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The WSTF-SNF2h Chromatin Remodeling Complex Interacts with Several Nuclear Proteins in Transcription*[S]

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The WSTF (Williams syndrome transcription factor) protein is involved in vitamin D-mediated transcription and replication as a component of two distinct ATP-dependent chromatin remodeling complexes, WINAC and WICH, respectively. We show here that the WICH complex (WSTF-SNF2h) interacts with several nuclear proteins as follows: Sf3b155/SAP155, RNA helicase II/Guα, Myb-binding protein 1a, CSB, the proto-oncogene Dek, and nuclear myosin 1 in a large 3-MDa assembly, B-WICH, during active transcription. B-WICH also contains RNAs, 45 S rRNA, 5 S rRNA, 7SL RNA, and traces of the U2 small nuclear RNA. The core proteins, WSTF, SNF2h, and nuclear myosin 1, are associated with the RNA polymerase III genes 5 S rRNA genes and 7SL, and post-transcriptional silencing of WSTF reduces the levels of these transcripts. Our results show that a WSTF-SNF2h assembly is involved in RNA polymerase III transcription, and we suggest that WSTF-SNF2h-NM1 forms a platform in transcription while providing chromatin remodeling.

ATP-dependent chromatin remodeling complexes are required in many nuclear processes, such as replication and transcription, in which factors need access to the DNA. Recent research has shown that ATP-dependent chromatin remodeling complexes are also required for chromatin maintenance and genome stability (for reviews see Refs. 1 and 2). Eukaryotic cells have several ATP-dependent chromatin remodeling complexes, which can be divided into at least four different classes depending on the ATPase in the complex as follows: SWI/SNF complexes, ISWI (Imitation of SWI)-containing complexes, Mi-2 complexes, and Ino80 (1, 2).

The ISWI-containing complexes are involved in a wide variety of nuclear processes depending on the binding partner (for review see Refs. 3 and 4). Many of the proteins of the WAL/BAZ/WAC family interact with ISWI proteins to form different nuclear complexes (3, 4), such as the ACF1/WCRF180 protein in ACF[3] and CHRAC (chromatin accessibility complex); the p305 NURF/FAC1 protein in NURF; and the Tip5 protein in NoRC. The WAL/BAZ/WAC proteins share the domain structure, which contains a C-terminal bromodomain adjacent to a PHD (plant homeodomain) finger (5), but they do not share an extended sequence homology. The individual proteins give specificity to the different complexes, and the ISWI protein provides the ATPase activity responsible for the remodeling of the nucleosome. WSTF (the Williams syndrome transcription factor) is a further WAL/BAZ/WAC protein (5, 6), but in contrast to other family members, it associates both with the ISWI protein (6–9) and with the SWI/SNF complexes (10–12). These two complexes, the WICH (WSTF-ISWI chromatin remodeling complex) and the WINAC (WSTF including the nucleosome assembly complex), are mainly involved in different processes. WICH is implicated in the replication of heterochromatin (6, 8), whereas WINAC is mainly implicated in transcription mediated by the vitamin D receptor but also in replication (10, 12). The *WSTF* gene was first identified as one of 15–16 genes heterozygously deleted in patients with William-Beuren syndrome, a neurodevelopmental disorder (13, 14), and an impaired vitamin D response caused by a low level of the WSTF protein may explain the hypercalcemia observed in these patients (10).

The WSTF in the WICH complex is recruited to replication foci by PCNA (8). However, studies in *Xenopus laevis* have shown that the DNA polymerase elongates in the absence of both WSTF and Acf1 (7). The WICH may play a role in the formation of chromatin after replication (8, 9). Silencing of WSTF by short interfering RNA in mammalian cells results in a delayed S-phase progression (10) and in the formation of cells with small nuclei with densely packed chromatin (8, 9). The function of WSTF in transcription is coupled to its association with mammalian SWI/SNF complexes in regulating the vitamin D response by binding to both activated and repressed gene promoters in a non-ligand-dependent manner (10, 11). The WSTF protein is recruited to the genes by its binding to acetylated histones, preferentially to acetylated histone H3 at lysine 14 (12) Because WSTF can be part of two ATP-dependent chromatin remodeling complexes, it has been suggested that WSTF acts as a platform to which other proteins bind depending on functional requirement (10, 11). Interestingly, WINAC is also involved in replication and contains the p150 CAF-1 protein (10), a subunit of CAF-1, a nucleosome assembly complex involved in replication (15).

The known role of WSTF in transcription has so far been restricted to its association to the human SWI/SNF. However, ISWI complexes, such as the NURF complex and the NoRC, also regulate transcription. These complexes act in transcription conducted by different RNA polymerases; NURF acts on developmental RNA polymerase II (RNA pol II) genes in *Drosophila* and man (16, 17), whereas NoRC is a silencer of rRNA genes, inhibiting RNA polymerase I (RNA pol I) transcription (18). RNA pol I transcribes the 47/45 S rRNA gene in the nucleolus (reviewed in Ref. 19). Similar to WSTF, TIP5 has binding sites for acetylated histones and recruits the NoRC to rDNA by binding to acetylated H4, specifically lysine 16, in the promoter (20). NoRC, in turn, targets

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. S1–S3 and additional Refs. 1–5.

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[3] The abbreviations used are: ACF, ATP-utilizing chromatin assembly and remodeling factor; pol, polymerase; snRNA, small nuclear RNA; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PCNA, proliferating cell nuclear antigen; WSTF, Williams syndrome transcription factor; WINAC, WSTF including nucleosome assembly Complex; WICH, WSTF-ISWI chromatin remodeling complex; NoRC, nucleolar remodeling complex; NURF, nucleosome remodeling factor; CSB, Cockayne syndrome protein B; NM1, nuclear myosin 1; shRNA, small hairpin RNA; ChIP, chromatin immunoprecipitation.

histone deacetylases, histone methyltransferases, and DNA methyltransferases to the rRNA gene to be silenced (18). So far, no ATP-dependent chromatin remodeling complex has been found to be associated with RNA polymerase III (RNA pol III) transcription. RNA pol III is the polymerase responsible for the transcription of short, untranslated RNAs, such as 5 S rRNA, tRNA, 7SL RNA (in the signal recognition particle), and U6 snRNA (reviewed in Ref. 21). Because RNA pol I and RNA pol III transcripts act in translation, the activities of these RNA polymerases are closely linked to cell growth. We report here the isolation of a high molecular weight WSTF assembly, designated B-WICH, that contains the human ISWI protein SNF2h and is involved in transcription.

