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NoRC—a novel member of mammalian ISWI-containing chromatin remodeling machines

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Transcription by RNA polymerase I on nucleosomal templates requires binding of the transcription termination factor TTF-I to a cognate site 160 bp upstream of the transcription start site. Binding of TTF-I is accompanied by changes in the chromatin architecture which suggests that TTF-I recruits a remodeling activity to the rDNA promoter. We have cloned a cDNA that encodes TIP5 (TTF-I-interacting protein 5), a 205 kDa protein that shares a number of important protein domains with WSTF (Williams syndrome transcription factor) and hAcf1/WCRF180, the largest subunits of human chromatin remodeling complexes hCHRAC and WCRF. TIP5 co-localizes with the basal RNA polymerase I transcription factor UBF in the nucleolus and is associated with SNF2h. The cellular TIP5–SNF2h complex, termed NoRC (nucleolar remodeling complex), induces nucleosome sliding in an ATP- and histone H4 tail-dependent fashion. The results suggest that NoRC is a novel nucleolar chromatin remodeling machine that may serve a role in the regulation of the rDNA locus.

Keywords: Acf1/chromatin remodeling/RNA polymerase I/SNF2h/TTF-I

Introduction

Transcriptional regulation can be brought about by dynamic changes in chromatin structure that facilitate or prevent the access of the transcriptional machinery to nucleosomal DNA (reviewed in Wolffe and Guschin, 2000). One mechanism by which chromatin structure can be altered involves disruption, stabilization and mobilization of the histone octamer by multiprotein complexes, leading to either repression or activation of transcription (reviewed in Flaus and Owen-Hughes, 2001). Genetic and biochemical analyses have identified a class of macromolecular chromatin remodeling complexes that play important roles in both the process of chromatin opening and the maintenance of higher order chromatin structure *in vivo*. Chromatin remodeling complexes hydrolyze ATP through a related subunit that belongs to the superfamily of SNF2-type ATPases (Eisen *et al*., 1995). The ATPase can be considered the catalytic core, or engine, which acts in concert with a subset of associated factors. Three classes of chromatin remodeling complexes have been characterized that are driven by either the SWI2/SNF2-type ATPases, the Mi-2 proteins or the ISWI-type ATPase (reviewed in Peterson, 2000). They are characterized by family-specific protein domains, i.e. the bromodomain, the chromodomain or SANT domain, which may reflect functional differences among distinct mammalian remodeling complexes. The different classes of remodeling complexes contain associated proteins that are likely to serve regulatory or targeting roles. Complexes that are related to the yeast SWI/SNF complex have been identified in *Drosophila*, *Xenopus laevis* and humans. Compared with the 2 MDa SWI/SNF complexes that consist of 9–12 subunits, the ISWI group of remodeling complexes are relatively small (300–650 kDa) containing 2–4 subunits. In *Drosophila*, three ISWI-containing complexes have been identified, i.e. NURF (Tsukiyama *et al*., 1995), CHRAC (Varga-Weisz *et al*., 1997) and ACF (Ito *et al*., 1997), which differ in their subunit composition and characteristic functions. Similar remodeling complexes containing apparent homologs of ISWI have also been found in yeast (Tsukiyama *et al*., 1999), *X.laevis* (Guschin *et al*., 2000) and humans (LeRoy *et al*., 1998, 2000; Bochar *et al*., 2000; Poot *et al*., 2000). The ATPase of the ISWI complexes is stimulated by nucleosomes and requires the N-terminus of histone H4, whereas SWI/SNF complexes are stimulated by DNA alone (Boyer *et al*., 2000; Clapier *et al*., 2001). Moreover, in contrast to the SWI/SNF family of complexes, where to date every complex displays a similar set of characteristic activities, individual members of the ISWI-family complexes display different activities that presumably reflect differences in their physiological role, such as chromatin assembly, transcription, replication and the maintenance of chromatin structure (Längst and Becker, 2001).

We have demonstrated recently that transcription of rRNA genes assembled into chromatin requires ATP-dependent remodeling of nucleosomes at the RNA polymerase I (Pol I) transcription start site. This remodeling reaction is triggered by the transcription termination factor TTF-I (Längst *et al*., 1997, 1998). Once bound to its target site upstream of the gene promoter, TTF-I can activate transcription, presumably by recruiting remodeling complexes to the rDNA promoter. In support of this, transcriptional activation is accompanied by ATP-dependent nucleosome remodeling. In an attempt to identify and isolate protein(s) that by specific interaction with TTF-I recruit chromatin-specific coactivators to the rDNA promoter, we performed a yeast two-hybrid screen with TTF-I

