Published online August 3, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 14 ***4091–4099***
doi:10.1093/nar/gkh732

The chromatin remodeling complex NoRC and TTF-I cooperate in the regulation of the mammalian rRNA genes *in vivo*

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Received May 25, 2004; Revised June 23, 2004; Accepted July 12, 2004

ABSTRACT

The transcription termination factor (TTF)-I is a multifunctional nucleolar protein that terminates ribosomal gene transcription, mediates replication fork arrest and regulates RNA polymerase I transcription on chromatin. TTF-I plays a dual role in rDNA regulation, being involved in both activation and silencing of rDNA transcription. The N-terminal part of TTF-I contains a negative regulatory domain (NRD) that inhibits DNA binding. Here we show that interactions between the NRD and the C-terminal part of TTF-I mask the DNA-binding domain of TTF-I. However, interaction with TIP5, a subunit of the nucleolar chromatin remodeling complex, NoRC, recovers DNA-binding activity. We have mapped the protein domains that mediate the interaction between TTF-I and TIP5. The association of TIP5 with the NRD facilitates DNA binding of TTF-I and leads to the recruitment of NoRC to the rDNA promoter. Thus, TTF-I and NoRC act in concert to silence rDNA transcription.

INTRODUCTION

Transcription of rRNA genes is efficiently regulated in response to metabolic and environmental challenges [for review, see (1)]. rRNA genes are present in multiple copies, and therefore, rRNA synthesis could be modulated by varying the transcription rate per gene or by varying the number of active genes. Although in yeast both of these mechanisms may operate under certain conditions [reviewed in (2)], in vertebrates the level of cellular rRNA is regulated by changing the rate of transcription initiation at active rRNA genes rather than by activating silent transcription units. *In vivo* psoralen-cross-linking studies, which can distinguish between transcriptionally active and inactive genes have revealed that even in exponentially growing mammalian cells that synthesize high levels of pre-rRNA, only half of the rRNA genes are transcriptionally active and maintained in an 'open' chromatin conformation. The other half that corresponds to inactive gene copies resides in a compact, heterochromatic structure. Previous studies have demonstrated that the transcription termination factor (TTF)-I plays a key role in the establishment of both the active and inactive state of rDNA (3–7). A terminator element, known as T_0, is located immediately upstream of the ribosomal RNA gene promoter (8). Binding of TTF-I to the promoter-proximal terminator triggers ATP-dependent nucleosome remodeling which correlates with efficient transcription initiation on otherwise repressed nucleosomal rDNA templates (5,6). This suggested that TTF-I activates transcription by recruiting remodeling factors that alter the chromatin structure at the rDNA promoter and allow transcription factor binding and initiation complex formation.

A search for proteins that interact with TTF-I revealed TIP5 (TTF-I interacting protein 5), a 205 kDa nucleolar protein that shares several domains with the large subunits of the human ACF, CHRAC and WSTF chromatin remodeling complexes (9). TIP5 was shown to be tightly associated with SNF2H in a complex, termed NoRC (*n*ucle*o*lar *r*emodeling *c*omplex). NoRC is localized within the nucleolus and mediates transcriptional silencing of rDNA (3,4,7). Like other members of ISWI-containing remodeling machines, NoRC can induce nucleosome sliding along a DNA fragment in an ATP-dependent and histone H4 tail-dependent fashion.

TTF-I exhibits a modular structure, consisting of a C-terminal DNA-binding domain and a central domain that is required for transcription termination (10), transcriptional activation on chromatin templates (5,6) and replication fork arrest (11,12). Interestingly, the DNA-binding activity of murine TTF-I is masked in the full-length protein. Removal of the N-terminal part of TTF-I enhanced DNA-binding activity (13,14), indicating that a negative regulatory domain (NRD) within the N-terminus of TTF-I inhibits the interaction of TTF-I with DNA *in vitro*. However, both full-length TTF-I and N-terminal deletion mutants terminated transcription with similar efficiency in crude cell extracts (14), suggesting that cellular proteins may modify TTF-I or induce conformational alterations that facilitate DNA binding and transcription termination.

In this study, we analyzed in detail the interaction between TTF-I and TIP5 and studied the molecular mechanism modulating TTF-I binding. We demonstrate that the N-terminal NRD interacts with the C-terminal DNA-binding domain (DBD) and this protein–protein interaction inhibits the

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DNA-binding activity of TTF-I. Significantly, DNA binding of TTF-I is restored by interaction with TIP5. The results suggest a synergistic function of TTF-I and NoRC in rDNA silencing. By interacting with the NRD, NoRC unmasks the DNA-binding domain of TTF-I and facilitates TTF-I binding to the promoter-proximal terminator T_0. TTF-I, on the other hand, is required for the recruitment of NoRC to rDNA. Thus, TTF-I and NoRC cooperate in targeting to the rDNA promoter, which, in turn, is the first step in rDNA silencing.

MATERIALS AND METHODS

Plasmids

Expression vectors encoding TTF-I, TIP5 and the respective mutants have been described (9,14,15). Recent sequence analysis (accession no. AAF43448) revealed a 25 amino acid repeat in the N-terminal part of TTF-I that is missing in the original TTF-I sequence submitted (accession no. CAA-58808). In contrast to published data, we use the designation TTFΔN210, TTFΔN348 and TTFΔN470 for the former denomination of the N-terminal TTF-I deletion constructs TTFΔN185, TTFΔN323 and TTFΔN445, respectively. Details on the construction of the vectors expressing TTF-I (pEGFP-TTF-I, pEGFP-TTFΔN210, pGEX-$TTF_{1–209}$, pRSET-TTFΔ 69-153, pRSET-TTFΔ30-153, pJC40-Flag-$TTF_{1–340}$) and TIP5 deletion mutants (pJC40-$hTIP5_{1–731}$, pJC40-$hTIP5_{1–593}$, pJC40-$hTIP5_{510–723}$, pJC40-$hTIP5_{611–723}$, pBS-TIP5Δ599-703, pcDNA-TIP5Δ599-703, pcDNA-$TIP5_{1–731}$) are available on request. The reporter plasmid pMr1930-BH (16) represents a fusion of a 5′-terminal mouse rDNA fragment (from −1930 to +292) with a 3′-terminal fragment containing two 'Sal box' terminator elements. The construct pHrD-IRES-Luc was a gift from S. T. Jacob (17).