We identified the following six novel association partners to WSTF-SNF2h by mass spectrometry and immunoprecipitation: the transcription factor Myb-binding protein 1a (Myb-bp1a) (22, 23); the splicing factor Sf3b/SAP155 (24); the RNA helicase II/Guα (25); the proto-oncogene Dek (25, 26); the nuclear excision repair enzyme Cockayne syndrome protein B (CSB) (28); and nuclear myosin 1 (NM1) (29). The formation of B-WICH depends on active transcription, which suggests that WSTF-SNF2h has a role in transcription, in addition to its role in replication. The B-WICH was also sensitive to RNase treatment, and we have shown that the 45 S rRNA, the 5 S rRNA, and the 7SL RNA are parts of the B-WICH assembly. This led us to suggest that the B-WICH is assembled upon RNA pol I and RNA pol III transcription. In support of a role for the WSTF-SNF2h in RNA pol III transcription, we found that these proteins, together with NM1, were present at the 5 S RNA and 7SL RNA genes and that silencing of WSTF by shRNA reduced the RNA levels of 5 S rRNA and 7SL RNA. The interactions between the WSTF-SNF2h core and nuclear proteins involved in replication, transcription, and DNA repair suggest a wide role for WSTF-SNF2h in the nucleus by acting as a platform for other proteins.

EXPERIMENTAL PROCEDURES

Cell Cultures—HeLa cells were grown in 5% fetal calf serum in Dulbecco's modified Eagle's medium under a 7% CO_2 atmosphere. Transfection of cells was performed using Lipofectamine Plus (Invitrogen) with an efficiency of ~50%.

Fractionation of HeLa Nuclei and Salt Extraction—HeLa cells were fractionated into a nucleoplasmic fraction and a chromatin (nuclear structures) fraction at different KCl concentrations as described by Remboutsika *et al.* (30).

Purification of WSTF-ISWI Complex (B-WICH)—HeLa cells were homogenized in a buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and a protease mixture containing 1 mM phenylmethanesulfonyl fluoride (PMSF), 1.6 μg/ml aprotinin, 0.7 μg/ml leupepstatin, and 1 mM dithiothreitol (DTT) in a Dounce homogenizer, ~12 strokes. The nuclei were collected by centrifugation at 2,800 rpm for 15 min at 4 °C. The nuclei were resuspended in extraction buffer containing 0.7 M KCl, 10 mM HEPES, pH 7.7, 0.1 mM EDTA, 10% glycerol, 1 mM PMSF, 1.6 μg/ml aprotinin, 0.7 μg/ml leupepstatin, 0.1 mM DTT; and the extract was centrifuged at 13,000 rpm for 20 min. The nuclear extract was then passed through a G-25 Sephadex column equilibrated with 0.1 M KCl in fractionation buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 20% glycerol, 0.1% Tween 20, 0.5 mM DTT, 0.2 mM PMSF), and the protein fraction was fractionated on a 20-ml phosphocellulose-11 (P-11) column equilibrated with 0.1 M KCl in fractionation buffer. The column was washed with 0.3 M KCl in fractionation buffer, and the ISWI-containing complexes were eluted by increasing the KCl concentration stepwise to 0.5, 0.65, and 0.85 M. The different P-11 fractions were subsequently separately passed through a Sephadex G-25 column equilibrated with 0.1 M KCl in fractionation buffer, and then applied to a 1-ml Mono Q column equilibrated with the same buffer. The protein complexes were eluted by a 20-ml KCl gradient from 0.2 to 0.8 M at a flow rate of 1 ml/min. Fractions of 0.5-ml volume were collected. Mono Q fractions containing WSTF were subjected to immunoprecipitation using a polyclonal C-terminal WSTF antibody (Cell Signaling) and collected for identification by MALDI-TOF mass spectrometry. Superose 6HR fractionation of the WSTF-enriched Mono Q fractions was performed in 0.3 M KCl in fractionation buffer.

Preparation of the HeLa Cell Nuclear Extracts—The protocol described above was followed when preparing 0.7 KCl HeLa cell nuclear extract. HeLa cell nuclear extract was also prepared using the buffer containing 0.42 M KCl as described by Dignam *et al.* (31). HeLa cell nuclear extracts were treated with RNase A (0.33 μg/μl) at room temperature for 20 min. RNaseOut (Invitrogen) was added to the untreated nuclear extracts. HeLa cells were treated with 1 μg/ml actinomycin D (Sigma) to obtain nuclear extract from transcriptionally inhibited cultures, and nuclear extracts were prepared after 30 min.

Immunoprecipitation of the WSTF-containing Complex—WSTF-containing complexes were immunoprecipitated from both whole cell extracts and nuclear extracts extracted with extraction buffer as described above containing 0.7 M KCl, without DTT. The whole cell extract (0.5 mg) and nuclear extract (0.3–0.4 mg) were quickly diluted to a final concentration of 0.35 M KCl with IP buffer (20 mM HEPES, pH 7.9, 0.01% Tween 20, 0.25 mM EDTA, 10% glycerol, and protease inhibitors as above) and incubated with antibody for 18 h at 4 °C. The immunocomplexes were precipitated using protein A/protein G-Sepharose. The pellet was washed six times with 0.25 M KCl or 0.35 M KCl IP wash buffer as indicated (0.25 or 0.35 M KCl, 20 mM HEPES, pH 7.9, 0.01% Nonidet P-40, 0.1 mM EDTA, 10% glycerol, and the protease mixture described above) and eluted with SDS-PAGE sample buffer. WSTF-containing complexes were immunoprecipitated from nuclear extract that had been treated with RNase A (0.5 and 1.0 mg/ml) for 20 min at room temperature before immunoprecipitation or from nuclear extracts from HeLa cells treated with 1 μg/ml actinomycin D.

SDS-PAGE and Immunoblotting—Samples were separated on 7% SDS-PAGE and transferred to an Immobilon membrane filter (Millipore) using a transfer buffer containing 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 5% methanol. The membranes were probed with polyclonal ISWI antibodies (kind gifts from P. Wade), polyclonal anti-Dek (kind gift from G. Grosveld), polyclonal anti-Myb-bp1a (kind gift form R. Keough), a polyclonal anti-RNA helicase II/Guα (kind gift from B. C. Valdez), a polyclonal Sf3b155/SAP155 antibody (kind gift of R. Lührmann), a polyclonal CSB antibody (Santa Cruz Biotechnology), polyclonal Acf1 (kind gift of T. Hirano), polyclonal anti-NM1 (32), monoclonal p150-CAF-1 (Abcam), monoclonal PCNA (Abcam), polyclonal anti-BRG1 (33), and the WSTF antibodies, against the N-terminal part (kind gift from P. Varga-Weisz) and one against the C-terminal part of the *Xenopus* WSTF (kind gift of T. Hirano). The signals were visualized by chemiluminescence (Pierce).