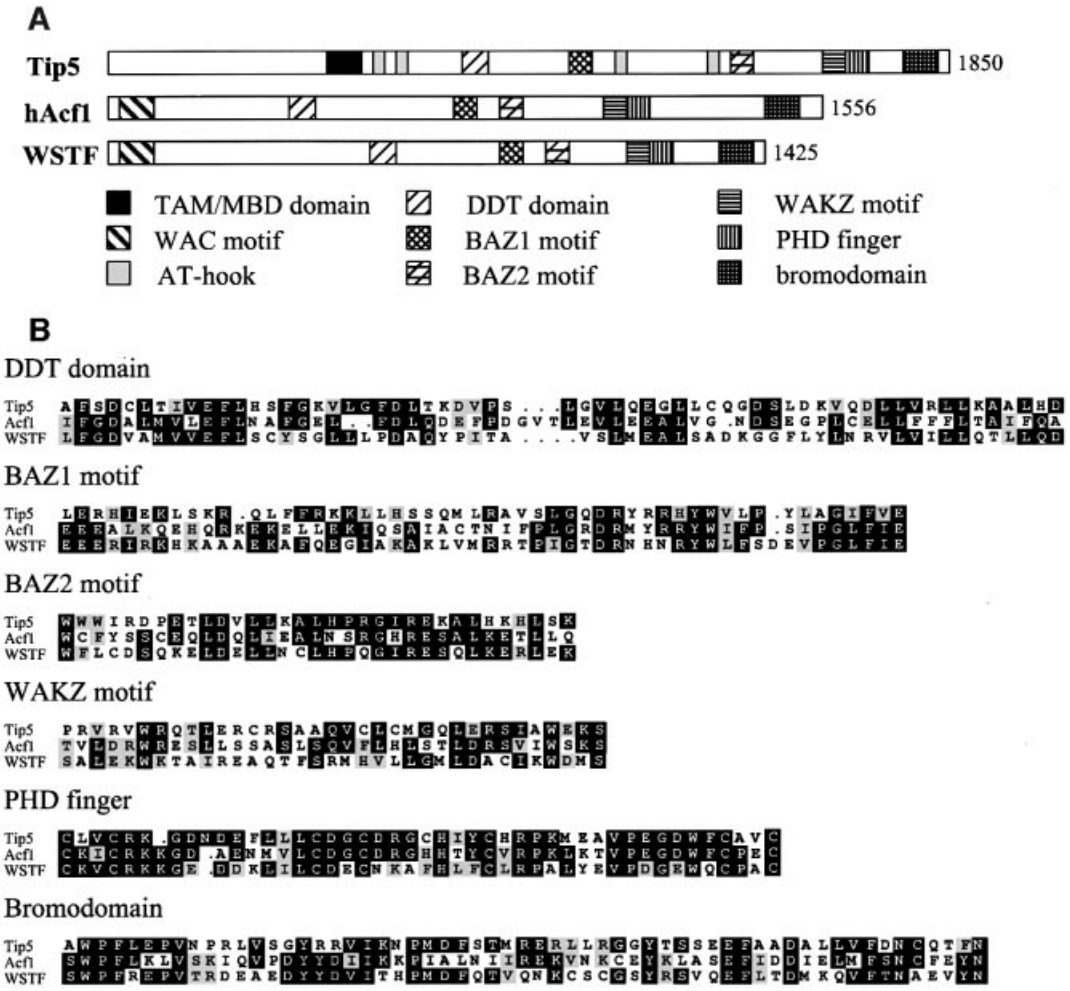

Fig. 1. TIP5 is a member of a family of chromatin remodeling factors. (**A**) Schematic diagram of mTip5, WSTF and hAcf1 showing the location of conserved sequence motifs. The cDNA sequence has been submitted to the DDBJ/EMBL/GenBank (accession No. AJ309544). The corresponding human clone is BAZ2A/WALp3 (accession No. AB032254). The numbers correspond to the numbers of amino acids in each protein. (**B**) Alignment of conserved sequence motifs in TIP5 with their counterparts in WSTF and hAcf1. For alignment of the TAM/MBD domain, see Aravind and Landsman (1998).

as bait. We have identified a novel gene, designated *Tip5* (TTF-I-interacting protein 5), which encodes a 205 kDa protein. TIP5 shares a number of important protein domains with Acf1, the largest subunit of both *Drosophila* and human ACF and CHRAC (Ito *et al*., 1999; Bochar *et al*., 2000; LeRoy *et al*., 2000; Poot *et al*., 2000) and WSTF (Williams syndrome transcription factor), a gene that is implicated in Williams syndrome (Lu *et al*., 1998), a complex developmental disorder with multisystemic effects. We demonstrate that the protein encoded by *Tip5* is contained in a macromolecular complex together with SNF2h, the mammalian homolog of ISWI. The results suggest that TIP5 represents a subunit of a novel remodeling machine, termed NoRC, that may be targeted to the ribosomal gene promoter by interaction with TTF-I bound to the upstream terminator.

Results

TIP5 belongs to a family of proteins related to Acf1 and WSTF

In an attempt to identify and isolate protein(s) that by specific interaction with TTF-I mediate Pol I transcription on chromatin templates, we performed a yeast two-hybrid screen. A fusion between the LexA protein (amino acids 1–202) and full-length TTF-I was used as a bait to screen a HeLa cell cDNA library fused to the B42 transcriptional activation domain. After screening of ~10^7 transformants, several positive clones were identified which correspond to previously uncharacterized genes. One of them, termed *Tip5*, encodes a 1.2 kb DNA corresponding to a human expressed sequence tag (EST) clone, KIAA0314 (Nagase *et al.*, 1997). Using cDNA screening, EST walking and conventional cloning techniques, we isolated a murine cDNA with a 5553 nucleotide open reading frame (ORF) that encodes a protein with a calculated mol. wt of 205 kDa.

The modular organization of TIP5 resembles that of proteins that have been associated with functions in chromatin structure and chromatin regulation (Figure 1). Acf1 is the largest subunit of the remodeling complexes ACF (ATP-utilizing chromatin assembly and remodeling factor) and CHRAC (chromatin accessibility complex) that have been identified in *Drosophila* (Varga-Weisz *et al*., 1997; Ito *et al*., 1999) and humans (Bochar *et al*., 2000; Poot *et al*., 2000; LeRoy *et al*., 2000). TIP5, Acf1