Expression and purification of recombinant proteins

Histidine-tagged TTF-I and the respective mutants were expressed in baculovirus-infected Sf9 cells and purified on NTA agarose (6). Proteins were eluted with 20 mM HEPES–KOH, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 200 mM imidazole, 1 mM PMSF, 1 mg/ml leupeptin and dialyzed against AM-100 (20 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 2 mM dithioerythritol (DTE), 100 mM KCl). Flag-tagged SNF2H was expressed in Sf9 cells and purified by affinity chromatography with antibodies that recognize the respective tag epitope. Recombinant NoRC was purified from Sf9 cells that express both TIP5 and Flag-tagged SNF2H. Glutathione *S*-transferase (GST), GST-$hTIP5_{332–726}$, GST-TTFΔN348, GST-$TTF_{1–209}$ and Flag-tagged $TTF_{1–340}$ were expressed in *Escherichia coli* BL21(DE3)pLysS. To be used in protein–protein interaction studies, wild-type and mutant TTF-I and TIP5 were synthesized *in vitro* using a TNT-Coupled Transcription/Translation Kit as specified by the manufacturer (Promega).

DNA binding assays

Reactions of 15 µl contained 5 fmol of a ^{32}P-labeled rDNA promoter fragment (from −232 to +16), 20 mM Tris–HCl, pH 8.0; 100 mM KCl; 5 mM $MgCl_2$; 0.2 mM EDTA; 2 mM TCEP; 10% glycerol, and 0.5 µg poly(dI–dC). After incubation for 30 min at 30°C, protein–DNA complexes were separated by electrophoresis on native 4.5% polyacrylamide gels. For proteolytic cleavage, DNA–protein complexes were incubated for 15 min with 100 ng V8 protease. Southwestern blots were performed as described (9).

Chromatin reconstitution and *in vitro* transcription assays

Purified histones were assembled into nucleosomes by salt gradient dialysis as described previously (18). Chromatin was purified by ultracentrifugation (SW-41, 45 000 r.p.m., 14 h) in a 15–30% sucrose gradient (4). Transcription assays were performed with pMrWT-T, a template containing a 5′-terminal murine rDNA fragment fused to a 3′-terminal fragment containing 10 terminator elements, spaced by 686 bp of plasmid sequences. Transcription assays of 25 µl contained 12 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2 mM $MgCl_2$, 80 mM KCl, 10 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP, UTP and GTP, 0.01 mM CTP and 1.5 µCi of [α-^{32}P]CTP, 20 ng of either naked DNA or chromatin and 5 µl of a DEAE-280 fraction (19). Transcription assays were incubated for 1 h at 30°C with purified TTF-I or TTFΔN210. Reactions were stopped by the addition of 25 µl stop buffer (20 µg glycogen, 2% SDS, 10 µg proteinase K, 100 mM EDTA) and incubation for 1 h at 40°C. Transcripts were purified by ethanol precipitation and analyzed on 4.5% polyacrylamide gels.

Yeast two-hybrid assay

Yeast two-hybrid screens were performed as described previously (20). Strain EGY48 harboring LexA-TTF-I or the respective mutants was transformed with pB42AD-$TIP5_{332–726}$ (9). Transformants were selected by culture on SD/galactose/-Ura/-His/-Trp/-Leu plates for 3 days and then patched onto SD/glucose/-Ura/-His/-Trp/-Leu plates.

Transient transfections, RNA analysis and luciferase reporter assay

NIH3T3 cells of 6×10^5 were transfected with pMr1930-BH, pEGFP-TTF-I or pEGFP-TTFNΔN210. RNA was isolated 48 h after transfection and analyzed on northern blots (21). To normalize for differences in RNA loading, the filters were also hybridized with a riboprobe that recognizes cytochrome *c* oxidase (cox) mRNA. To measure Pol I transcription by the luciferase assay, 5×10^5 HeLa cells were transfected with 1.5 µg pHrD-IRES-Luc (17), phRL-TK and pcDNA-TIP5, using the Polyfect (Qiagen) transfection reagent. After 24–48 h, cells were lysed and luciferase activity was measured using the Dual Luciferase Assay kit (Promega) in a Luminometer (Lumat LB9501; Berthold).

Protein–protein interaction assays

GST fusion proteins (GST-$hTIP5_{332–726}$, GST-$TTF_{1–209}$ and GST-TTFΔN348) were expressed in *E.coli* BL21(DE3)pLysS and purified on glutathione–Sepharose. Twenty microliters of glutathione–Sepharose containing 1 mg/ml of immobilized GST or GST fusion protein were incubated with 2.5 µl of ^{35}S-labeled protein in 22.5 µl buffer AM-100. Flag-tagged recombinant proteins were immobilized on M2-agarose. Control beads were saturated with 1 mg/ml of a mixture of Flag-epitope peptide, BSA, insulin and phosphatidyl–choline.

The beads were incubated for 4 h at 4°C in buffer AM-150/0.1% NP-40 with 2.5 µl of ^{35}S-labeled proteins in 22.5 µl buffer AM-150. After washing with 10 volumes of buffer AM-100, AM-300 or AM-500, bound proteins were separated on SDS–polyacrylamide gels and visualized by autoradiography.

RESULTS

Full-length TTF-I does not bind to DNA *in vitro*

In previous studies we have used an N-terminally truncated form of murine TTF-I (TTFΔN210) rather than the intact protein, because the truncated protein binds efficiently to the 'Sal box' target sequence *in vitro*, whereas the full-length protein does not (13). Surprisingly, in most functional assays, such as transcription termination (14) and replication fork arrest (11) both full-length and truncated TTF-I exhibited similar activities. As TTF-I-mediated functions are known to require binding of TTF-I to the 'Sal box' target sites upstream and downstream of the rDNA transcription unit (22), the disparity between DNA-binding activity and TTF-I function was obscure. To investigate the mechanism underlying masking of DNA-binding activity, full-length TTF-I and TTFΔN210 were expressed in insect cells and the DNA-binding activity of affinity-purified proteins was compared in electrophoretic mobility assays (Figure 1). Consistent with previous results (14), TTFΔN210 efficiently bound to its target sequence, whereas almost no DNA–protein complexes were observed with full-length TTF-I (Figure 1A). Limited digestion with protease V8 yielded a distinct DNA–protein complex whose amount and mobility was similar in full-length TTF-I and TTFΔN210. The finding that both proteolytic clipping and deletion of the N-terminus unmasked DNA binding of TTF-I indicates that sequences within amino acids 1–209 act as a negative regulatory domain that inhibits DNA-binding and may control TTF-I function.

It is conceivable that binding of full-length TTF-I to DNA may not be revealed in electrophoretic mobility shift assays. We therefore compared DNA binding of wild-type and mutant TTF-I on Southwestern blots. TTF-I and two deletion mutants (TTFΔN210 and TTFΔN348) were separated on SDS–polyacrylamide gels, transferred to a nitrocellulose membrane. Proteins were renatured, and probed with a radiolabeled 'Sal box' probe (Figure 1B, lanes 4–6), or probed with antibodies against TTF-I (Figure 1B, lanes 1–3). Again, the deletion mutants, but not full-length TTF-I, bound to rDNA, demonstrating the role of the N-terminus in masking the DNA-binding domain of TTF-I.