Mass Spectrometry Analysis—Coomassie-stained bands were excised from the SDS-polyacrylamide gradient gel and subjected to in-gel digestion with trypsin. The peptides were extracted from the gel, desalted on Zip-Tip (Millipore), and analyzed by MALDI-TOF mass spectrometry (34).

RNA Preparation and WSTF-shRNA—The RNAs in B-WICH were isolated from the immunoprecipitated complex by extraction by Trizol reagent (Invitrogen) and DNase-treated (Invitrogen). The RNA was then converted to cDNA by reverse transcriptase using random primers (Invitrogen), in the presence of RNaseOut, and the cDNA was analyzed

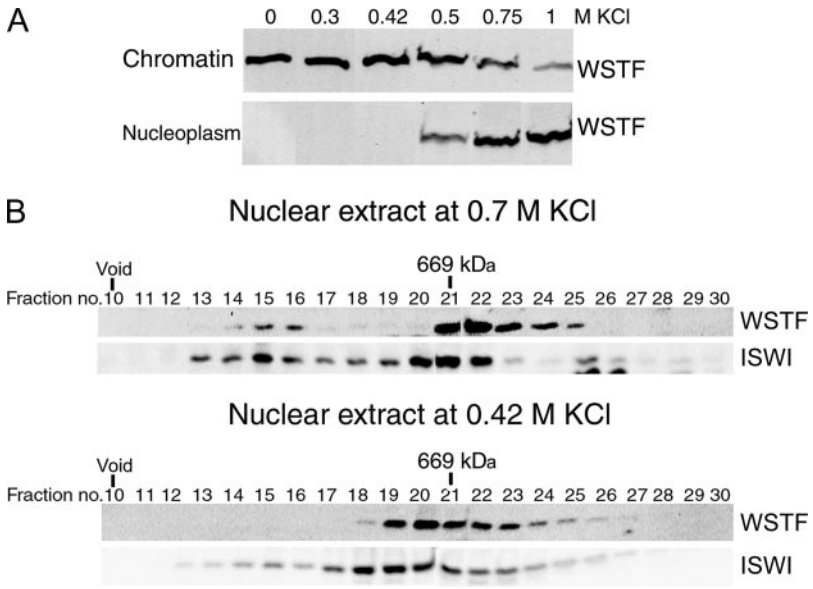

FIGURE 1. **WSTF is tightly associated with chromatin and is extracted in a high molecular weight complex.** *A,* immunoblot of the WSTF protein in the nucleoplasmic and chromatin fractions of nuclear extracts prepared at different KCl concentrations. The KCl concentration is indicated *above* the lanes. The amount loaded in each lane corresponds to 40 × 10^5 cells. *B,* immunoblots of fractions from a Superose 6HR size exclusion column of crude nuclear extract (2 mg) prepared using 0.7 M KCl (*top panel*) or 0.42 M KCl as described by Dignam *et al.* (31) (*bottom panel*). The immunoblots show the molecular weight distribution of the WSTF protein and the ISWI proteins, as indicated to the *right*. The fraction numbers are marked *above* the lanes and the size marker and void volume are marked at the *top*.

using PCR with primers for 45 S RNA, 18 S RNA, 28 S RNA (35), 5 S RNA, $tRNA^{Tyr}$, $tRNA^{Leu}$, 7SL RNA (36), U6 snRNA, U2 snRNA, and U1 snRNA (37). The protein level of WSTF was knocked down in cells using WSTF-shRNA (expressed from the pSuper vector) with the sequence 5′-GAACAGGSSGTTGCTGAGC. Total RNA was prepared 36 h after transfection using Trizol (Invitrogen) and then treated with DNase (Invitrogen). The RNA was converted to cDNA by reverse transcriptase (Superscript III; Invitrogen) using random primers, followed by PCR using specific primers for 5 S, 7SL, tRNA (36), U6 snRNA, U2 snRNA, and U1 snRNA (37). All primer pairs were optimized for PCR. The signals were quantified using Quantity One (Bio-Rad).

Chromatin Immunoprecipitation—ChIP analyses were performed as described previously (38) and repeated in six independent experiments. Chromatin was prepared from HeLa cells after cross-linking with formaldehyde. The chromatin was sheared by sonication to a DNA size of 500–1500 bp. Chromatin fragments were precipitated with antibodies against WSTF (Cell Signaling), SNF2h (Abcam), NM1 (32), and TFIIIC220 (Santa Cruz Biotechnology) bound to protein A/G-Sepharose beads (50% of each). Two controls were used: beads only and donkey IgG (Abcam) bound to the beads. The resulting precipitated DNA fragments were amplified by PCR with specific primers for the genes for 5 S rRNA, 7SL RNA, $tRNA^{Tyr}$, $tRNA^{Leu}$ (36), $tRNA^{Lys}$ (47), U6-1, U6-4 (39), U1, and U2 (37).

Protein Determination—Protein concentrations were determined using the Bradford reagent (Bio-Rad).

RESULTS

The WSTF Protein Associates Tightly with Chromatin—Extraction of HeLa cell nuclei at different salt concentrations revealed that the WSTF protein was tightly associated with the chromatin fraction. The major part of the WSTF was extracted at concentrations above 0.42 M KCl, and most of the protein was released at 1 M KCl (Fig. 1*A*). This finding prompted us to determine in which constellations the WSTF protein was released at different KCl concentrations, as it interacts both with the SNF2h protein and with the SWI/SNF complexes. The WSTF protein extracted from nuclei at 0.7 M KCl eluted from a Superose 6HR size exclusion column in two distinct peaks, one at 700–800 kDa and one at 2–3 MDa (Fig. 1*B*). The same two WSTF peaks were also seen with two other polyclonal antibodies against WSTF, one against the N-terminal part (6) and one against the C-terminal part (7) (not shown). Only the WSTF peak at the lower mass, 800 kDa, was present in the nuclear extract prepared at 0.42 M KCl (Fig. 1*B*). The WICH complex has a molecular mass of 700 kDa (6), to which the lower molecular mass peak corresponds. The ISWI protein eluted in two major peaks from 0.7 M KCl nuclear extracts, one peak co-eluting with the high molecular weight WSTF peak and the other peak at 600–900 kDa, partially co-eluting with the low molecular weight WSTF peak. The 0.42 M KCl extract also contained high molecular weight ISWI complexes, but the main protein peak appeared at 700 kDa to 1 MDa. The lower ISWI peak co-eluted with different proteins found in ISWI complexes, Acf1 and TIP5, whereas the large peak co-eluted with SMC1 (not shown), the cohesin present in a large ISWI-NuRD-cohesin complex involved in sister-chromatin segregation (40).