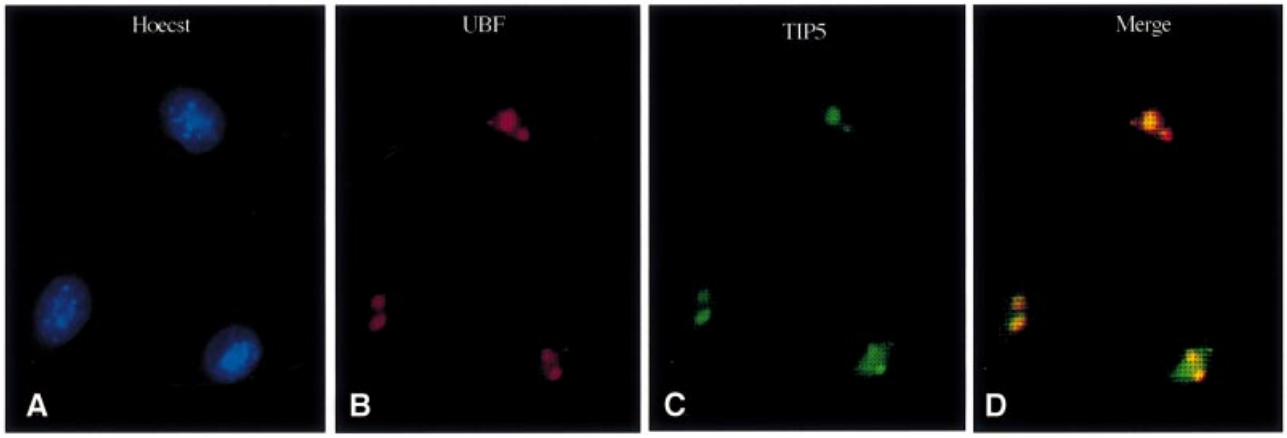

Fig. 2. TIP5 co-localizes with UBF in the nucleolus. Interphase NIH-3T3 cells were fixed, and either stained with Hoechst 33258 (**A**) or immunostained with antibodies against UBF (**B**) and TIP5 (**C**). Co-localization of TIP5 and UBF are shown as yellow in the merged image (**D**).

and WSTF, a gene that is deleted in Williams syndrome individuals (Lu *et al*., 1998), share six motif sequences, i.e. a bromodomain, one or two PHD (plant homeodomain) fingers, a WAKZ (WSTF/Acf1/KIAA0314/ZK783.4) motif, a BAZ 1 and a BAZ 2 motif (Jones *et al*., 2000) as well as DDT, a helical ~60 amino acid domain that is present in different transcription and chromosome remodeling factors and is predicted to play a role in DNA binding (Doerks *et al*., 2001). The order of the distinct modules along Acf1, WSTF and TIP5 is the same, suggesting that these proteins are related and may serve similar functions. Despite this similarity, the WAC motif, a novel protein domain of unknown function (Ito *et al*., 1999), which is present at the N-terminus of WSTF and Acf1, is not contained in TIP5. Conversely, there are distinct modules in TIP5 that are not contained in Acf1 and WSTF. These include a domain, known as TAM (TIP5/ARBP/MBD), and four AT-hooks, a motif that is known to mediate binding to the minor groove in DNA (Aravind and Landsman, 1998). AT-hooks are thought to co-regulate transcription by modifying the architecture of DNA, thereby enhancing the accessibility of promoters to transcription factors. The presence of multiple sequence motifs that have been found in chromatin-associated proteins suggests a role for TIP5 in chromatin-mediated processes and indicates that different members of this protein family serve distinct functions.

Nucleolar localization of TIP5

Given that TIP5 interacts with TTF-I, it should be localized within the nucleolus. To examine the intranuclear localization of cellular TIP5, NIH-3T3 cells were immunostained with antibodies that recognize an N-terminal epitope of TIP5 (TIP5N1–18). The staining pattern of TIP5 was compared with that of UBF, a basal transcription factor for Pol I that is associated with both active and inactive rRNA genes (Zatsepina *et al*., 1993). Indirect immunofluorescence microscopy using anti-UBF antibodies revealed a bright staining of the nucleolus in interphase cells (Figure 2B). The nucleolar fluorescence of UBF is speckled, rather than uniform, showing necklace-like structures composed of numerous small beads (Zatsepina *et al*., 1993). Significantly, the fluorescence pattern observed with anti-TIP5 antibodies closely resembles UBF staining (Figure 2C). When the two images were merged, nucleolar regions of TIP5 and UBF staining coincided in yellow, indicating that both proteins co-localize *in vivo* (Figure 2D). The co-localization of TIP5 with UBF in nucleolar foci suggests a function for TIP5 in rDNA transcription.

Interaction of TTF-I and TIP5

The yeast two-hybrid system revealed that TTF-I interacts with a region of TIP5 harboring amino acids 332–726. To demonstrate the interaction between cellular TTF-I and this part of TIP5 *in vitro*, we incubated an affinity matrix containing GST fused to TIP5/332–726 with nuclear extracts from mouse cells. The unbound proteins (FT), the wash (W) and the eluate (E) were analyzed on immunoblots using a serum raised against TTFΔN323 (Evers *et al*., 1995). As shown in Figure 3A, a significant amount of cellular TTF-I was bound to and could be eluted from the matrix containing GST–TIP5/332–726 but not GST alone (compare lanes 3 and 5). We also used bead-bound hTIP5/332–726 and ^{35}S-labeled TTF-I to demonstrate the ability of TIP5 to interact with TTF-I (Figure 3B). In several independent experiments, ~10% of TTF-I bound to the resin containing GST–hTIP5/332–726 but not to control beads containing GST alone. Thus, part of TIP5 encompassing amino acids 332–726 mediates the association between TTF-I and TIP5.

The TAM domain and AT-hooks synergize in mediating DNA binding of TIP5

TIP5 contains a TAM (TIP5/ARBP/MBD) domain, i.e. a motif with substantial sequence homology to the MBD domain that has been identified in a number of methyl-CpG-binding proteins (Hendrich and Bird, 1998). Both the MBD and the AT-hooks are known to play a role in DNA binding. To examine the DNA-binding properties of the TAM domain and AT-hooks, histidine-tagged polypeptides harboring either the TAM domain alone (TIP5-TAM, amino acids 510–611) or, additionally, two adjacent AT-hooks (TIP5-TAM-AT, amino acids 510–723) were

