. In those cases where the rDNA promoter was activated, the nucleosome covered both rDNA promoter elements, the upstream control element (UCE) and the core, and the transcription start site. The nucleosome was defined by the diagnostic resistance of associated DNA to restriction enzymes and MNase, both at low and single nucleotide resolution (Längst *et al.*, 1997; this work). Neither the binding of TTF-I or UBF alone, nor that of the entire transcription machinery, created a MNase cleavage profile that resembled that in chromatin (data not shown). Since a significant fraction of templates are active under these conditions (at least 30%; Längst *et al.*, 1997), the nucleosomal promoter appears to be compatible with the interaction of the Pol I transcription factors. The activation of rDNA transcription by TTF-I was chromatin specific and not observed on protein-free DNA, which leads us to envisage a scenario in which the TTF-I-induced repositioning of a nucleosome on the promoter leads to the establishment of a specific promoter architecture that is compatible with, if not a prerequisite for, pre-initiation complex formation. This was unexpected because nucleosomes positioned over promoters are usually inhibitory to transcription complex assembly (Knezetic and Luse, 1986; Felts *et al.*, 1990; Clark and Wolffe, 1991; Lorch *et al.*, 1992; Owen-Hughes and Workman, 1994) and, in general, positioned nucleosomes frequently repress transcription by blocking the ability of DNA-binding proteins or basal transcription factors to bind to the promoter.

While the experimental data can be interpreted in various ways, we would like to discuss in more detail one model that we find attractive and that is compatible with the data. The wrapping of DNA around a histone octamer in two tight circles of ~80 bp brings distant DNA sequences into close proximity on the nucleosomal surface and hence may facilitate the interaction of transcription factors (Wolffe, 1994). By analogy, winding the rDNA promoter around a histone complex would bring the UCE and the core element into close proximity, which might facilitate specific interactions between the TBP-containing promoter selectivity factor TIF-IB/SL1 and the HMG box-containing architectural factor UBF. In this scenario, the nucleosome positioned at the rDNA promoter may provide the correct scaffolding for productive interactions between TIF-IB/SL1 and UBF bound at the two recognition sites which are separated by 120 base pairs. This is an attractive hypothesis because it is in accord with early findings demonstrating that spacing changes between the core and the UCE affect both the cooperatively of the two elements and transcriptional activity. These previous studies revealed a strict requirement for the UCE *in vivo*, whereas *in vitro* the effect of this control element was much less pronounced (Schnapp *et al.*, 1990). Consistent with the rDNA promoter architecture exerting an indispensable function in rDNA transcriptional activity, analysis of interactions of UBF with *X. laevis* rDNA by electron spectroscopic imaging demonstrated that a single dimer of xUBF can organize ~180 bp of DNA into a loop of almost 360°. DNA looping required only the proximal part of UBF including the N-terminal dimerization domain and HMG-boxes 1–3 (Bazett-Jones *et al.*, 1994). Thus, both the histone octamer and UBF are capable of wrapping DNA and juxtaposing remote protein-binding sites. The recent finding that UBF binds to nucleosomes without displacing the core histones (Kermekchiev *et al.*, 1997) raises the interesting possibility that not only TTF-I, but also UBF, may serve an important role in Pol I transcription on nucleosomal templates. However, our data do not address the issue of whether the nucleosome remains structurally unaltered upon transcription factor binding or whether transient structural changes or even histone loss occurs.

The concept of architectural nucleosomes that influence transcription initiation positively by facilitating the interaction between distant transcription factors has been suggested previously from analyses of promoters that contain a positioned nucleosomes between proximal and distal transcription factor-binding sites (Schild *et al.*, 1993; Wallrath *et al.*, 1994; Quivy and Becker, 1996; Stünkel *et al.*, 1997). In the scenario we discussed for the rDNA promoter (see above), the nucleosome facilitates the interaction of transcription factors bound on the surface of the nucleosome. There are several examples in the literature of ternary complexes between DNA, transcription factors and modified nucleosomal particles (reviewed by Owen-Hughes and Workman, 1994; Beato and Eisfeld, 1997). Ternary complex formation between transcription factors and nucleosomes usually depends on precise rotational positioning of recognition sequences on the surface of the nucleosome (for examples see Li and Wrange, 1995; Wong *et al.*, 1997b). The hormone regulatory element that governs glucocorticoid induction of the mouse mammary tumor virus long terminal repeat (LTR) is organized into such a rotationally positioned nucleosome. Hormone-induced binding of the glucocorticoid receptor to its nucleosomal binding sites triggers an undefined transition in nucleosome structure that leads to increased accessibility of the neighboring DNA, but does not lead to complete nucleosome disassembly (Yamamoto, 1984; Zaret and Bresnick *et al.*, 1992; Fragoso *et al.*, 1995). It has been argued that the bending of the DNA over this nucleosome surface may allow the binding of an array of transcription factors without steric clashes (Truss *et al.*, 1995). The active albumin enhancer provides a further example for which a specific arrangement of transcription factors on the surface of a nucleosome has been suggested (McPherson *et al.*, 1993). Moreover, biochemical analyses of transcription factor binding and nucleosome remodeling at the human immunodeficiency virus 1 (HIV-1) LTR revealed that the binding of several transcription factors to nucleosomal DNA increased the DNase I sensitivity of the nucleosome, but apparently did not displace the nucleosome, suggesting that ternary complexes composed of transcriptional activators, histones and DNA may be functional (Steger and Workman, 1997). Collectively, these findings indicate that under certain circumstances