WSTF-SNF2h Associates with Several Other Nuclear Proteins—We subsequently purified the large WSTF-containing assembly through three chromatographic steps, using a purification scheme as shown in Fig. 2*A*. Nuclear extract was first applied on a P-11 column, from which proteins were eluted in several steps at different KCl concentrations as shown in Fig. 2*A*, before being separated on a Mono Q column and a final Superose 6HR size exclusion column. The large WSTF-containing peak was only detected in the 0.5–0.65 M KCl fraction from the P-11 column. The fraction eluting at 0.65–0.85 M KCl contained only the 700-kDa WSTF peak (not shown). The WSTF protein eluted from the final Superose 6 HR column in a broad peak, centered at a molecular mass of 3 MDa, with a further smaller peak at 1 MDa (Fig. 2*B*). The ISWI protein also eluted at a molecular mass of 3 MDa, but the main peak eluted at 1 MDa (Fig. 2*B*). Other ISWI complexes, such as ACF/WCRF and NoRC, eluted at 1 MDa (supplemental Fig. 1), and the large ISWI-Mi2-cohesin eluted in a broad peak at 1–3 MDa (supplemental Fig. 1). The WSTF in the 3-MDa peak interacted with ISWI. Immunoprecipitation using the WSTF antibody of the 3 MDa peak (fractions 15 and 16 from the Superose of nuclear extract prepared at 0.7 M KCl, presented in Fig. 1) precipitated ISWI protein (Fig. 2*C*).

We performed immunoprecipitations of WSTF-containing Mono Q fractions (supplemental Fig. 2) to identify binding partners to the WSTF protein. The precipitate contained several proteins (Fig. 2*D*), the identities of which were determined by MALDI-TOF mass spectrometry. The 170-kDa protein band was the WSTF, and the 130-kDa protein band was SNF2h (stained strongly with Coomassie, suggesting more than one protein), indicating that the WICH complex was in the assem-

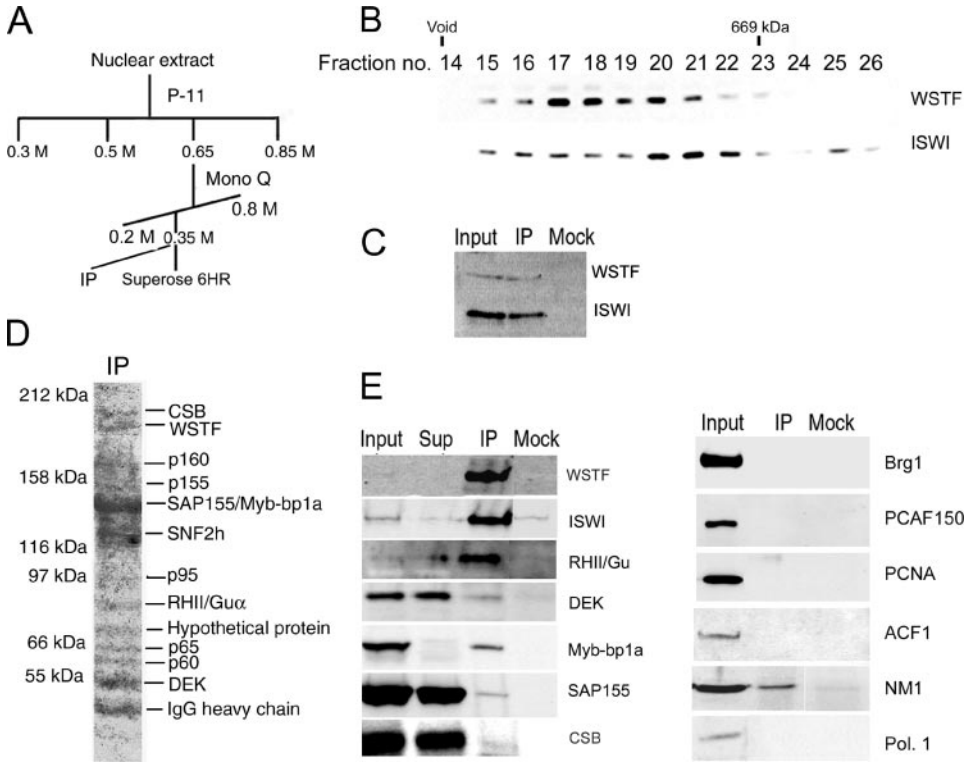

FIGURE 2. **The WSTF protein is isolated as a component of a large ISWI assembly.** *A,* purification scheme used for the isolation of WSTF-containing assemblies. *B,* immunoblots of the fractions from the final Superose 6HR purification step show the size distributions of the WSTF protein and the ISWI protein, as indicated on the *right*. The proteins in the different fractions were separated on a 7% SDS-PAGE. The size markers are indicated on *top*, together with the void volume and the fraction number. *C,* immunoblot of proteins precipitated from the high molecular weight WSTF peak from nuclear extract by the anti-WSTF. The *Input* is 1/10 of the input fraction; *IP* is the precipitated proteins, and *Mock* is an precipitation with only beads and without antibody. The immunoblot was probed for the WSTF protein and the ISWI protein as indicated to the *right. D,* Coomassie-stained gel of the immunoprecipitated WSTF assembly from the WSTF peak from Mono Q. The proteins were separated on a 5–20% SDS-polyacrylamide gradient gel and Coomassie-stained, and the bands were excised for analysis by MALDI-TOF mass spectrometry. The size markers are indicated on the *left*. The identities of the proteins are marked on the *right,* and protein bands marked *p160*, *p155*, *p95*, *p65*, and *p60* have been provisionally identified. *E,* immunoblot of the proteins in immunoprecipitation using the WSTF antibody from whole cell extracts prepared using 0.7 M KCl in the lysis buffer. The immunoblot was probed with antibodies against the WSTF protein, the ISWI proteins, the RNA helicase II/Guα (RHII/Gu), Dek, Myb-bp1a, Sf3b155/SAP155, and the CSB protein as indicated on the *right*. In addition, immunoblots were probed for known interacting partners to WSTF, ISWI, or to proteins involved in RNA pol I transcription as follows: Brg1, pCAF-150, PCNA, ACF1, NM1, or RNA pol I, as indicated on the *right*. *Input* is 1/10 of the input; *sup* is 1/10 of the supernatant, and *IP* is the immunoprecipitated pellet. *Mock* is a precipitation of nuclear extract without antibodies.

bly. The additional proteins precipitated by the WSTF antibody were the 168-kDa CSB, the 155-kDa Sf3b155/SAP155, the 149-kDa Myb-bp1a (Sf3b155/SAP155 and Myb-bp1a in a mixture in a strongly stained band at position 150 kDa), RNA helicase II/GUα with a molecular mass of 87 kDa, and the 45-kDa proto-oncogene Dek. One further protein was the 95-kDa uncharacterized U1 small ribonucleoprotein 1SNRP homologue, and the protein with a molecular mass of 70 kDa was denoted as protein FLJ23164 from an open reading frame. No subunits of the WINAC, such as BRG1, p150 CAF-1, FACT140, and topoisomerase II (10), were identified in the immunoprecipitate, demonstrating that the large molecular weight WSTF-containing assembly consisted of the WICH complex associated with several other nuclear proteins in a novel constellation, designated B-WICH.