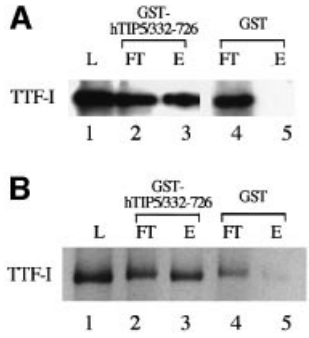

Fig. 3. TTF-I interacts with TIP5 *in vitro* and *in vivo*. (**A**) Cellular TTF-I interacts with immobilized TIP5. A 25 µl aliquot of nuclear extract was applied onto 20 µl columns of glutathione–Sepharose containing 1 mg/ml immobilized GST–hTIP5/332–726 (lanes 2 and 3) or GST (lanes 4 and 5). After washing with buffer AM-100, bound proteins were eluted with buffer AM-1000 (E) and 50% of the eluate was analyzed on western blots using anti-TTF-I antibodies. Ten percent of the input nuclear extract (NE) is shown in lane 1. (**B**) Pull-down assay with recombinant TTF-I. A 10 µl aliquot of bead-bound GST–hTIP5/332–726 (lanes 2 and 3) or GST (lanes 4 and 5) was incubated with 10 ng of ^{35}S-labeled TTF-I, and 50% of captured TTF-I was analyzed by electrophoresis in 10% SDS–polyacrylamide gels and by autoradiography. Ten percent of the reaction (L) is shown in lane 1.

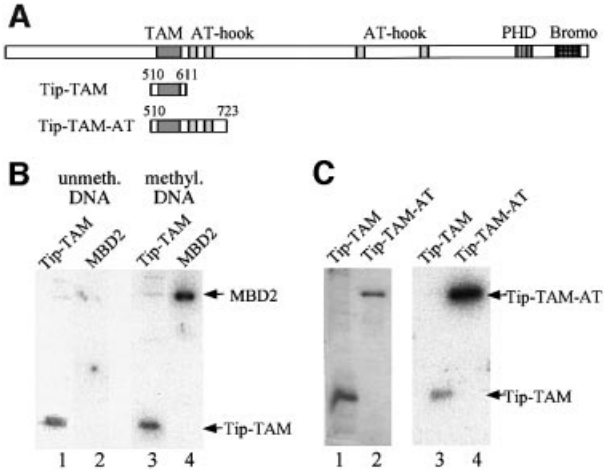

Fig. 4. The TAM domain and AT-hooks mediate DNA binding of TIP5. (**A**) Scheme showing the subclones of Tip5 used in the southwestern blot assay. The numbers refer to the amino acids that are encoded by the subclones of Tip5. (**B**) Binding of the MBD-like domain of TIP5 and MBD2 to unmethylated and methylated DNA. Histidine-tagged TIP5-TAM (lanes 1 and 3) and MBD2 (lanes 2 and 4) were expressed in *E.coli*, purified on Ni–agarose columns, separated by 15% SDS–PAGE and assayed for binding to ^{32}P-labeled unmethylated oligonucleotides as described (Hendrich and Bird, 1998). (**C**) AT-hooks enhance DNA-binding activity. TIP5-TAM (100 pmol) and TIP5-TAM-AT (25 pmol) were separated on an SDS–polyacrylamide gel and either stained with Coomassie Blue (lanes 1 and 2) or assayed by southwestern blotting for binding to a labeled oligonucleotide (5′-GTTCCTTTGAGGTCCGGTTCTTTTCGTTATGGGGTCATTT-3′) harboring the upstream control element of the murine rDNA promoter (lanes 3 and 4).

separated on SDS–polyacrylamide gels and probed with radiolabeled DNA probes. To test whether the TAM domain would bind preferentially to methylated DNA, we used a synthetic oligonucleotide that is recognized by members of the MBD protein family in a methylation-dependent manner (Hendrich and Bird, 1998). As expected, the methyl-CpG-binding protein MBD2 bound with high specificity and efficiency to the methylated probe (Figure 4B, lane 4). In contrast, binding of TIP5-TAM to DNA was not affected by DNA methylation. This result suggests that, despite the sequence homology to MBD, the TAM domain of TIP5 does not mediate specific interaction with methylated DNA. If the TAM domain was fused to two AT-hooks (TIP5-TAM-AT), the overall DNA-binding efficiency increased by almost two orders of magnitude (Figure 4C). Thus, the central part of TIP5 including the TAM domain and AT-hooks mediates the interaction with DNA.

Cellular TIP5 is present in a high molecular mass protein complex containing hISWI

Relatives of TIP5, e.g. hAcf1/WCRF180, are subunits of ISWI-containing chromatin remodeling complexes. To assess whether cellular TIP5 is contained in a macromolecular protein complex, we purified TIP5 from mouse whole-cell extracts by chromatography on DEAE–Sepharose, SP-Sepharose and heparin–Ultrogel, and determined the apparent molecular mass by gel filtration on Superose 6 (Figure 5B). TIP5 eluted with a native molecular mass of ~800 kDa, suggesting that it is contained within a macromolecular protein complex. The structural homology of TIP5 and Acf1 prompted us to examine whether SNF2h, the mammalian homolog of *Drosophila* ISWI (Aihara *et al*., 1998), would co-fractionate with TIP5. If fractions from the sizing column were probed with anti-SNF2h antibodies, the majority of SNF2h was found to elute at 500–600 kDa (Figure 5B, lower panel), presumably representing mammalian ACF, CHRAC or WCRF. A significant part of SNF2h, however, co-eluted with TIP5, suggesting that SNF2h may be associated with TIP5.

If cellular TIP5 is contained in a complex with SNF2h, then it should be co-precipitated with anti-TIP5 antibodies. To test this, a partially purified fraction that contains TIP5, Acf1 and SNF2h was incubated with α-TIP5N1–18 antibodies, eluted with the epitope peptide and analyzed on western blots (Figure 5). After precipitation with anti-TIP5 antibodies, Acf1 remained in the supernatant (lane 4), whereas both TIP5 and SNF2h were eluted from the immunoprecipitate (lane 5). This result demonstrates that TIP5 and SNF2h are subunits of a protein complex that is distinct from ACF. As TIP5 localizes within the nucleolus and, as will be shown below, catalyzes nucleosome movement, we termed the TIP5–SNF2h complex NoRC (nucleolar remodeling complex). To define the subunit composition of cellular NoRC, the affinity-purified complex was subjected to SDS–PAGE and the polypeptides were visualized by silver staining. As shown in Figure 5D, NoRC contains stoichiometric amounts of TIP5 and SNF2h. Whether or not the ~80 kDa and 50 kDa proteins are genuine components of the cellular TIP5-containing protein complex remains to be determined.

