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Nucleolin Is Required for RNA Polymerase I Transcription In Vivo▽

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Eukaryotic genomes are packaged with histones and accessory proteins in the form of chromatin. RNA polymerases and their accessory proteins are sufficient for transcription of naked DNA, but not of chromatin, templates in vitro. In this study, we purified and identified nucleolin as a protein that allows RNA polymerase II to transcribe nucleosomal templates in vitro. As immunofluorescence confirmed that nucleolin localizes primarily to nucleoli with RNA polymerase I, we demonstrated that nucleolin allows RNA polymerase I transcription of chromatin templates in vitro. The results of chromatin immunoprecipitation experiments established that nucleolin is associated with chromatin containing rRNA genes transcribed by RNA polymerase I but not with genes transcribed by RNA polymerase II or III. Knockdown of nucleolin by RNA interference resulted in specific inhibition of RNA polymerase I transcription. We therefore propose that an important function of nucleolin is to permit RNA polymerase I to transcribe nucleolar chromatin.

Eukaryotic cells contain three nuclear enzymes that transcribe DNA, RNA polymerases I, II, and III, which are responsible for transcription of rRNA genes, all protein coding genes, and genes encoding various small RNAs, respectively. Although these are large, multisubunit enzymes, none alone is capable of specific initiation of transcription. Rather, initiation from their cognate promoters requires the participation of polymerase-dedicated initiation proteins such as UBF and SL1 in the case of RNA polymerase I and the six general transcription factors (GTFs) for RNA polymerase II (see references 25, 56, 60, 67, 70, and 75 for reviews). The biochemical studies that led to the identification of the RNA polymerases and these accessory proteins focused on initiation and used naked templates. However, in eukaryotes, genomic DNA is packaged with histones and non-histone-associated proteins in the form of chromatin. Consequently, RNA polymerases must negotiate chromatin during transcription in vivo.

The basic repeating unit of chromatin, the nucleosome, comprises 147 bp of DNA wrapped around a histone octamer consisting of two copies of the core histones H2A, H2B, H3, and H4 (46). The majority of nucleosomes in mammalian cells are periodically spaced, with an average repeat length of 190 bp (54). In the presence of linker histones such as histone H1, nucleosomal arrays form the more compact 30-nm fiber, in which nucleosomes are stacked in a helical arrangement (87). The 30-nm chromatin fiber can be further condensed to form higher-order structures, a property that allows the storage of large DNA genomes in an organized manner. However, such condensation reduces the access of transcription machines to DNA templates. Consequently, actively transcribed genes are generally associated with less-condensed chromatin that is devoid of linker histones and contains histones carrying specific modifications (65, 81).

Regions of transcriptionally active chromatin are enriched in histones that are posttranslationally modified by the covalent addition of acetyl groups to specific lysine residues (27, 28). Several histone acetyltransferases, such as GCN5, PCAF, and Tip60, have been found to be components of multiprotein transcriptional activators (62). Conversely, histone deacetylases, which remove acetyl groups from histones, have been shown to participate in the repression of transcription and several have been identified as subunits of transcriptional corepressors (29, 39, 52, 90, 91). Another common histone modification that regulates access to chromatin templates is lysine methylation (37, 38, 80). Depending on the specific lysine residues that are methylated, this modification can either repress or activate transcription (49). ATP-dependent chromatin remodeling enzymes form another class of proteins that manipulate the structure of chromatin. The first to be identified, the SWI/SNF complex of *Saccharomyces cerevisiae*, has been directly implicated in the transcription of some genes (11, 17, 32, 36). Biochemical studies have established that members of the SWI/SNF family use ATP hydrolysis to alter the conformation of histones within a nucleosome (32, 36, 42, 74). Members of the ISWI family, which all possess a homolog of the *Drosophila* ISWI protein, have been identified in many organisms and use energy from ATP hydrolysis to slide nucleosomes translationally along DNA (33, 40, 41, 63, 84).

In vitro, RNA polymerase II requires only the GTFs to initiate and elongate transcription from a naked DNA template. However, these components are incapable of transcribing a DNA template that has been assembled into chromatin (59). This observation led to the discovery of the heterodimeric protein FACT (*fa*cilitates *c*hromatin *t*ranscription), which permits RNA polymerase II to elongate transcription through nucleosomes (57). FACT is composed of a human homologue of *S. cerevisiae* protein Spt16 and HMG1-like protein SSRP1. Biochemical studies have demonstrated that FACT interacts specifically with histones H2A and H2B (4, 58). The Spt16 subunit possesses histone chaperone activity that is thought to destabilize the nucleosome and allow transient displacement of

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H2A and H2B during RNA polymerase II elongation (4). It has not yet been firmly established whether FACT is the only mammalian protein that allows chromatin transcription by RNA polymerase II or whether FACT functions with the other RNA polymerases. To address such issues, we set out to identify additional proteins that stimulate transcription through nucleosomes in the presence of limiting concentrations of FACT. Here we report the purification and identification one such protein, nucleolin, which we demonstrate is essential for RNA polymerase I transcription in vivo.