The NRD does not impair the sequence specific functions of TTF-I in *in vitro* and *in vivo* transcription assays

As truncation of the N-terminus recovers the DNA-binding activity of TTF-I, we hypothesized that inhibition of DNA binding could be mediated by a direct interaction between the N- and C-terminal part of TTF-I. To examine whether the N-terminus can interact with the DNA-binding domain of TTF-I, pull-down assays were performed using ^{35}S-labeled TTF-I$_{1–209}$ and immobilized GST-TTFΔN348, a fusion between GST and the C-terminal half of TTF-I harboring the DBD (Figure 2A, lanes 1–3). Clearly, TTF-I$_{1–209}$ interacted with GST-TTFΔN348 but not with GST alone. In a reciprocal approach, ^{35}S-labeled TTFΔN348 was retained by both bead-bound TTF-I$_{1–209}$ and TTF-I$_{1–340}$ (lanes 4–9). These results indicate that the NRD interacts with the DBD of TTF-I and suggests that this interaction inhibits DNA binding.

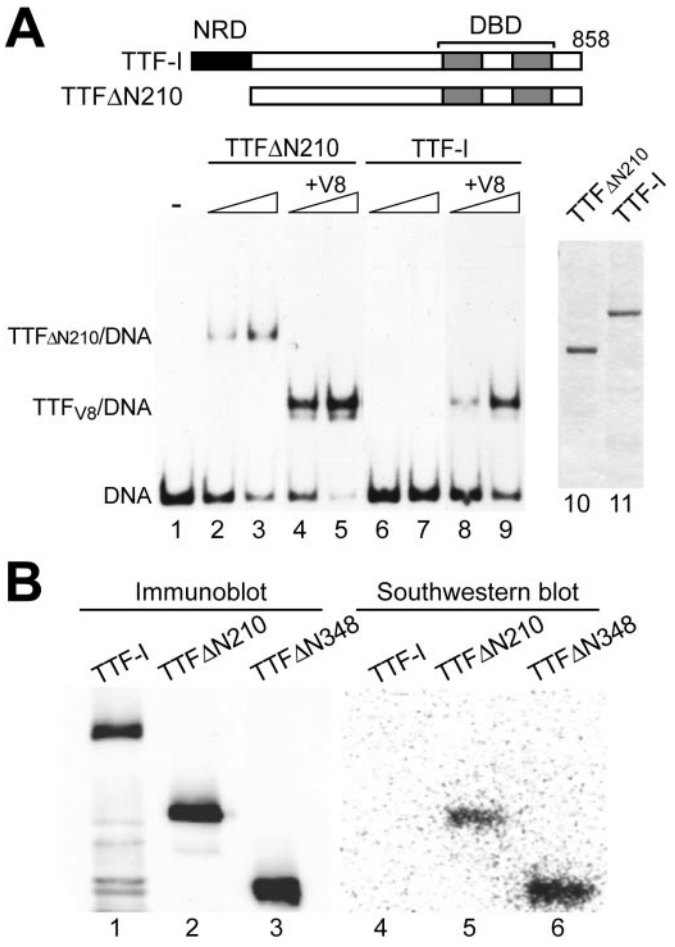

Figure 1. The negative regulatory domain masks the DNA activity of TTF-I. (**A**) Electrophoretic mobility shift assay. Increasing amounts (2.5 and 5 fmol) of recombinant TTFΔN210 (lanes 2–5) or TTF-I (lanes 6–9) were incubated with a ^{32}P-labeled rDNA fragment (from −232 to +16) in the absence or presence of 100 ng V8 protease as indicated. Protein–DNA complexes were resolved on a native 4.5% polyacrylamide gel and analyzed by autoradiography. TTF-I and TTFΔN210 were expressed in sf9 cells, purified, resolved by SDS–PAGE and stained with Coomassie Blue (lanes 10 and 11). A scheme of the TTF-I domain structure is presented above. The two gray boxes mark regions of homology with the DNA-binding domain of c-Myb. (**B**) Southwestern blot. Histidine-tagged TTF-I, TTFΔN210 and TTFΔN348 were expressed in *E.coli*, purified on Ni^{++}-agarose and separated by 8% SDS–PAGE. Proteins were analyzed on immunoblots with anti-TTF-I antibodies (lanes 1–3) and assayed for binding to a ^{32}P-labeled 'Sal box' probe (lanes 4–6).

We have previously shown that TTF-I efficiently terminates transcription in crude extracts (14). No proteolytic cleavage of TTF-I could be detected, indicating that intact TTF-I is capable of binding to DNA in the presence of auxiliary cellular proteins. To analyze the capability of full-length TTF-I to activate rDNA transcription in chromatin, an rDNA minigene was assembled into chromatin and transcription on the nucleosomal template was compared with transcription on naked DNA. A transcription extract was used [DEAE-280 fraction;