We immunoprecipitated WSTF-containing complexes from whole cell extracts prepared at 0.7 M KCl to verify the interactions between these proteins and WSTF. We detected the ISWI protein, RNA helicase II/Guα, Dek, the Myb-bp1a, the Sf3b155/SAP155, and CSB in the immunoprecipitations using the C-terminal WSTF-antibody (Fig. 2*E*). Only minor fractions of the CSB, Sf3b155/SAP155, and Dek were immunoprecipitated by the WSTF antibody, suggesting that these proteins also exist in other complexes or particles. Other proteins that interact with WSTF, such as BRG1, p150 CAF-1, or PCNA, were not detected in the immunoprecipitation nor was the ISWI-interacting protein Acf1 (Fig. 2*E*). Because many of the interacting proteins were involved in RNA processing during transcription, in particular during RNA pol I transcription, we also examined WSTF immunoprecipitates for the presence of RNA pol I and NM1. Of these, NM1, which is involved in both RNA pol I and RNA pol II transcription, interacted with the WSTF assembly under native conditions (Fig. 2*E*).

Interactions in the B-WICH Depend on RNA and Active Transcription—We subsequently examined whether DNA mediated the interactions in the high molecular weight WSTF assembly by treating 0.7 M KCl nuclear extract with ethidium bromide or DNase I. The high molecular weight WSTF peak remained after both ethidium bromide treatment (Fig. 3*A*, *EtBr*) and after DNase I digestion (not shown), demonstrating that different proteins assembling on DNA did not form the high molecular weight WSTF assembly. Treatment of HeLa cell nuclear extract by RNase A, however, revealed that RNA mediated interactions in B-WICH. The large molecular weight WSTF peak disappeared, and only one WSTF peak at a molecular mass of 700 kDa to 1 MDa remained in the RNase A-treated nuclear extract when fractionated on a Superose 6 HR column (Fig. 3*A*, *RNase*). The ISWI protein eluted in a peak at a molecular mass of 2–3 MDa and in a broad peak at 700 kDa, after the RNase A treatment. We next examined whether the formation of B-WICH depends upon active transcription, because RNA mediated some of the interactions in the B-WICH assembly. HeLa cells were treated with 1 μg/ml actinomycin D before nuclear extracts were prepared. In the subsequent Superose 6HR fractionation, only one WSTF peak was present, with a molecular weight that was the same as that of