the occupancy of a regulatory region by a nucleosome is not necessarily repressive for transcription, but may sometimes even facilitate the assembly of transcription factor complexes.

The site-specific nucleosome remodeling at the rDNA promoter requires the binding of TTF-I and ATP-dependent cofactors present in both the *Drosophila* embryo extract and the murine transcription factor fraction. Energy-consuming enzyme complexes that modify nucleosome structures, like the NURF, CHRAC or ACF complexes, have been isolated from *Drosophila* embryo extracts (Tsukiyama and Wu, 1995; Ito *et al.*, 1997; Varga-Weisz *et al.*, 1997). We now show that functionally equivalent factors also exist in a fraction of mouse proteins, highly enriched for Pol I transcription factors. The Pol I transcription system is, however, not sufficiently defined to allow a separation of the nucleosome remodeling activities from the basal transcription apparatus. Therefore, we were not able to determine whether the observed nucleosome remodeling was an absolute requirement for transcription initiation. By contrast, the spacing experiment demonstrated that nucleosome remodeling occurs under conditions where the rDNA promoter is repressed. Remodeling is, therefore, not a consequence of transcription. Our finding that TTF-I is unable to stimulate transcription from chromatin-free templates argues that a chromatin component must be involved in activation. Although we have been unable to prove rigorously that the nucleosome remodeling is causal to the observed transcriptional activation, due to the presence of chromatin remodeling activities in the Pol I transcription system, a scenario where nucleosome positioning creates a defined promoter architecture required for transcription is clearly attractive.

The observed synergism between TTF-I and remodeling factors may be explained by either of two possibilities. TTF-I may take advantage of a transient remodeling of nucleosomes by an untargeted remodeling machine. In this scenario, TTF-I binding requires a prior remodeling event for stable interaction with chromatin. Alternatively, TTF-I is capable of interacting with chromatin by itself and can initiate a remodeling event with the help of cofactors. Since TTF-I is capable of binding to its target site T_0 in nucleosomal DNA in the absence of additional factors, it is possible that TTF-I is the cardinal transcription factor whose ability to interact with chromatin triggers a series of events leading to efficient transcription in chromatin. It is tempting to speculate that TTF-I might target specific remodeling complexes to the rDNA promoter which would cause a repositioning of nucleosomes, and this positioned nucleosome in turn facilitates transcription complex assembly on nucleosomal templates.

A comparison of the mechanisms that govern rDNA transcription in mouse and in yeast is instructive. The yeast transcription termination factor Reb1, also known as GRF2, not only binds to its target sites upstream and downstream of the ribosomal transcription unit, but also functions as an auxiliary activator of the *GAL* gene by creating a nucleosome-free region (Chasman *et al.*, 1990). Our data may therefore explain why terminator elements precede all eukaryotic ribosomal transcription units studied so far and why their position relative to the respective promoters is practically identical (Morgan *et al.*, 1983; Moss, 1983; Grummt *et al.*, 1986b; Henderson and Sollner-Webb, 1986). Previously, the promoter-proximal terminator has been proposed to function as a 'fail-safe' signal to prevent promoter occlusion by RNA polymerases coming from upstream (Bateman and Paule, 1988; Henderson *et al.*, 1989). However, our results suggest that the main function of the upstream terminator is to establish a defined nucleosomal structure of the rDNA promoter which facilitates binding and interactions of basal transcription factors to yield productive initiation complexes. This view is supported by elegant *in vivo* cross-linking and electron microscopic studies in yeast showing that newly replicated rDNA is packaged regularly into nucleosomes, indicating that the active chromatin structure is not inherited directly at the replication fork (Lucchini and Sogo, 1995). The establishment of an open chromatin structure at the promoter is a post-replicative process which involves disruption of pre-formed nucleosomes. By analogy to the situation in mammals, the yeast Pol I termination factor Reb1p may be involved in defining a transcription-competent chromatin architecture, a hypothesis that remains to be tested.