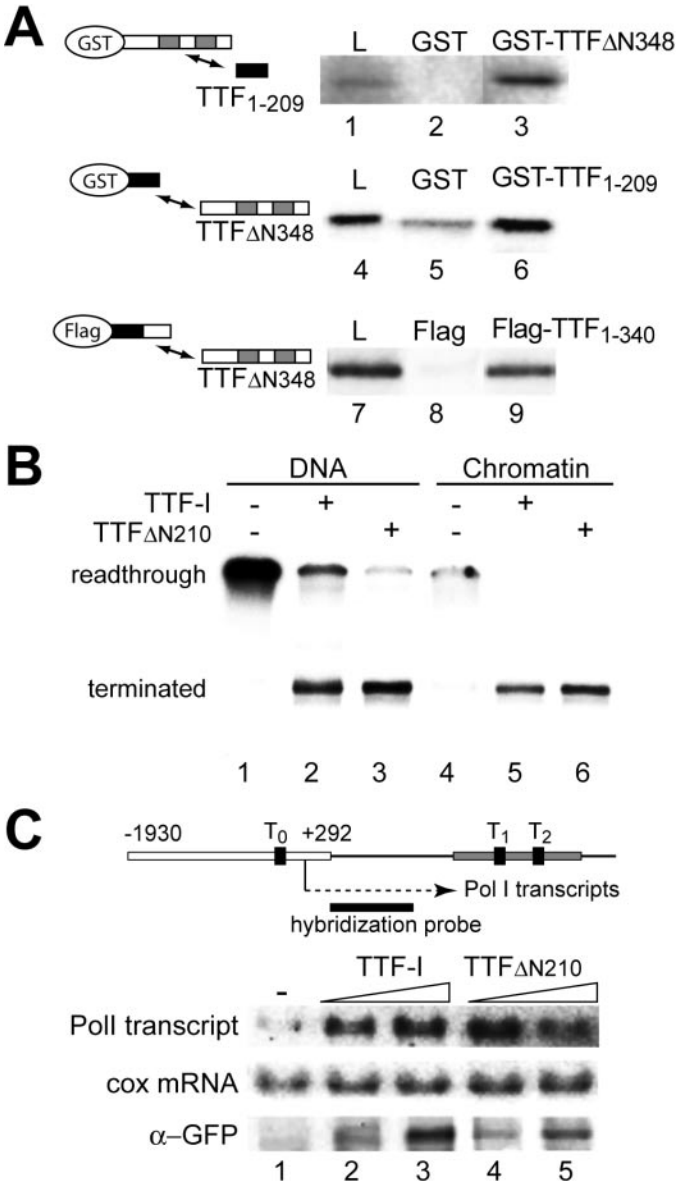

Figure 2. The NRD does not impair TTF-I functions in transcription assays. (**A**) Interaction between the N- and C-terminal part of TTF-I. Twenty microliters of immobilized GST (lanes 2 and 5), GST-TTFΔN348 (lane 3), GST-$TTF_{1–209}$ (lane 6), 20 µl of M2-agarose (lane 8) and FLAG-$TTF_{1–340}$ (lane 9) were incubated with 10 ng of ^{35}S-labeled $TTF_{1–209}$ (lanes 1–3) or TTFΔN348 (lanes 4–6). After washing, bound proteins were eluted with SDS–PAGE loading buffer. Ten percent of the load (L) and 50% of captured and eluted TTF-I were analyzed by SDS–PAGE and autoradiography. The immobilized proteins are marked by a labeled circle, the DBD of TTF-I is indicated by gray boxes and the NRD as a black box. (**B**) *In vitro* transcription and termination assay. A minigene (pMrWT-T) containing the rDNA promoter and the termination region was used for *in vitro* transcription. DNA was incubated with the transcription extract in the absence or presence of TTFΔN210 and TTF-I (lanes 1–3). Transcription on the minigene assembled into chromatin is shown in lanes 4–6. Readthrough transcription in the absence and terminated transcription in the presence of TTF-I is indicated on the left. (**C**) Transfection assay. NIH3T3 cells were co-transfected with 2.5 µg of pMr1930-BH and 0.5 and 1 µg of pEGFP-TTF-I or pEGFP-TTFΔN210. Transcripts from the reporter plasmid were analyzed on northern blots using a plasmid-specific riboprobe (upper panel). To normalize for variations of RNA loading, the membrane was hybridized with a riboprobe detecting cytochrome *c* oxidase mRNA (cox, middle panel). TTF expression was analyzed on immunoblots using α-GFP antibodies (lower panel). A scheme showing the reporter pMr1930-BH is shown above.

(19)] that contains all factors required for transcription but lacks TTF-I. On naked DNA templates, long read-through transcripts were synthesized in the absence of exogenous TTF-I (Figure 2B). In the presence of full-length TTF-I and TTFΔN210 specifically terminated transcripts were synthesized (lanes 2 and 3), though the efficiency of termination was lower in TTF-I compared to TTFΔN210. In chromatin, binding of TTF-I to the promoter-proximal terminator T_0 is required to counteract repressive chromatin structures and activate transcription (23). Nevertheless, chromatin-mediated transcriptional repression was relieved with similar efficiency both by TTFΔN210 and TTF-I (Figure 2B, lanes 4–6), indicating that in this assay the NRD did not impair TTF-I binding to rDNA. The observation that full-length TTF-I-mediated transcription termination and transcriptional activation in chromatin suggests that the transcription extract contains activities that facilitate sequence-specific binding of TTF-I to DNA.

To compare the activity of TTF-I and TTFΔN210 *in vivo*, transient transfection assays were performed. A reporter construct was used that represents a fusion of the promoter and the terminator region of mouse rDNA (16). As shown in Figure 2C, transcription of the Pol I reporter plasmid was enhanced by overexpression of TTF-I or TTFΔN210. The fact that both wild-type and mutant TTF-I promote Pol I transcription activation and transcription termination *in vivo* indicates that the DBD is accessible in the full-length protein. The data suggest that the inhibitory effect of the NRD is relieved by either auxiliary cellular protein(s) or by specific post-translational modifications.

The NRD of TTF-I interacts with TIP5

In the search for protein(s) that by specific interaction with the NRD may facilitate DNA binding of TTF-I, we screened for TTF-I interacting proteins in a yeast two-hybrid assay. One of the positive clones, termed *TIP5* (for TTF-interacting protein #5), encodes a 205 kDa subunit of a SNF2H-containing chromatin remodeling complex, termed NoRC (9). The initial cDNA identified in the two-hybrid screen encoded an internal region of TIP5 (amino acids 332–726), indicating that this part of TIP5 interacts with TTF-I. To determine whether the N-terminus of TTF-I is required for interaction with TIP5, the yeast strain EGY48 was co-transfected with plasmids pB42-$TIP5_{332–726}$ and LexA/TTF containing various fragments of TTF-I, and the β-galactosidase activity was measured (Figure 3A). This experiment revealed that all constructs that contained the N-terminal 210 amino acids of TTF-I strongly interacted with $TIP5_{332–726}$, whereas fusion proteins that lack the N-terminus were inactive in the β-galactosidase assay. This result indicates that sequences within the NRD of TTF-I are required for the interaction with TIP5.

To demonstrate a physical interaction between the N-terminus of TTF-I with TIP5 in pull-down assays, binding of ^{35}S-labeled TTF-I derivatives to GST-$TIP5_{332–726}$ was analyzed. The results in Figure 3B confirm the results obtained in the two-hybrid screen in that all TTF-I mutants that harbor the NRD bound to GST-$TIP5_{332–726}$, whereas mutants that lack the NRD did not. This demonstrates that the NRD of TTF-I interacts with the part of TIP5 harboring amino acids 332–726.