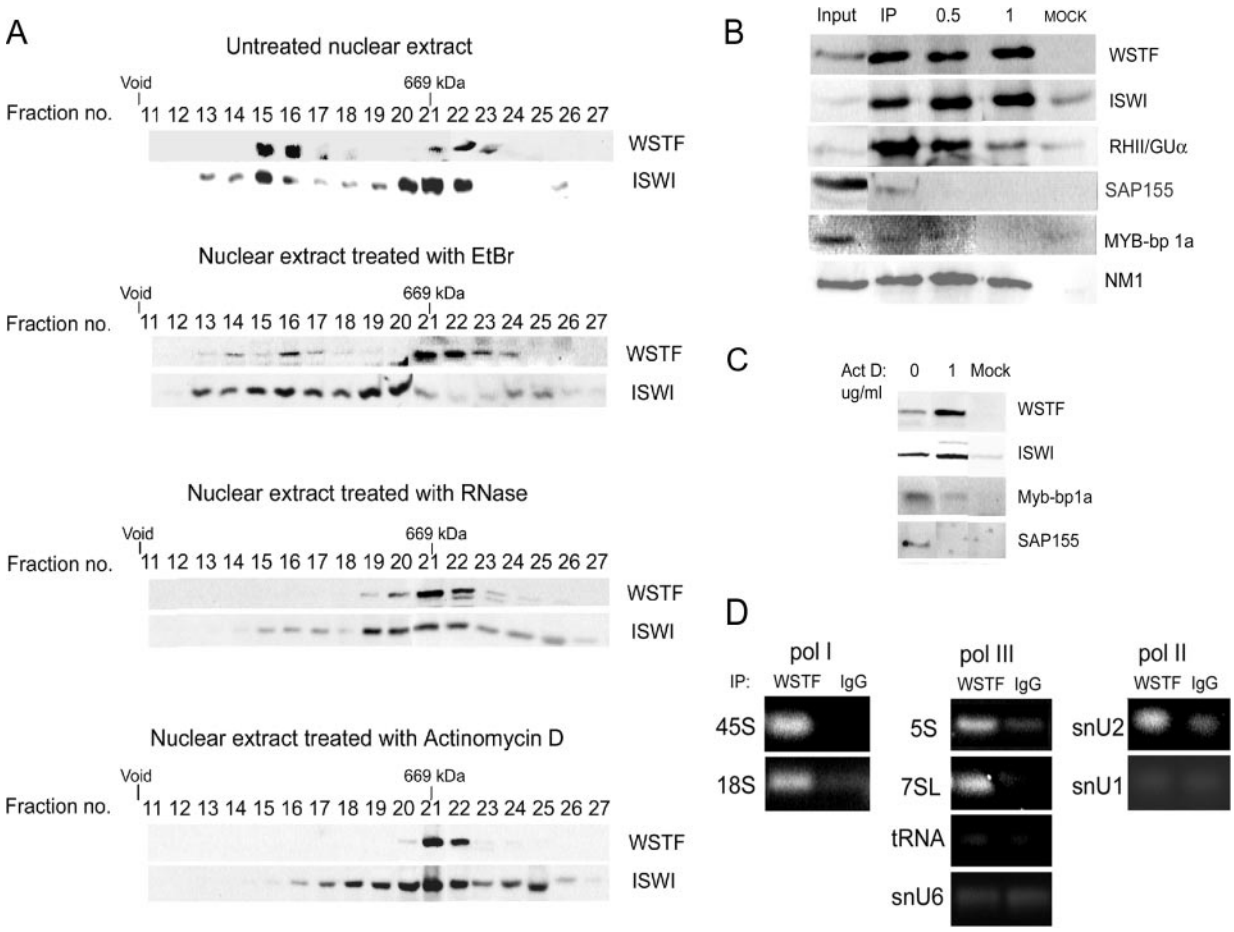

FIGURE 3. **The B-WICH complex is sensitive to RNase A and active transcription.** *A,* the size distribution of WSTF protein in untreated crude nuclear extract prepared at 0.7 M KCl (2 mg) was compared with that found in extracts treated with ethidium bromide (*EtBr*), RNase A (0.33 mg/ml) (*RNase*), or in extracts from cells treated with actinomycin D (1 μg/ml) for 30 min (*Actinomycin D*). Immunoblots of the fractions from a Superose 6HR size exclusion column show WSTF and ISWI as indicated on the *right*. The fraction number, the void volume, and the 669-kDa size marker are marked on the *top*. *B,* immunoprecipitations of the WSTF protein and WSTF-interacting proteins in untreated 0.7 M KCl nuclear extracts and extracts treated with RNase A, 0.5 and 1 mg/ml, as indicated *above* the lanes, for 20 min. The proteins in the precipitates were separated on SDS-PAGE (7%), transferred to an Immobilon membrane, and probed with antibodies against WSTF, ISWI, RNA helicase II/GUα (RHII/Gu), Sf3b155/SAP155, Myb-bp1a, and NM1as indicated on the *right*. 1/10 of the input is marked as *Input*, *IP* is the immunoprecipitate, and the nuclear extract incubated with only protein A/G-Sepharose beads is marked *Mock*. *C,* immunoprecipitation of WSTF and WSTF-associated proteins from cells treated with 1 μg/ml actinomycin D (*Act D*) for 30 min. The membrane was probed with antibodies against WSTF, ISWI, Myb-bp1a, Sf3b155 and SAP155, as indicated on the *right*. No antibody is marked *Mock*, and the treatments are indicated *above* the lanes. *D,* the C-terminal WSTF antibody was used to immunoprecipitate the high molecular weight assembly from nuclear extracts from growing HeLa cells. RNA was prepared from the precipitate, and the cDNA was used in PCR with specific primers for the 5′-external transcribed spacer and 18 S of the 45 S rRNA, the 5 S rRNA, tRNA, 7SL RNA, U6 snRNA, U2 snRNA, and U1 snRNA, as indicated to the *left* of the *panels*. RNAs immunoprecipitate from nuclear extracts exposed to unspecific IgG was used as control (*IgG*). The RNA polymerase responsible for the transcription is indicated at the *top*.

the WICH complex, at 900 kDa to 1 MDa (Fig. 3*A*, *actinomycin D*). The ISWI eluted in a broad peak from 500 kDa to 2 MDa.

The Interactions with RNA Helicase II/Guα, Sf3b155/SAP155, and Myb-bp1a Are RNA-sensitive—We examined the different interactions in B-WICH in more detail by immunoprecipitating WSTF-containing complexes from 0.7 M nuclear extract from HeLa cells that had been treated with RNase A (0.5 and 1 mg/ml for 20 min at room temperature). The interactions between the WSTF protein, the ISWI protein, and NM1 were unaffected by RNase A treatment (Fig. 3*B*) demonstrating that these proteins associate by protein-protein interactions. On the other hand, the interactions with RNA helicase II/Guα, Sf3b155/SAP155, and Myb-bp1a were disrupted by the RNase A treatment (Fig. 3*B*). The RNA helicase II/Gu required a concentration higher than 0.5 mg/ml RNase A to dissociate from the WSTF, whereas the Sf3b155/SAP155 and the Myb-bp1a dissociated at a concentration of 0.5 mg/ml.

The Interactions with Myb-bp1 and Sf3b155/SAP155 Depend on Transcription—In order to investigate whether interaction partners in the B-WICH assembly required transcription to assemble, WSTF complexes were immunoprecipitated from nuclear extracts from cells treated with 1 μg/ml actinomycin D. The interaction with ISWI was not affected by actinomycin D, again confirming that this is the core in the WSTF assembly. Immunoblots of the proteins in the precipitate revealed that the associations with Myb-bp1 and Sf3b155/SAP155 depended upon active transcription (Fig. 3*C*), because lower amounts of these proteins were found in immunoprecipitates from nuclear extracts of cells treated with actinomycin D.

The B-WICH Complex Contains RNA pol I and III Transcripts—The identity of the RNA species in B-WICH was determined by preparing RNA from immunoprecipitated B-WICH from HeLa cell nuclear extract. Because some of the proteins in the B-WICH assembly, such as RNA helicase II/Guα, play a role in nucleolar rRNA synthesis or processing, we used primers in the PCR for different parts of the RNA pol I 45 S transcript. The 5′-external transcribed spacer and the 18 S were found in the precipitate, indicating that the whole 45 S precursor transcript was a part of B-WICH (Fig. 3*D*, *pol I* transcripts). In addition, two RNA pol III transcripts, 5 S rRNA and the 7SL RNA, were enriched in the WSTF precipitate, whereas two other types of RNA pol III transcript, tRNA and U6 snRNA, were not (Fig. 3*D*, *pol III* transcripts). We also examined the B-WICH precipitate for two abundant RNA pol II RNAs, the U1 snRNA and the U2 snRNA. U1 snRNA was not enriched