NoRC mobilizes mononucleosomes

The structural similarity of TIP5 to Acf1 and WCRF180 as well as the presence of SNF2h in the purified complex

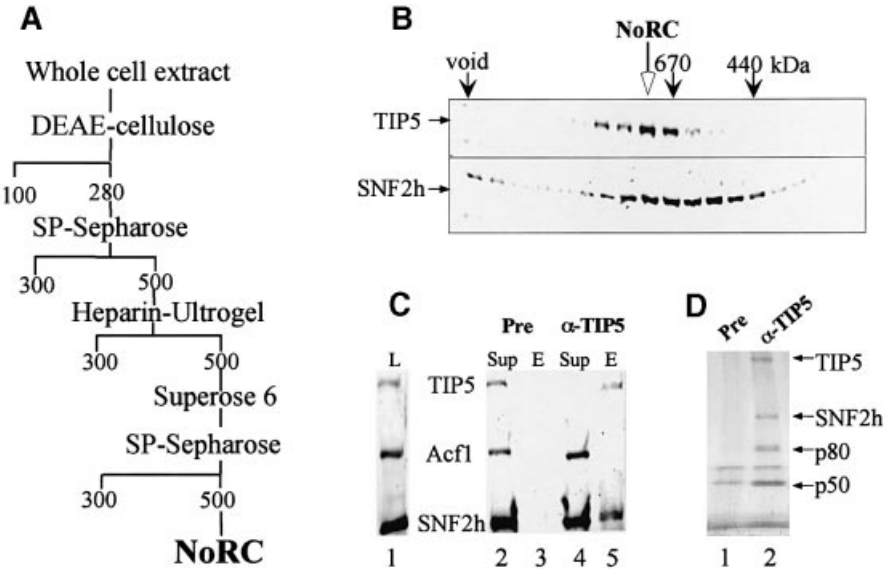

Fig. 5. Purification of NoRC. (**A**) Fractionation scheme used to purify cellular NoRC. (**B**) Resolution of TIP5-containing complexes by gel filtration. Proteins present in the H-500 fraction were size fractionated on Superose 6 and analyzed on immunoblots using anti-TIP5/N1–18 (upper panel) and anti-SNF2h (lower panel) antibodies. The position of molecular weight standards (thyroglobulin, 670 kDa; apoferritin, 440 kDa; aldolase, 158 kDa) is indicated at the top of the panel. (**C**) Western blot. NoRC was precipitated from a partially purified fraction (SP-500) with affinity-purified anti-TIP5/N1–18 antibodies and eluted with the epitope peptide. As a control, pre-immune serum (pre) was used. Western blot analysis of 2.5% of the load (L, lane 1) and the supernatants (Sup, lanes 2 and 4), and 15% of the eluate (E, lanes 3 and 5) was performed using antibodies against TIP5, SNF2h and Acf1. (**D**) Subunit composition of NoRC. A silver-stained 4–12% polyacrylamide gel of immunopurified NoRC is shown (lane 2). TIP5 and SNF2h were identified by mass spectroscopy.

suggest that NoRC may remodel chromatin. To assess the ability of NoRC to alter the nucleosome positioning at the rDNA promoter, we used an octamer mobilization assay that has been used previously to demonstrate ISWI- and CHRAC-mediated nucleosome movement (Längst *et al*., 1999; Brehm *et al*., 2000). This assay relies on the fact that mononucleosomes reconstituted from pure histones exhibit distinct positions that can be separated by gel electrophoresis (Linxweiler and Hörz, 1984). Nucleosomes located at the end of a DNA fragment migrate faster than those that are positioned more centrally. In the experiments in Figure 6, nucleosomes were reconstituted on a 248 bp mouse rDNA promoter fragment (from –232 to +16) and different translational positions were used as substrates in the nucleosome mobility assay. Consistent with previous results, CHRAC catalyzed an energy-dependent shift of fast migrating to slow migrating nucleosomes, which is due to sliding of nucleosomes from the peripheral to the central position of the DNA fragment (Figure 6A, lanes 1–6). Significantly, the immunopurified TIP5 complex behaved as CHRAC, i.e. it induced movement of peripheral nucleosomes to the center of the DNA fragment (lanes 7–9). Again, this reaction was dependent on the amount of NoRC in the assay and required ATP (lanes 10–11). Bead-bound control antibodies that were incubated with mouse whole-cell extract did not support nucleosome sliding (lanes 12–14), demonstrating that nucleosome mobilization was brought about by NoRC and not by a remodeling activity that bound to protein A–Sepharose non-specifically. Significantly, NoRC-driven octamer sliding was