MATERIALS AND METHODS

Purification of proteins required for RNA polymerase II transcription and chromatin assembly. The purification of RNA polymerase II and transcription factor IIH (TFIIH) from mammalian cell nuclear extract and of TATA-binding protein (TBP), TFIIB, TFIIE, and TFIIF synthesized in *Escherichia coli* was done as described previously (47). Core histones, RSF, and FACT were purified from HeLa cell nuclei by following previously described protocols (40, 57, 79). A chimeric activator (GAL4-AD) composed of a FLAG-tagged GAL4 DNA-binding domain fused to the activation domain of c-Myc was synthesized in Hi-5 insect cells and purified by immunoaffinity chromatography as described previously (50).

In vitro chromatin assembly. RSF-mediated chromatin assembly was performed essentially as described previously (44, 45), typically by using 4 μg of template DNA and empirically optimized ratios of core histones to DNA. Assembly was monitored by limited digestion with micrococcal nuclease (Sigma), and the DNA products were purified and examined by agarose gel electrophoresis (44). Chromatin assembled in this way was typically frozen at −80°C until use.

In vitro transcription by RNA polymerase II. The pG5MLP plasmid contains five binding sites for the yeast GAL4 protein fused to the adenovirus major late promoter and a G-less cassette (71). Transcription reaction mixtures were prepared as described previously (15, 57) and contained 200 ng of pG5MLP naked or assembled into chromatin as indicated, 10 ng of TBP, 10 ng of TFIIB, 10 ng of TFIIF, 50 ng of TFIIE, 150 ng of TFIIH, and 100 ng of RNA polymerase II. For transcription of chromatin templates, reaction mixtures also contained various concentrations of FACT, as indicated (0.25× is 375 ng, 0.5× is 750 ng, 1× is 1.5 μg, 2× is 3 μg, and 3× is 4.5 μg of FACT, respectively). Reaction mixtures containing nucleolin contained 1.2 μg of purified protein. Following incubation at 30°C for 1 h, transcription products were digested with 50 U of RNase T1 (Roche) for 30 min at 30°C. The RNA was then purified and analyzed by electrophoresis in 6% polyacrylamide gels containing 8 M urea, cast, and run in 0.09 M Tris-borate, pH 7.5, containing 2 mM EDTA (1× TBE). The gels were dried and subjected to autoradiography at −80°C with intensifying screens.

Immunoblotting. Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon (Millipore Incorporated). The membranes were blocked by incubation in 20 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 0.5% (vol/vol) Tween 20 (TBST), and 10% (wt/vol) nonfat milk for 1 h at 25°C. Antibodies used in this study were anti-FLAG M2 (Sigma), anti-hSpt16 (Transduction Laboratories), antinucleolin (Stressgen), anti-β-actin (Abcam), and anti-mouse secondary antibodies conjugated to horseradish peroxidase (Promega). Antibody incubations were performed with TBST containing 1% (wt/vol) milk, and the blots were developed by enhanced chemiluminescence (Amersham Biosciences).