Materials and methods

Plasmids

Plasmids of the pMr series contain murine rDNA sequences cloned into pUC9. pMrWT contains mouse rDNA sequences from –170 to +155 including the upstream terminator T_0 at position –170. To be used as template in the cell-free transcription system, pMrWT was linearized with *Nar*I to produce 332 bp run-off transcripts. pMrΔT_0 is the same as pMrWT, but the T_0 site was inactivated by deletion of three nucleotides (see Figure 2A). pMrΔ-144 contains rDNA sequences from –144 to +155. In pMrT_1, an oligonucleotide containing the sequence of the downstream terminator T_1 was inserted into pMrΔ-144 to replace T_0 with the downstream terminator T_1. In pMrT_1* the oligonucleotide is inserted in the opposite orientation with respect to the transcription start site.

The minigene construct pMrT_2 represents a fusion of the promoter fragment contained in pMrWT and a fragment from the 3′-terminal spacer (from +603 to +686 with respect to the 3′ end of 28S rRNA) including the terminator element T_2. pMr$T_{1–10}$ is similar to pMrT_2 except that a 3.5 kb 3′-terminal rDNA fragment (from +57 to +3643) containing 10 terminator elements ($T_{1–10}$) was inserted downstream of the promoter. The same terminator fragment was inserted downstream of pMrT_1 and pMrT_1* to yield the minigenes pMrT_1-$T_{1–10}$ and pMrT_1*-$T_{1–10}$.

To construct the push-apart (PA) series of minigenes, a *Bam*HI site was introduced into pMrWT at position –155 to yield pMrPA_0. This site was used to change the distance between the terminator and the promoter. The distance between T_0 and the promoter was reduced by digesting the *Bam*HI overhangs with S1 exonuclease to produce pMrP$A_{–4}$. To introduce four nucleotides, the *Bam*HI site was filled in by the Klenow fragment of DNA polymerase (pMrPA_{+4}). In pMrPA_{+10}, pMrPA_{+15}, pMrPA_{+30}, pMrPA_{+60} and pMrPA_{+90}, single or multiple copies of 10 and 15 bp oligonucleotides were inserted.

Purification of recombinant TTF-I

cDNA encoding histidine-tagged TTF-I was inserted into the baculovirus expression vector pBacPAK9 (Clontech) and expressed in Sf9 cells. At 48 h after infection, extracts were prepared by sonifying 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 successively with lysis buffer containing 0.5% NP-40/1 mM imidazole and 0.5% NP-40/10 mM imidazole, respectively, and TTF-I was eluted 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, 20% glycerol]. All experiments were performed with an N-terminally truncated version of TTF-I, i.e. TTFΔN185, because full-length recombinant TTF-I is hard to express

in sufficient amounts and exhibits low DNA-binding activity (Sander *et al.*, 1996).

Assembly of nucleosome core particles

Histones were extracted from chromatin of HeLa cells and purified by chromatography on a hydroxylapatite column as described (Simon and Felsenfeld, 1979). Mononucleosome core particles were assembled using purified core histones and polyglutamic acid (PGA, Sigma) according to Stein *et al.* (1989). A 176 bp DNA fragment spanning mouse rDNA sequences from –232 to –56 was synthesized by PCR using an end-labeled primer. Octamers were assembled by incubation of 6 µg of histones and 12 µg of PGA for 2 h at room temperature in 200 µl of 0.125 M NaCl. Precipitates were removed by centrifugation, and 1 µl of the histone–PGA (HP) mix was incubated with 200 ng of the end-labeled rDNA fragment at 125 mM NaCl for 3 h at 37°C. To select for mononucleosomes, free DNA was cleaved with *Eco*RII (40 U/µg DNA). The integrity of the nucleosomes was assessed by electrophoresis of an aliquot of the reaction on a 4% polyacrylamide gel in 0.5× TBE. TTF-I binding was assayed in 20 µl volumes containing 0.2 pmol of nucleosomes and 0.4 pmol of TTF-I in 10 mM Tris–HCl (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$ and 10% glycerol. After incubation at 20°C for 10 min, the reactions were subjected to DNase I footprinting.