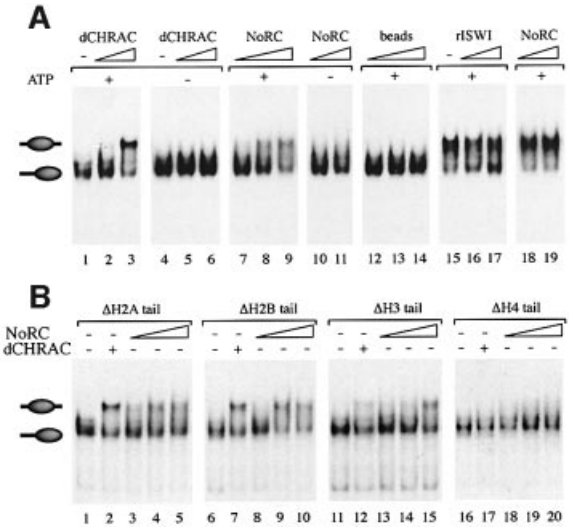

Fig. 6. NoRC triggers movement of mononucleosomes. (**A**) Nucleosome mobilization by affinity-purified NoRC. Increasing amounts of CHRAC, immunoprecipitated NoRC and control beads were incubated with end-positioned nucleosomes (lanes 1–14) in the presence or absence of ATP as indicated. In lanes 15–19, recombinant ISWI and NoRC were incubated with nucleosomes positioned centrally on the fragment. Nucleosome positions were analyzed by electrophoresis on a native polyacrylamide gel. The positions of nucleosomes are shown on the left. (**B**) The tail of histone H4 is required for NoRC-dependent nucleosome sliding. Increasing amounts of immunoprecipitated NoRC and purified CHRAC were incubated with end-positioned nucleosomes reconstituted from octamers containing three recombinant full-length histones and one tail-less histone. All reactions contained ATP. Nucleosome sliding was analyzed by native polyacrylamide gel electrophoresis.

restricted to nucleosomes that are positioned at DNA ends. When incubated with central nucleosomes, the translational position of octamers was not affected by NoRC (lanes 18 and 19). Recombinant ISWI, on the other hand, moved centrally positioned nucleosomes to the end of the DNA fragment (lanes 15–17).

ISWI-dependent chromatin remodeling machines require nucleosomal substrates with intact histone H4 tails for both stimulation of ATPase activity and nucleosome movement (Clapier *et al*., 2001). To examine whether NoRC-mediated nucleosome sliding requires the N-terminal part of histones, we performed the remodeling assays with recombinant nucleosomes that lack individual histone tails. In the experiment shown in Figure 6B, reconstituted nucleosomes that are positioned at the end of the DNA fragment were isolated and used as substrates for both CHRAC and NoRC. Consistent with previous results, CHRAC-dependent mobilization of nucleosomes did not require the tails of histones H2A, H2B and H3 (Figure 6B, lanes 2, 7 and 12). If, however, the N-terminal tail of histone H4 was deleted, CHRAC failed to move the nucleosome towards the center of the DNA fragment (lane 17). If NoRC was assayed on nucleosomal substrates lacking individual histone tails, a similar result was obtained. Like CHRAC, NoRC was able to mobilize nucleosomes lacking the N-termini of histones H2A, H2B and H3 (lanes 3–5, 8–10 and 13–15). However, deletion of the tail of histone H4 completely abolished nucleosome sliding (lanes 18–20). These functional assays demonstrate that the TIP5 complex is a novel member of ACF/CHRAC-like nucleosome remodeling machines.

Discussion

In this communication, we have identified and characterized NoRC, a novel member of mammalian SNF2h-containing remodeling complexes. The largest subunit of NoRC, i.e. TIP5, is a member of a growing family of proteins with similar domain architecture. This family of proteins has been named BAZ, 'bromodomain adjacent zinc finger' and WAL (WSTF-, Acf1-like) proteins, respectively (Jones *et al*., 2000; Poot *et al*., 2000). The most prominent members of this family are Acf1, the largest subunit of the chromatin remodeling complexes ACF and CHRAC (Ito *et al*., 1997; Varga-Weisz *et al*., 1997; Poot *et al*., 2000), and WSTF, a gene that is deleted in patients with William Beuren syndrome (Lu *et al*., 1998). BAZ2A/hWALp3 corresponds to TIP5. All members of the WSTF/ACF-like protein family are characterized by a distinct modular organization, i.e. they contain the WAKZ motif (Ito *et al*., 1999), followed by one or two PHD fingers and a bromodomain near the C-terminus. The 110 amino acid bromodomain is present in a growing number of transcriptional coactivators, many of which are known to reside in large multiprotein complexes and serve a role in chromatin modification. It is possible that tandem PHD fingers and a bromodomain form a cooperative interaction unit, as has been suggested recently for the KRAB proteins (Schultz *et al*., 2001). Some bromodomain proteins are subunits of chromatin remodeling complexes, like Acf1, Brg1 and Brm (Ito *et al*., 1999; Kingston and Narlikar, 1999; Peterson, 2000). Moreover, most proteins with histone acetyltransferase (HAT) activity contain a bromodomain. The structure of the bromodomain recently has been shown to allow specific interactions with acetylated histone H4 tails (Owen *et al*., 2000). The recognition of specific N- and C-terminal modifications in the histone tails has been postulated to be one of the key interactions determining the transcriptional competence of chromatin (Strahl and Allis, 2000). It is therefore possible that TIP5 function is sensitive to the acetylation state of specific lysine residues that are required to anchor TIP5 to chromatin.

It is intriguing that TIP5 shares domains with Acf1 and WSTF that are implicated in protein–protein interactions, such as PHD fingers and the BAZ1 motif. The PHD finger is a 50–80 amino acid zinc finger-like motif with a unique Cys4–His–Cys3 pattern that has been identified in numerous proteins, many of which are implicated in chromatin-mediated transcriptional control (Aasland *et al*., 1995). The BAZ1 motif has been reported to be involved in the interaction with SNF2h, the human homolog of ISWI (Jones *et al*., 2000). WSTF and hAcf1, but not TIP5, contain at their N-terminal region a WAC motif, a novel protein domain that appears to target ACF to pericentric heterochromatin. Moreover, TIP5 contains a module, termed TAM, a motif that is related to the MBD motif present in methyl-CpG-binding proteins (Nan *et al*., 1993). It is tempting to speculate that the role of TAM is to localize NoRC adjacent to the binding site of TTF-I on the rDNA promoter by virtue of interacting with DNA. Indeed, the TAM domain of TIP5 binds to DNA. However, binding was not very efficient and we did not detect preferential binding either to the rDNA promoter (data not shown) or to methylated DNA. The two AT-hooks that are adjacent to the TAM domain enhance DNA-binding activity by almost two orders of magnitude. This suggests that the AT-hooks, which have been shown to affect association of proteins with chromatin (Bourachot *et al*., 1999), may collaborate with bromodomains to specify interactions with particular nucleosomes.