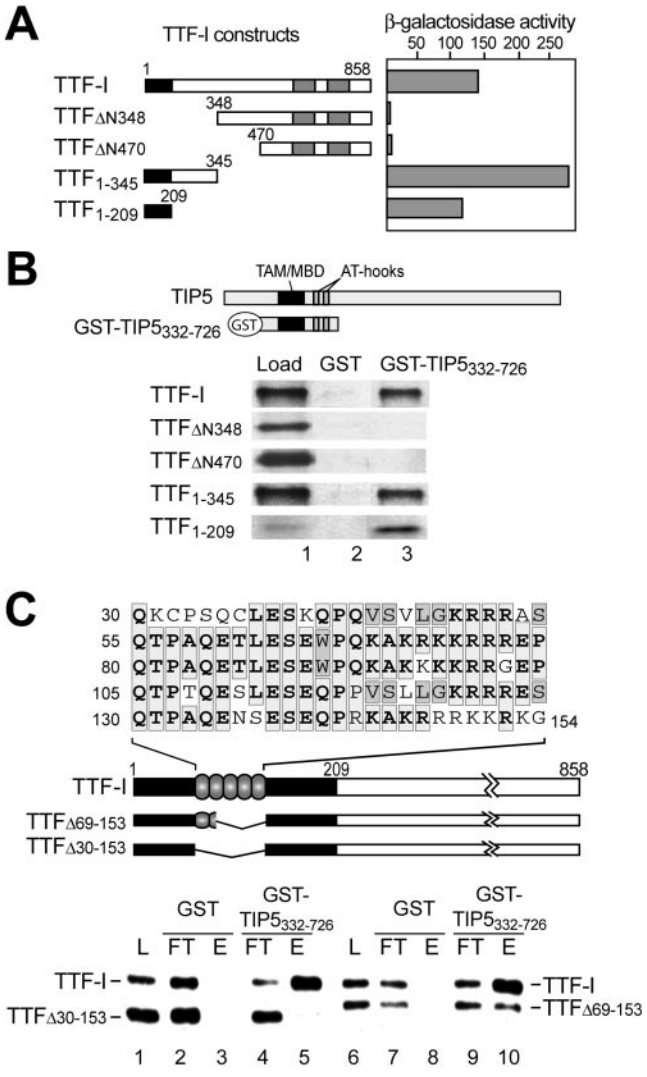

Figure 3. A repeated sequence in the N-terminus of TTF-I interacts with TIP5. (**A**) Yeast two-hybrid assay. Full-length TTF-I, TTFΔN348, TTFΔN470, $TTF_{1–345}$ and $TTF_{1–209}$ fused in frame to the LexA DNA-binding domain were expressed in the yeast strain EGY48 together with B42-$TIP5_{332–726}$. Activation of the *LacZ* reporter gene was quantified by a liquid β-galactosidase assay. Values are averages of duplicate assays of two independent transformations. (**B**) The N-terminal part of TTF-I harboring amino acids 1–209 interacts with TIP5. The indicated ^{35}S-labeled TTF-I proteins were passed over a 20 µl microcolumn containing GST (lane 2) or GST-$TIP5_{332–726}$ (lane 3). After washing, bound proteins were eluted with SDS–PAGE loading buffer. Ten percent of the load (lane 1) and 50% of the eluates (lane 3) were analyzed by SDS–PAGE and autoradiography. A scheme of the GST-$TIP5_{332–726}$ protein and the domain structure is shown above. (**C**) TIP5 associates with a 5-fold repeat of 25 amino acids in the N-terminal part of TTF-I. ^{35}S-labeled TTF-I (lanes 1–10), TTFΔ30-153 (lanes 1–5) and TTFΔ69-153 (lanes 6–10) were mixed as indicated and analyzed for binding to GST-$TIP5_{332–726}$. Ten percent of the load (L), the flow-through fraction (FT) and 50% of eluted TTF-I (E) were analyzed by SDS–PAGE and autoradiography. The sequence of the 5-fold repeat of 25 amino acids in TTF-I and a scheme of the TTF-I constructs is shown above. The gray boxes indicate conserved amino acids within the 5-fold repeat.

To pinpoint the region that mediates the interaction with TIP5, we assayed several NRD mutants for their ability to interact with TIP5. There are five 25 amino acid repeats within the N-terminal part of TTF (amino acids 30–154), all of which are required for efficient binding of TTF-I to TIP5. Deletion of 3.5 or all 5 repeats in the NRD led to a progressive loss of the interaction between TTF-I and TIP5 (Figure 3C). This result indicates that TTF-I interacts with TIP5 via the repeats within the NRD, each repeat contributing to the efficiency of binding.

Interaction of TTF-I with TIP5 restores the DNA-binding activity of TTF-I

The results above revealed that the part of TIP5 that interacts with TTF-I harbors the evolutionary conserved TAM (TIP5/ARBP/MBD) domain as well as two AT-hooks. To narrow the region of TIP5 that mediates the interaction with TTF-I, pull-down experiments were performed with the immobilized NRD of TTF-I (GST-$TTF_{1–209}$) and ^{35}S-labeled TIP5 derivatives. The results in Figure 4A demonstrate that all constructs harboring residues 611–723 interact with the NRD of TTF-I. This suggests that an internal region that includes the AT-hooks mediates the interaction between TTF-I and TIP5.

The interaction between the N-terminus of TTF-I and TIP5 may serve as a molecular switch that unmasks the DNA-binding domain of TTF-I, thereby facilitating TTF-I binding to rDNA. To examine whether interaction with TIP5 would enable TTF-I to bind to DNA, we used electrophoretic mobility assays to analyze the DNA-binding activity of TTF-I in the absence and presence of $TIP5_{611–723}$ (Figure 4B). Noteworthy, in the presence of $TIP5_{611–723}$ full-length TTF-I was capable of binding to rDNA (lanes 4–6). The DNA-binding activity of TTFΔN210, on the other hand, was not affected by $TIP5_{611–723}$ (lanes 12 and 13). This result demonstrates that the NRD-mediated inhibition of DNA binding of TTF-I is relieved by interaction with TIP5. Switching TTF-I from the inactive- to the active DNA binding form may also be induced by the NoRC complex. The interaction of TTF-I with TIP5 would unmask the TTF-I DNA-binding site and co-target TTF-I and NoRC to the rDNA promoter.

NoRC-mediated repression of Pol I transcription requires the interaction of TIP5 with TTF-I

Next, we monitored the interaction of TTF-I and TTFΔ69-153 with reconstituted NoRC, using TTFΔN348 as an internal control in pull-down assays. The results in Figure 5A reveal that the part of TTF-I that mediates the interaction with TIP5 is also required for the interaction with the reconstituted NoRC complex (lanes 1–10). Moreover, we analyzed whether the TIP5 mutants were able to interact with SNF2H, forming the NoRC complex. TIP5Δ599-703 that lacks the TTF-I interaction domain was still able to interact with SNF2H, forming a functional NoRC complex (data not shown). In contrast, the N-terminal part of TIP5 ($TIP5_{1–731}$), failed to interact with SNF2H, but still contains the TTF-I interaction domain and was shown to interact with Dnmt1 (3).