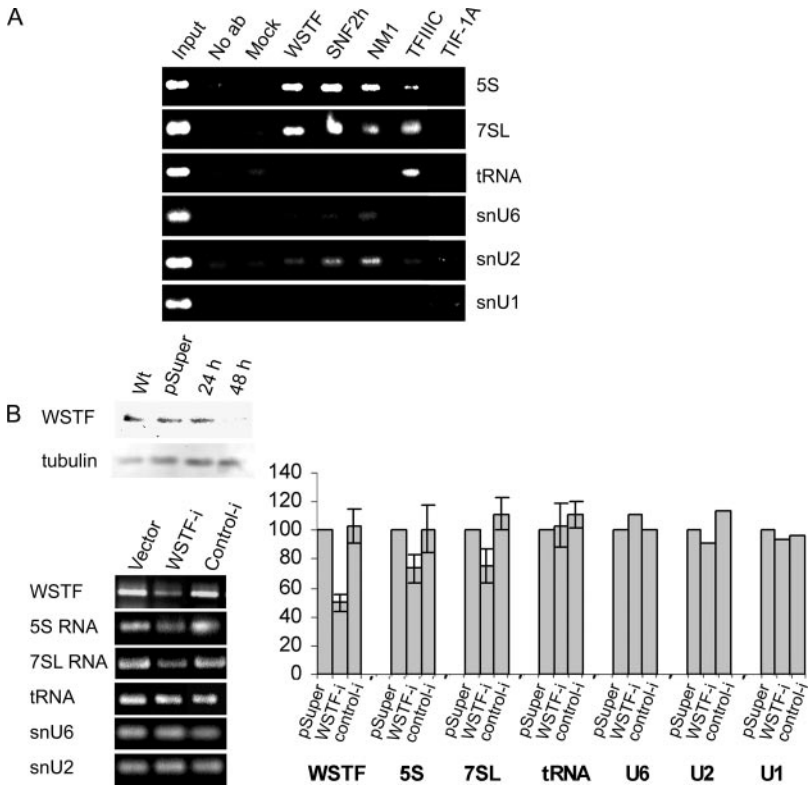

FIGURE 4. **The WSTF, SNF2h, and NM1 are at the 5 S and 7SL genes, and reduced levels of WSTF reduce the level of 5 S rRNA and 7SL RNA.** *A,* ChIP of the WSTF, SNF2h, NM1, TFIIIC220, and TIF-1A, as indicated *above* the lanes, at RNA pol III and RNA pol II snRNA genes. The genes, 5 S rRNA, 7SL RNA, tRNA, U6 snRNA, U2 snRNA, and U1 snRNA, are marked to the *right* of the lanes. No antibody (*No ab*) and unspecific IgG were used as control, and *input* is 0.5% of the input chromatin. *B,* WSTF was silenced by expression of shRNA from the pSuper vector in HeLa. Immunoblot of the WSTF protein level at different times, indicated *above* the lanes, in total cell lysate (50 μg/lane) shows that a highly reduced level is obtained after 48 h. The α-tubulin was used as a loading control. To determine the effect on different RNAs, total RNA was prepared 36 h after transfection and transferred into cDNA, and the levels investigated by PCR with specific primers for WSTF, 5 S rRNA, 7SL RNA, tRNA, snU6, snU2, and SnU1 are as indicated to the *right*. The transfection efficiency was estimated to 50%. The lane with RNA from cells transfected only with the pSuper vector is marked *pSuper*, and *WSTF-i* is RNA from cells transfected with pSuper-shWSTF, and *control* is RNA from cells transfected with vector expressing an unrelated shRNA. Quantification of the RNA levels is presented in the graph and expressed as % of the RNA level in cells transfected with only pSuper in each experiment with standard deviation. The number of independent experiments are as follows: WSTF $n = 3$; 5 S $n = 5$; 7SL $n = 4$; tRNA $n = 4$; and for U6, U2, U1 $n = 2$.

in the WSTF precipitate, although traces of the U2 snRNA were present (Fig. 3*D*, *pol II* transcripts). Immunoprecipitations of fractions of the low molecular weight and the high molecular weight (B-WICH) WSTF peaks demonstrated that the RNAs were only present in the B-WICH assembly, and the low molecular weight peak was devoid of RNA (not shown).

The WSTF, SNF2h, and NM1 Proteins Are Present at RNA pol III Genes—Because RNA pol III transcripts were found in the B-WICH assembly, we examined the occupancy of the WSTF, SNF2h, and NM1 at the four types of RNA pol III gene as follows: the 5 S rRNA gene, the 7SL RNA gene, tRNA gene, and U6 gene. WSTF, SNF2h, and NM1 were associated with the genes for 5 S rRNA and 7SL RNA but not with the genes for $tRNA^{Leu}$, the active U6-1 (Fig. 4*A*), or the inactive U6-4 (not shown). Two other tRNA genes were investigated, the genes for $tRNA^{Lys}$ and $tRNA^{Tyr}$, but no WSTF, SNF2h or NM1 was present on these genes. The RNA pol III transcription factor TFIIIC220 in TFIIIC2 was found at the 5 S rRNA gene, the 7SL RNA gene, and the tRNA genes (Fig. 4*A*). TFIIIC220 is not as tightly linked to transcription of the U6 snRNA genes (41) and was not detected at this gene. In addition, we performed ChIP on the U2 snRNA and U1 snRNA genes, both of which are transcribed by RNA pol II. None of the proteins WSTF, SNF2h, or NM1 was associated with the U1 snRNA gene, but low levels of the proteins were consistently found associated with the U2 snRNA gene (Fig. 4*A*).

Reducing the Level of the WSTF Protein Reduces the Levels of 5 S rRNA and 7SL RNA—Next, we investigated whether the WSTFs are involved in RNA pol III transcription by silencing of the WSTF protein using shRNA from the pSUPER vector for 36 h. The protein level of WSTF decreased after 24 h and was highly reduced 48 h after transcription (Fig. 4*B*). However, after longer time periods, cells changed morphology and lost their contact with the surface. Therefore, samples were harvested after 36 h. Even after this short time of exposure to shWSTF, the cellular levels of 5 S rRNA and 7SL RNA were reduced by ~25% (Fig. 4*B*). Control cells were transfected with either only the pSuper vector or pSuper expressing an unrelated control shRNA, neither affecting the levels of 5 S rRNA and 7 SL RNA in cells (Fig. 4*B*). The levels of the $tRNA^{Tyr}$, U6 snRNA, U2 snRNA, and U1 snRNA were not reduced in cells expressing the shWSTF (Fig. 4*B*).

DISCUSSION

Here we show that an ATP-dependent chromatin remodeling assembly consisting of WSTF-ISWI is involved in RNA pol III transcription. We have isolated the WSTF-SNF2h complex associated with several nuclear proteins and RNAs in an assembly denoted B-WICH. The WSTF protein has been purified previously as part of two distinct types of ATP-dependent chromatin remodeling complex: associated with BRG1- and BRM-containing SWI/SNF complexes in the WINAC (10), and associated with only the SNF2h protein in WICH (6). We used a KCl concentration higher than that used when purifying either the WICH or the WINAC when preparing the initial nuclear extract. Many nuclear proteins are not extracted from chromatin and other nuclear structures at 0.42 M KCl, and higher salt concentrations are needed for their release. The hyperphosphorylated RNA pol II requires high NaCl concentrations (31), whereas many histone deacetylases require latrunculin treatment of the cells before they are released (42). Higher KCl concentrations than 0.5 M are required to release the major part of the WSTF, a fact that suggests that it is tightly associated with the chromatin fraction. The 0.7 M KCl concentration used during nuclear extraction allowed other proteins connected to WSTF-SNF2h to be released from chromatin. PCNA, which recruits WICH to replication foci (8), was not present in this extracted assembly. PCNA interacts with the C-terminal part of WSTF, and the final step in our purification of the B-WICH assembly was an immunoprecipitation using an antibody against the C-terminal part of WSTF. Thus, the B-WICH may represent a different pool of WSTF-SNF2h than the pool that functions in replication.

The proteins identified as associating partners with the WSTF-ISWI assembly have been ascribed functions in different nuclear processes, and they are present in different nuclear compartments. Four of the proteins are found in the nucleolus, the Myb-bp1a (22, 43), the RNA helicase II/Guα (25), the CSB (44), and NM1 and may play a role in rRNA processing. Despite the localization of the Myb-bp1a, it has mainly been associated with the regulation of RNA pol II transcription (23, 45). Its orthologue in yeast, Pol5, however, plays a major role in rRNA processing (46, 47). The CSB protein is involved in several nuclear processes, mainly in nuclear excision repair and transcription-coupled repair (27) and in RNA pol I transcription (44). Two other proteins involved in RNA processes were identified in the large B-WICH assembly as follows: Sf3b155/SAP155, which is part of two mRNA splice factors, the major U2 small ribonucleoprotein and the minor U11/U12 small ribonucleoprotein heterodimer (24, 48), and the proto-oncogene Dek, a chromatin protein involved in transcription (49) and found at exon-exon junctions (27).