Studies of mammalian remodeling complexes initially focused on the human SWI/SNF homologs, the Brg-1 and Brm proteins. A third family, which includes the nucleosome remodeling and deacetylase (NuRD) complexes, contains a CHD/Mi-2 protein(s) as the central ATPase(s). Another complex containing ISWI and a 325 kDa subunit (hRSF) was purified from human cells based on its ability to facilitate transcription from chromatin templates (LeRoy *et al*., 1998). All of these complexes have been shown to remodel chromatin *in vitro* and to endow chromatin with dynamic properties to regulate many nuclear processes, such as transcription, replication, recombination and DNA repair. In this communication, we have identified and characterized a novel ACF-like remodeling complex that contains TIP5, SNF2h, and perhaps two additional proteins, p50 and p80. Importantly, TIP5 interacts with the Pol I transcription termination factor TTF-I and the majority of TIP5 co-localizes with UBF in the nucleolus. Therefore, it is reasonable to predict that NoRC serves a specific role in maintaining or altering the chromatin structure of the rDNA locus. Consistent with a specialized function for NoRC, current data indicate that each remodeling complex can perform only a subset of known remodeling activities.

These include the ability to transfer histone octamers *in trans*, disorder nucleosome arrays, space nucleosomes evenly along an array, perturb or reposition individual nucleosomes and facilitate the access of proteins to nucleosomal DNA (for a review see Kingston and Narlikar, 1999). For example, excess NURF perturbs the regular positioning of histone octamers relative to one another within an assembled nucleosomal array, while ACF and CHRAC promote the opposite reaction, i.e. they improve the assembly of regularly spaced nucleosomes (Tsukiyama *et al*., 1995; Ito *et al*., 1997; Varga-Weisz *et al*., 1997; Poot *et al*., 2000). Moreover, the ATPase activity of SWI2/SNF2 is stimulated by DNA, Mi-2 by nucleosomes and ISWI by both, i.e. is partially stimulated by DNA and further activated by nucleosomes. Additional differences between remodeling complexes have been observed with mononucleosome substrates. SWI2 and Mi-2 trigger nucleosome movement in the absence of histone tails, whereas both ISWI and NoRC require the tail of histone H4 for activity (Boyer *et al*., 2000; Brehm *et al*., 2000). Finally, ISWI moves a histone octamer from a central position to the end of a DNA fragment, whereas CHRAC directs nucleosome movement in the opposite direction (Längst *et al*., 1999). Significantly, immunoprecipitated NoRC behaves like CHRAC, i.e. it moves nucleosomes from the end of a DNA fragment to a central position of the DNA.

The marked conservation of ISWI homologs in species as diverse as yeast and humans suggests that they serve important functions. Apparently, the cell uses different complexes to assemble specialized chromatin structures, and the different types of complexes may be targeted to different sets of cellular genes. The analysis of NoRC, a novel member of ISWI/SNF2h-based chromatin remodeling complexes, supports this view. Our finding that NoRC both interacts with TTF-I and co-localizes with the basal Pol I transcription factor UBF suggests a function in rDNA transcription. We have shown previously that binding of TTF-I to its cognate site upstream of the rDNA promoter induces chromatin remodeling, which is a prerequisite for Pol I transcription initiation (Längst *et al*., 1997, 1998). Thus, TTF-I can activate transcription on chromatin templates, presumably by recruiting remodeling complexes to the rDNA promoter. Whether or not the TIP5 complex or another remodeling machine that is present in the partially purified transcription system is responsible for TTF-I-mediated transcriptional activation is not yet known. We can not even exclude the possibility that NoRC is associated with inactive ribosomal gene copies and exerts a repressive effect on Pol I transcription. Further investigation of the role of NoRC on Pol I transcription using a defined *in vitro* system consisting of reconstituted nucleosomal arrays and purified transcription factors is clearly warranted. The reconstitution of NoRC from recombinant subunits will constitute a major milestone on this track. Regardless of whether NoRC exerts a stimulatory or repressive effect on rDNA transcription, NoRC has the potential to regulate cellular rRNA synthetic activity and, with that, ribosome biogenesis. Future studies will address the role of TIP5 in modulating transcriptional activity that occurs as the cell responds to external signals or progresses through the cell cycle.

Materials and methods

Cloning of cDNA encoding Tip5

The yeast two-hybrid screening was performed essentially as described (Gyuris *et al.*, 1993). The yeast strain EGY48 harboring LexA-TTF-I was transformed with a human fibroblast (Wi-38) cDNA fusion library cloned into the vector pB42AD (Clontech), which contains the B42 activation domain. Transformants were selected on SD/galactose/–Ura/–His/–Trp/–Leu plates for 3 days and then patched onto SD/glucose/–Ura/–His/–Trp/–Leu plates. About 10^7 colonies were screened. Five positive clones were identified and sequenced. One of them, designated *Tip5*, contained a 1.2 kb cDNA that corresponds to a human EST clone, KIAA0314 (Nagase *et al.*, 1997). The partial cDNA was used to screen a mouse cDNA library to yield a partial clone encompassing amino acids 1–598 of murine TIP5. Finally, conventional techniques were used to isolate a murine cDNA with a 5553 nucleotide ORF that encodes full-length TIP5 with a calculated molecular mass of 205 kDa. The cDNA sequence has been submitted to the DDBJ/EMBL/GenBank (accession No. AJ309544). The corresponding human gene (accession No. AB032254) has been termed BAZ2A (Jones *et al*., 2000) and hWALp3 (Poot *et al*., 2000), respectively. The full-length cDNA was inserted into diverse vectors to facilitate expression in *Escherichia coli* and in mammalian cells.