NoRC has been shown to play an essential role in rDNA silencing. Overexpression of TIP5 represses transcription of both endogenous rDNA repeats and co-transfected Pol I reporter plasmids (3,7). To analyze the effect of TIP5Δ599-703 on Pol I transcription, NIH3T3 cells were co-transfected with the reporter plasmid and expression vectors encoding TIP5 or TIP5Δ599-703. As shown in Figure 5B, transcription of the reporter plasmid was reduced by overexpression of TIP5. In contrast, transcription was not affected by TIP5Δ599-703,

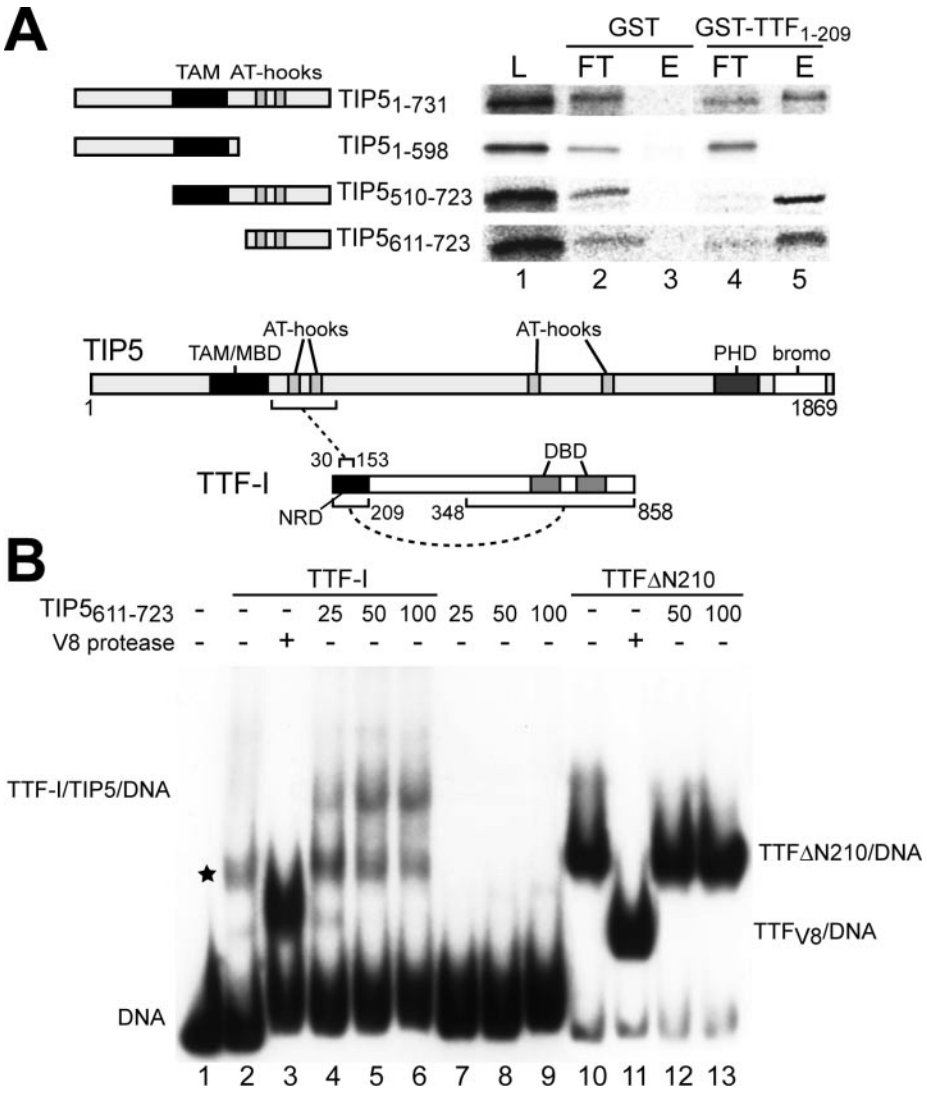

Figure 4. Interaction of the NRD with TIP5 restores the DNA-binding activity of TTF-I. (**A**) GST pull-down experiment. 20 µl of immobilized GST (lanes 2 and 3) and GST-TTF$_{1–209}$ (lanes 4 and 5) were incubated with the indicated, ^{35}S-labeled TIP5 derivatives. Ten percent of the load (L), the flow-through fraction (FT) and 50% of the eluted proteins (E) were analyzed by SDS–PAGE and autoradiography. A scheme indicating the position of the TAM domain and AT-hooks of TIP5 is shown on the left. At the bottom, a scheme depicts the intermolecular (TTF-I-TIP5) and TTF-I-TTF-I interacting regions in TIP5 and TTF-I. The interacting regions are connected by dashed lines. (**B**) Electrophoretic mobility shift assay. Full-length TTF-I (lanes 2–6) or TTFΔN210 (lanes 10–13) were pre-incubated with increasing amounts (25, 50 and 100 ng) of purified TIP5$_{611–723}$ and assayed for binding to a ^{32}P-labeled fragment harboring nucleotides from −232 to +16 of mouse rDNA promoter. TTF-I/DNA complexes were separated from free DNA on 4.5% native polyacrylamide gels. The asterisk indicates a DNA–protein complex containing either a non-specific DNA-binding protein present in the TTF-I preparation or a proteolytic fragment of TTF-I.

indicating that the interaction of TIP5 with TTF-I is required for transcriptional repression *in vivo*.

A similar result was obtained with a luciferase reporter that contains an internal ribosome entry site (IRES) downstream of the human rDNA promoter, allowing translation of a Pol I transcript (17). Consistent with the results obtained in the northern blot assay, TIP5 but not TIP5Δ599-703 repressed rDNA transcription (Figure 5C, lanes 1–3). To verify whether rDNA transcriptional repression depends on the molecular switch in TTF-I, or repression is mediated by TIP5, we analyzed TIP5$_{1–731}$ in the rDNA luciferase reporter assay (Figure 5C, lanes 4 and 5). Overexpression of this construct did not repress rDNA transcription, suggesting that switching TTF-I to its DNA-binding mode by interaction with TIP5 is required, but not sufficient for rDNA repression. Direct or indirect effects mediated by the C-terminus of TIP5 play an important role in transcriptional repression of the rDNA genes.