The formation of the high molecular weight B-WICH assembly required active transcription, with the WSTF and SNF2h forming a core that was independent of ongoing transcription. We identified three of the interacting proteins, Myb-bp1a, Sf3b155/SAP155, and NM1, whose interactions with the B-WICH were reduced or lost when transcription was inhibited. In addition to proteins, the assembly contained the 45 S rRNA, 5 S rRNA, and 7SL RNA, suggesting that the B-WICH has a function in RNA pol I and RNA pol III transcription. At least three of the interacting proteins were associated through RNA as follows: RNA helicase II/GUα, Myb-bp1a, and Sf3b155/SAP155. It is tempting to speculate that these proteins associate with the B-WICH core via the growing RNA during transcriptional elongation. Some of the proteins, such as the RNA helicase II/GUα and the Myb-bp1a, are nucleolar and may associate via the 45 S rRNA, indicating a role for WSTF-SNF2h in rRNA synthesis. The presence of NM1 in B-WICH further supports the idea that WSTF-SNF2h plays a role in RNA pol I transcription. NM1 associates with the nucleolar rRNA gene where it acts as a molecular switch and constitutes a link with RNA pol I via TIF 1A and actin (32, 35). By associating with the WSTF-SNF2h assembly, NM1 connects chromatin remodeling to the RNA pol I machinery at transcriptional initiation and elongation of the 45 S rRNA genes (50). The B-WICH assembly also contained traces of U2 snRNA, the RNA to which Sf3b155/SAP155 binds. However, we cannot determine whether the presence of Sf3b155/SAP155 and the U2 RNA in B-WICH reflects an interaction of the U2 splice factor or is because of an association during transcription of the U2 snRNA gene. It is possible that all proteins identified that interact with B-WICH do not associate simultaneously with WSTF-SNF2h, and that the B-WICH assembly represents a number of constellations of proteins with WSTF-SNF2h as a core. The nuclear localization varies between the identified proteins as do the processes involved. Furthermore, it is unclear how the formations of the different WSTF complexes, such as WINAC, WICH, and B-WICH, are regulated. The localization of the WSTF protein in the nucleus, the state of the cell, the cell cycle phase, and the processes initiated may be factors that determine which protein it is that assembles.

The presence of the 5 S rRNA and the 7SL RNA in the B-WICH assembly suggested that it also was involved in RNA pol III transcription. We found the WSTF-SNF2h-NM1 proteins associated with the RNA pol III genes 5 S rRNA and 7SL RNA. The occupancies of the proteins were specific for these genes, and they were not found on tRNA genes or U6 snRNA genes. In addition, post-transcriptional silencing of WSTF reduced the levels of 5 S rRNA and 7SL RNA specifically, further underscoring a role in RNA pol III transcription. RNA pol III genes have been classified into four types based on promoter organization and factor requirement. The 5 S rRNA gene and the 7SL gene are of different types; the 5 S rRNA gene has an internal promoter that requires TFIIIA, TFIIIC, and TFIIIB, whereas the 7SL gene has both an internal promoter that requires TFIIIC and TFIIIB and external upstream elements that include a TATA box-like element (for review see Ref. 21). The other two types are tRNA genes, which have an internal promoter that requires TFIIIC and TFIIIB, and U6 snRNA genes/7SK genes, which have an external promoter that uses TFIIIB. Regulatory factors of RNA pol III transcription, such as the proto-oncogene c-Myc, the retinoblastoma protein, and p53 (51), are all recruited to the genes by TFIIIB, the RNA pol III-specific factor that contains TATA-box binding protein (51). Given the presence of the WSTF, SNF2h, and NM1 proteins at only the 5 S rRNA genes and 7SL genes, the TFIIIB does not provide the necessary specificity. Instead, the underlying chromatin architecture at the different RNA pol III genes may be a factor that determines the requirement for ATP-dependent chromatin remodeling. In the 5 S promoter, TFIIIA binding is increased by histone tail modification, and it is helped by the intrinsic histone acetyltransferase activity of TFIIIC2 *in vitro* (52). Earlier reports show, however, that ATP is required for full transcription of the gene (53). The nucleosome architecture at the U6 snRNA gene group is different from the architecture at the 5 S gene, with a positioned nucleosome upstream of the gene during active transcription (54, 55). The WSTF-SNF2h-NM1 assembly may be required when a nucleosome is positioned over the gene, as is the case for the 5 S gene, and may allow for histone modifications at these sites. Because of its architecture, the U6 gene may not require the WSTF-SNF2h-NM1 activity. We propose that the function of a chromatin remodeling complex at the 5 S RNA and 7SL RNA pol III genes alters the nucleosome pattern over the region where these genes are located, to give a basal transcription level. We cannot exclude, however, that redundant chromatin remodeling complexes exist in RNA pol III transcription because the effect on the levels of 5 SrRNA and 7SL RNA by post-trancriptional silencing of WSTF is not complete.

The activities of RNA pol I and RNA pol III are closely coupled, and coordination of the two RNA polymerases is essential for balancing RNAs involved in translation. c-Myc activates both RNA pol I genes and RNA pol III genes, suggesting that it plays a role in the coordination of the growth response (56, 57). WSTF, SNF2h, and NM1 can act in a similar manner, as these proteins are found both at the nucleolar 45 S rDNA (50), at the 5 S rRNA, and at the 7SL RNA and thereby coordinate protein synthesis and trafficking. In contrast to c-Myc, the WSTF, SNF2h, and NM1 proteins are not present at tRNA genes nor is the level of tRNA altered in cells expressing shRNA-WSTF. tRNA genes are ISWI target genes in yeast, recruited by a subunit of the RNA pol III transcription factor TFIIIB, Bdp1 (58). However, the yeast Isw2p protein complex is required for the integration of the yeast transposon Ty1 at certain tRNA genes and does not affect the tRNA transcription (59).

Kitagawa *et al.* (10) have proposed that WSTF acts as a platform for other proteins, either SWI/SNF complexes or the ISWI protein, depending on the chromatin environment and the process activated. The WSTF-SNF2h complex is now shown to also act as a platform, with chromatin remodeling activity; it operates with PCNA in replication, and it associates with NM1 and several other nuclear proteins during active RNA pol I and RNA pol III transcription.

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