Expression and purification of recombinant proteins

To express subclones of TIP5, histidine-tagged TIP5-TAM (amino acids 510–611) or TIP5-TAM-AT (amino acids 510–723) were expressed in *E.coli* (DE3)pLysS and purified on Ni^{2+}-NTA–agarose. Briefly, extracts were centrifuged at 12 000 *g* at 4°C for 30 min and the supernatant was incubated for 3 h at 4°C with Ni^{2+}-NTA–agarose (Qiagen) in lysis buffer [300 mM KCl, 20 mM Tris–HCl pH 8.0, 1% NP-40, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM imidazole]. The resin was washed with lysis buffer containing 0.5% NP-40 and 20 mM imidazole and proteins were eluted with 200 mM imidazole.

Protein–protein interaction assays

The region of human TIP5 that was identified in the yeast two-hybrid screen was fused to GST to yield pGEX1N-hTip/332–726. pGEX1N-hTip5/332–726 was expressed in *E.coli* BL21(DE3) and purified on glutathione–Sepharose beads as specified by the manufacturer (Pharmacia). To monitor interaction of TTF-I with immobilized TIP5, 25 µl of nuclear extract or 2.5 µl of ^{35}S-labeled TTF-I (Evers *et al*., 1995) in 22.5 µl of buffer AM-100 were applied onto 20 µl columns of glutathione–Sepharose containing 1 mg/ml immobilized GST–hTIP5/332–726. After washing with 10 vols of buffer AM-100, bound proteins were eluted with high-salt buffer AM-1000 and TTF-I was visualized on western blots or by autoradiography.

Southwestern assay

Recombinant proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes and DNA binding was monitored as described (Lewis *et al*., 1992). Briefly, after denaturation for 5 min in 6 M guanidine hydrochloride in binding buffer (20 mM HEPES–KOH pH 7.9, 5 mM $MgCl_2$, 40 mM KCl, 10 mM β-mercaptoethanol), membrane-bound proteins were washed sequentially with 3, 1.5, 0.75 and 0.375 M guanidine hydrochloride in binding buffer. The membrane was washed twice with binding buffer, blocked with 2% milk and washed again. The filters were incubated for 1 h at room temperature with ~100 000 c.p.m./ml of specific ^{32}P-labeled oligonucleotides and 5 µg/ml pBS-SK DNA in binding buffer plus 0.1% NP-40. The membrane was washed with five changes (5 min each) of binding buffer supplemented with 0.01% NP-40. Protein-bound DNA was monitored by a PhosphorImager.

Purification of NoRC

TIP5-containing protein complexes were purified from mouse cell extracts using a combination of conventional and immunoaffinity chromatography. An 800 mg aliquot of whole-cell extract proteins was first fractionated on a DEAE–Sepharose™ Fast Flow column (Pharmacia) in buffer AM-100 [100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl pH 7.9, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol and protease inhibitors (Complete™, Roche)]. After elution with buffer AM-280, TIP5-containing fractions were chromatographed on SP-Sepharose™ Fast Flow (Pharmacia). Fractions eluting at 500 mM KCl (SP-500) were purified further on heparin–Ultrogel. After washing with buffer AM-300, NoRC was step-eluted with 500 mM KCl, precipitated with 70% ammonium sulfate, resuspended in buffer AM-300/0.01% NP-40 and size-fractionated on a Superose 6 HR 10/30 (Pharmacia) gel filtration

column. After concentration on SP-Sepharose™ Fast Flow, fractions containing TIP5 were incubated for 2 h at 4°C with 70 µl of protein G-bound anti-TIP5 antibodies, transferred into columns and washed with 20 column volumes of AM-300, 5 vols of AM-500, and NoRC was eluted with the epitope peptide (3 µg/µl) in 80 µl of buffer AM-300/0.1% NP-40 and protease inhibitors.

Immunofluorescence

NIH-3T3 cells grown on coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 15 min at room temperature, washed with PBS and incubated for 90 s with cold methanol. The coverslips were incubated for 1 h at 37°C with primary antibodies and washed three times before being incubated for 1 h at 37°C with the appropriate secondary antibodies. Finally, the coverslips were mounted in Fluoromount-G (Southern Biotechnology Associated, Inc.). Immunofluorescence was monitored by microscopy and images were digitally recorded. Primary antibodies were α-mTIP5N1-18 (rabbit) and α-hUBF (human), which were diluted 1:100 and 1:2400, respectively, in PBS containing 0.1% bovine serum albumin (BSA). Secondary antibodies (Dianova GmbH) were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1:500) or Texas red-conjugated goat anti-human IgG (dilution 1:200). DNA was stained with 5 ng/ml Hoechst 33258 (Boehringer Mannheim).

Nucleosome mobilization assay

Mononucleosomes were reconstituted on a labeled 248 bp murine rDNA fragment (from –232 and +16) with either recombinant histones using salt gradient dialysis or with purified *Drosophila* histones using polyglutamic acid (Brehm *et al*., 2000). NoRC was immunopurified by incubating bead-bound anti-TIP5N1–18 antibodies (3–5 µg of IgGs per 10 µl of protein A–agarose) with 10 µg/µl whole-cell extract proteins in buffer AM-300 containing 0.1% NP-40 and protease inhibitors. Nucleosome mobility was assayed as described (Brehm *et al*., 2000). Typical reactions contained 60 fmol of purified nucleosomes, 1 mM ATP, 200 ng/µl BSA in buffer AM-100. Positioned mononucleosomes were incubated with recombinant ISWI (2–6 fmol), purified CHRAC (0.2–0.6 fmol) and increasing amounts of NoRC for 90 min at 26°C. The nucleosome positions were analyzed by electrophoresis in 4.5% polyacrylamide gels in 0.4× TBE.

Antibodies

Polyclonal antibodies recognizing residues 1–18 of TIP5 were raised in rabbits using a keyhole limpet hemocyanin-conjugated synthetic peptide (α-mTIP5N1–18). The anti-hISWI and anti-hACF1 antibodies have been described (Poot *et al*., 2000). Antibodies were affinity-purified using the corresponding immunogen cross-linked to an UltraLink™ Iodoacetyl column (Pierce) according to the manufacturer's instructions.

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