DISCUSSION

In this study, we have investigated the molecular mechanisms that regulate the DNA-binding activity of TTF-I and the role of TTF-I in NoRC-mediated repression of rDNA transcription. Previous studies have established that TTF-I does not bind to the 'Sal box' target sequence *in vitro* unless the N-terminus has been deleted or DNA binding was 'activated' by limited protease treatment. This suggested that the DNA-binding activity

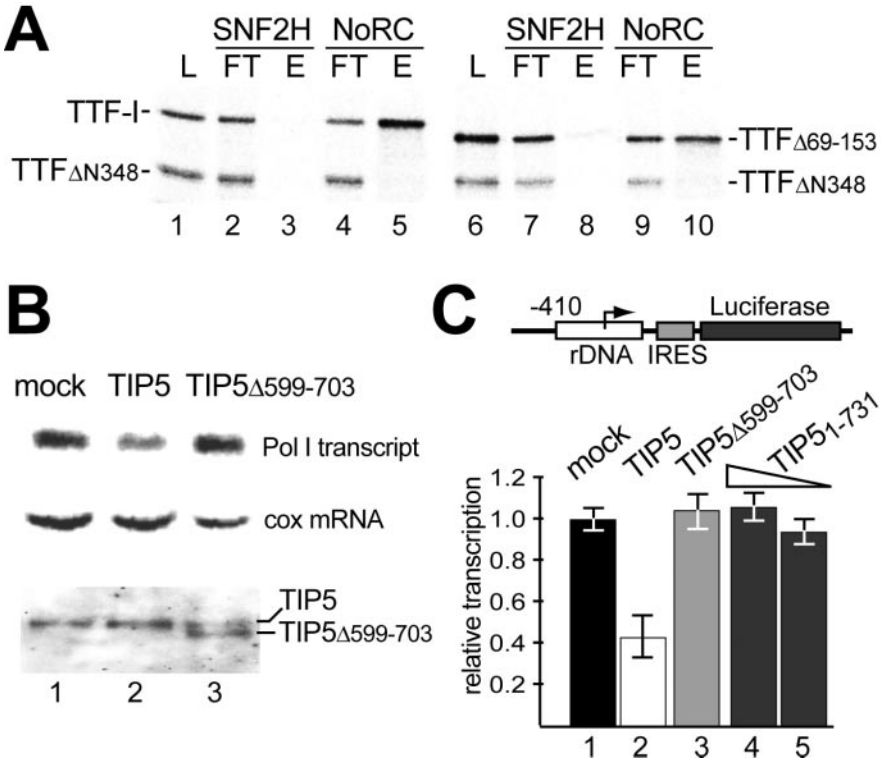

Figure 5. Interaction of TIP5 with TTF-I is required for transcriptional repression of rDNA. (**A**) The 5-fold repeat in the NRD of TTF-I interacts with TIP5 in the NoRC complex. Of immobilized FLAG-SNF2H or reconstituted NoRC, 20 µl was bound to M2-agarose and incubated with the indicated combinations of ^{35}S-labeled TTF constructs. Ten percent of the load (L), the flow-through fraction (FT) and 50% of the eluted TTF (E) were analyzed by SDS–PAGE and autoradiography. (**B**) Transfection assay. NIH3T3 cells were co-transfected with 0.5 µg of pMr1930-BH and 1 µg of TIP5 or TIP5Δ599-703. Transcripts from the reporter plasmid were analyzed on northern blots using a plasmid-specific riboprobe (Pol I transcript, upper panel). To normalize for variations of RNA loading, the membrane was hybridized with a riboprobe detecting cytochrome *c* oxidase mRNA (cox mRNA, middle panel). Protein expression levels were analyzed on immunoblots with anti-TIP5 antibodies, showing the endogenous TIP5 protein (lower panel, lane 1) and weak overexpression of the indicated TIP5 proteins (lanes 2 and 3). (**C**) Luciferase reporter assay. HeLa cells were co-transfected with the Pol I driven pHrD-IRES-Luc construct, a Pol II driven Renilla-Luciferase construct and the indicated TIP5 protein expression constructs. After 48 h, both firefly and Renilla luciferase activity was measured using the Dual luciferase Assay kit (Promega). A graphical representation of the relative transcription activity of pHrD-IRES-Luc is presented. The error bars represent the variations of three independent experiments. A scheme of the Pol I specific human rDNA promoter containing pHrD-IRES-Luc construct is shown above.

of TTF-I is masked in the full-length protein and the N-terminal part of TTF-I prevents DNA binding. This indicated that the N-terminus of TTF-I inhibits DNA binding via interaction with the C-terminus. Consistent with this, the N-terminal 209 amino acids of TTF-I can form stable oligomers in solution and repress DNA binding when fused to a heterologous DNA-binding domain (14), indicating that the N-terminal negative regulatory domain of TTF-I represses both its own and heterologous DNA-binding activity. Remarkably, full-length TTF-I is functionally active in crude extracts, i.e. it binds to its target sequence and terminates Pol I transcription (14). This suggests that either modifications of the NRD, interaction with cellular proteins or intracellular proteolytic cleavage may unmask DNA binding. We exclude the latter possibility, because we failed to detect N-terminally truncated TTF-I in cell lysates and never observed proteolysis of TTF-I in crude extracts. With regard to post-translational modifications, TTF-I is phosphorylated at multiple sites (24), and TTF-I is modified by acetylation (A. Németh, unpublished data). Though the significance of these modifications for TTF-I function remain to be investigated, our results are consistent with the NRD interacting with the C-terminal part of TTF-I, and this interaction blocks binding of TTF-I to DNA.

Several other transcription factors have been shown to be subject to autoinhibition by intramolecular masking of their DNA-binding domain. These include c-Myb (25), numerous Ets family members [reviewed in (26)], basal RNA polymerase III transcription factors (27), Swi4 (28) and POZ domain proteins (29). Like TTF-I, the DNA-binding activity of c-Myb is inhibited by intramolecular protein interactions and truncation leads to activation of the c-Myb protein. Only truncated versions of c-Myb are able to efficiently transform cells or induce tumors in animals (30). In this study, we have used a variety of protein–protein interaction assays to show that the NRD of TTF-I interacts both with the C-terminal part of TTF-I harboring the DNA-binding domain, and with TIP5, a subunit of the nucleolar SNF2H-containing chromatin remodeling complex NoRC (4,9). Interaction with TIP5 counteracts the repressive function of the NRD. The NRD contains five repeated 25 amino acid sequence elements that mediate protein–protein interactions. Progressive deletion of these repeats impairs the interaction between TTF-I and TIP5 and abrogates NoRC-mediated repression of rDNA transcription. The data suggest that the interaction with TIP5 alters the structure of TTF-I, unmasking the DNA-binding domain and co-targeting a TTF-I-NoRC complex to the rDNA promoter. We have

previously shown that the TTF-I-NoRC interaction is required for specific chromatin binding of TTF-I, nucleosome remodeling and transcriptional repression of the rDNA promoter *in vitro* (4).