Assembly and analysis of chromatin

Preparation of cytoplasmic extracts from 0–90 min *Drosophila* embryos and chromatin reconstitution was performed as described (Becker and Wu, 1992; Becker, 1994). Assembly reactions (40 µl) contained 11 µl of *Drosophila* embryo extract, 200 ng of plasmid DNA, 3 mM ATP, 30 mM creatine phosphate and 1 µg/ml creatine kinase. Plasmids were either used as circular DNA or were linearized with *Nde*I, filled in with biotin-16-dUTP, cleaved with *Nar*I and immobilized on magnetic beads (Dynabeads-streptavidin M 280, Dynal). If the reactions were complemented with TTF-I, 0.1 pmol of TTFΔN185 was added 5.5 h after the onset of chromatin assembly and the assays were incubated for another 30 min at 26°C.

For indirect end-labeling, chromatin (100 µl) was digested with 10 U of MNase (Sigma) for 10, 30 and 90 s in the presence of 3 mM $CaCl_2$. The reactions were stopped by 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 derived from pUC9.

Sarkosyl treatment of chromatin

Sarkosyl treatment of chromatin was performed as described (Tsukiyama and Wu, 1995). Sarkosyl was added to assembled chromatin to yield a final concentration of 0.08% and was incubated at room temperature for 5 min. Sarkosyl and other low molecular weight components including ATP were removed by spinning 100 µl reactions (1 min, 1100 *g*) through a 1 ml Sephacryl S300-HR resin in 20 mM Tris–HCl (pH 7.6), 100 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM dithiothreitol (DTT). To analyze the remodeling activity of extracts or partially purified proteins, Sarkosyl-treated chromatin was incubated with 1 pmol of TTF-I, 3 mM ATP, 30 mM creatine phosphate and 1 µg/ml creatine kinase for 30 min at 26°C in the absence or presence of the protein fraction to be tested. For indirect end-labeling, the purified chromatin was digested for 10, 30 and 90 s with 1–10 U of MNase.

MNase and DNase I footprinting of chromatin

Circular DNA was reconstituted into chromatin in the absence or presence of TTF-I, and 100 ng aliquots were digested at room temperature in a total volume of 60 µl with increasing amounts (1–10 U) of DNase I (Worthington) or MNase for 20 and 40 s. Protein-free DNA was digested with 0.002 U of DNase I or 0.002 U MNase. The reaction was stopped by adding 0.2 volumes of 4% SDS/0.1 M EDTA and purified DNA was analyzed by 30 cycles of a PCR with 0.15 U of *Taq* polymerase (Boehringer) and 0.2–0.4 pmol (7×10^5 c.p.m.) of a labeled primer encompassing rDNA sequences from +111 to +130. 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 DTT, 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, 1–2 µCi of [α-^{32}P]UTP, 20–25 ng of either naked DNA or reconstituted chromatin, and 5 µl (15 µg protein) of a murine nuclear extract that had been fractionated by chromatography on DEAE–Sepharose CL-6B (DEAE-280 fraction). The purification of Pol I and specific transcription factors has been described (Schnapp *et al.*, 1996). In the reconstituted transcription system used in Figure 7, the reactions contained 4 µl of Pol I (H-400 fraction), 3 µl of TIF-IB (CM-400 fraction), 3 µl of TIF-IA/TIF-IC (PL-650 fraction) and 8 ng of affinity-purified recombinant UBF. 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 4.0% polyacrylamide gels.

For primer extension analysis, RNA was precipitated, dissolved in 25 µl of hybridization buffer [80% formamide, 0.4 M NaCl, 0.04 M PIPES (pH 6.4), 1 mM EDTA] and hybridized for 3 h at 37°C to a labeled primer (from +111 to +130). After transcription with avian myeloblastosis virus reverse transcriptase, the cDNAs were analyzed on polyacrylamide–urea gels as described (Grummt and Skinner, 1985).

Restriction site protection analysis

pMrWT was linearized with *Nde*I, filled in with biotin-16-dUTP, digested with *Nar*I and bound to Dynabeads. The immobilized DNA was assembled into chromatin in the absence or presence of 1 pmol of TTF-I. Protein-free DNA and chromatin templates were incubated with 20 U of *Rsa*I at 30°C in 25 µl standard transcription assays in the absence or presence of 5 µl of the DEAE-280 fraction. After 5, 15 and 45 min, the supernatants were magnetically separated from the bead-bound DNA, purified by proteinase K treatment, dotted onto nylon filters and hybridized with a ^{32}P-labeled oligonucleotide covering rDNA sequences from +111 to +130.

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