Switching TTF-I from the inactive to the active DNA binding form by TIP5 is not sufficient to repress rDNA transcription. The C-terminal deletion $TIP5_{1–731}$, harboring the TTF-I interaction region did not repress rDNA transcription in the reporter assay. The results suggest that TTF-I targets NoRC to the rDNA promoter and that the C-terminal part of TIP5 mediates transcriptional repression. The C-terminus of TIP5 interacts with SNF2H and was previously shown to interact with HDAC1 (7). Together with the observation that the molecular motor SNF2H requires the histone H4 tail for nucleosome remodeling (31) and that the NoRC mediated repression requires the histone H4 tail (9), we suggest that nucleosome remodeling and histone modification are crucial steps in transcriptional silencing (Figure 6).

Besides the effect of TIP5 on TTF-I function, TTF-I serves an essential role in the recruitment of NoRC to rDNA. Since distinct nucleosome remodeling events lead to activation and repression of rDNA transcription in chromatin, this mechanism presents an elegant way to target nucleosome remodeling activities to a specific site. Targeting of chromatin remodeling complexes to gene promoters appears to be a common theme. In yeast, Ume6-dependent targeting of the ISW2 chromatin remodeling complex was shown to repress transcription of early meiotic genes (32). Similarly, the *Drosophila* Nurf

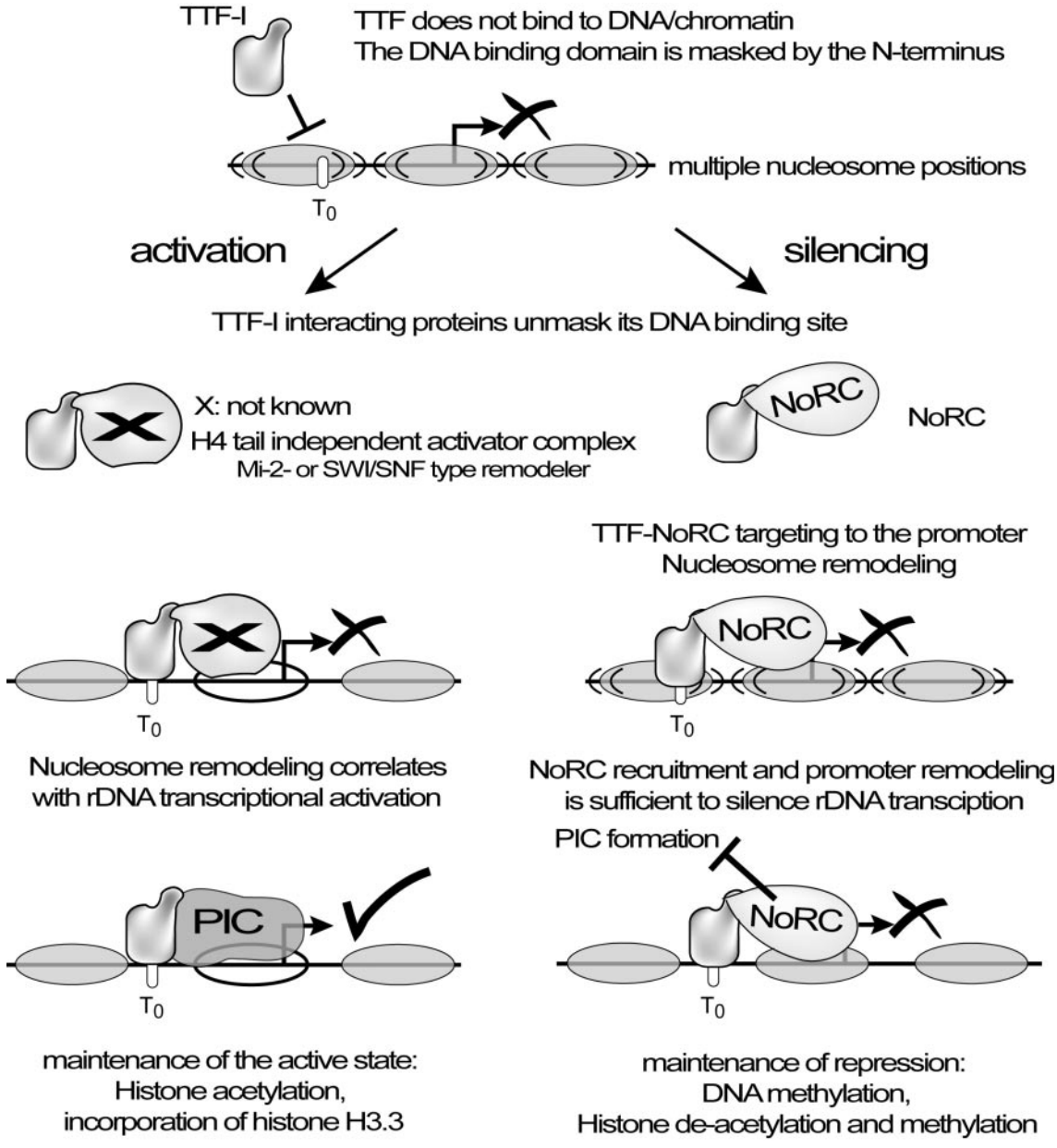

Figure 6. Working model showing the regulation of the rDNA genes in chromatin. TTF-I plays a dual role in rDNA regulation in that it either establishes the active or the repressed state of the gene. The functional role of TTF-I is defined by interacting proteins, which restore the DNA-binding activity of TTF-I and regulate the formation of the preinitiation complex (PIC).

complex was shown to be recruited by specific interactions of the Nurf301 subunit with the transcription factors GAGA and HSF (33). In mammals, the transcription factor EKLF specifically recruits the SWI/SNF complex to the β-globin promoter, generating specific DNase I hypersensitive sites (34).

TTF-I is a multivalent protein which plays an essential role both in transcription termination of RNA polymerase I and in transcription activation on chromatin (5,6). TTF-I plays a dual role in rDNA transcription. On the one hand, it is required to activate rDNA transcription on chromatin templates (6). On the other hand, TTF-I plays an essential role in NoRC-mediated repression of rDNA transcription (3,4). Moreover, TTF-I has been shown to be a polar contrahelicase that causes pausing of replication forks and blocks replication fork progression (11,12). Given the multiple functions of TTF-I in rDNA transcription and replication, it is conceivable that other cellular proteins interact with the NRD, unmask the DNA-binding domain and facilitate DNA binding of TTF-I. In this scenario, interaction with specific proteins would be a means to regulate the DNA-binding activity and hence the role of TTF-I in Pol I transcription and DNA replication.

ACKNOWLEDGEMENTS

We thank Urs Hoffmann-Rohrer for help in performing the yeast two-hybrid experiments. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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