Nucleolin purification. Human nuclear extract (800 mg of protein) was prepared from HeLa cells grown in suspension culture as described previously (12). The extract was dialyzed into 20 mM Tris-HCl, pH 7.9, containing 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20% (vol/vol) glycerol (BC buffer), and 50 mM KCl (BC50). The extract was applied to a DEAE-cellulose column (DE52; Whatman), and bound proteins were eluted with BC500, dialyzed into BC100, and loaded onto a phosphocellulose column (P11; Whatman). The P11 column was eluted sequentially with BC buffer containing 0.35, 0.65, and 1.0 M KCl. The flowthrough fraction was dialyzed into BC50 and loaded onto an SP-Sepharose column (Pharmacia). This column was eluted sequentially with BC buffer containing 0.1, 0.2, 0.3, 0.5, and 1 M KCl. The peak activity was detected in the 0.2 M eluate, which was dialyzed, loaded onto a DEAE-5PW column (TosoHaas), and eluted with a linear gradient of 50 mM KCl to 0.7 M $(NH_4)_2SO_4$ in BC buffer. The chromatin-specific stimulatory activity eluted at ~300 mM $(NH_4)_2SO_4$. The active fractions were pooled and adjusted to 60% saturation with $(NH_4)_2SO_4$. The soluble fraction was dialyzed into BC50, and the proteins were concentrated by chromatography on a DE52 column. They were then fractionated on a Superdex 200 (Pharmacia) gel filtration column run in BC100. The active fractions eluted with an apparent molecular mass of 125 kDa. The stimulatory activity present in the 0.35 M KCl P11 fraction was dialyzed into BC50 and subjected to chromatography on SP-Sepharose and DEAE-5PW columns exactly as described above. The active fractions from the DEAE-5PW column [eluting at ~0.3 M $(NH_4)_2SO_4$] were pooled and subjected to gel filtration with a Superose 6 column (Pharmacia). The activity eluted with an apparent molecular mass of 125 kDa. The 125-kDa proteins from both purifications were excised from SDS-polyacrylamide gels and subjected to mass spectrometry by ProtTech Incorporated.

Nucleolin immunodepletion. The antinucleolin antibody described previously or the B6 monoclonal antibody against an adenoviral protein (64) was bound to protein A/G beads (Santa Cruz Biotechnology, Inc.) that had been preblocked by incubation with 1 μg/ml bovine serum albumen by rotation at 4°C overnight. The antibody-coupled beads, as well as preblocked beads alone, were washed with phosphate-buffered saline, added to purified nucleolin that had been dialyzed into BC100 without β-mercaptoethanol, and rotated at 4°C for 2 h. The supernatants from the immunodepletions were collected and assayed for transcriptional activity and by immunoblotting as described above.

Production of FLAG-nucleolin. Nucleolin cDNA (26), a kind gift of Les Hanakahi, Johns Hopkins University, was subcloned into a CβF expression vector that includes the cytomegalovirus promoter and an amino-terminal FLAG tag (53). This vector was introduced into 293 cells by the calcium phosphate precipitation method, and nuclei were prepared 24 h later by hypotonic lysis in 10 mM Tris-HCl, pH 7.8, containing 1.5 mM $MgCl_2$, 0.25 M sucrose, and 0.5 mM PMSF (10). Nuclei were collected by centrifugation at 3,000 rpm and lysed by Dounce homogenization in BC buffer containing 0.5 M NaCl without β-mercaptoethanol. The extract was cleared by centrifugation at 13,000 rpm for 15 min and applied to anti-FLAG M2 resin (Sigma) overnight at 4°C with rotation. The beads were washed with the same buffer, and proteins were eluted by competition with FLAG peptide in BC buffer containing 50 mM NaCl without β-mercaptoethanol for 12 h at 4°C. The eluted proteins were analyzed by SDS-PAGE and stored at −80°C.

Immunofluorescence. HeLa cells were grown on glass slides, fixed, and probed as described previously (23, 55). Nucleolin, RNA polymerase I (RPA194 subunit), TFIIF (RAP 30 subunit), and RNA polymerase II were detected with rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc.) and Cy5-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). The DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI) by the standard protocol. Stained cells were examined by confocal microscopy with a Zeiss LSM 510 confocal microscope with a 40× water immersion C-Apochromat objective.

In vitro RNA polymerase I transcription assays. RNA polymerase I and SL-1 were purified from HeLa cell nuclear extracts by combining previously described protocols (16, 51, 66). For RNA polymerase I, HeLa cell nuclear extracts were prepared as described previously (8) and fractionated over DEAE-5PW (TosoHaas), CM-Sepharose (Pharmacia), heparin-Sepharose HiTrap (Pharmacia), and Superose 6 (Pharmacia). Purification of RNA polymerase I was followed by immunoblotting for RPA194, the largest subunit, with a rabbit polyclonal antibody (Santa Cruz) and by nonspecific transcription (66). SL-1 was purified from HeLa cell nuclear extract by chromatography on DEAE-cellulose (Whatman), heparin-agarose (Sigma), SP-Sepharose, and Superdex 200. Its purification was monitored by immunoblotting for TBP with an anti-TBP monoclonal antibody (9). The FLAG-UBF1 expression plasmid was a kind gift from Ingrid Grummt and Renate Voit. FLAG-UBF1 protein was synthesized in 293 cells and purified by immunoaffinity chromatography.

The template for transcription was the plasmid $pHrP_2$, which contains the human rRNA gene from positions −411 to +375. Transcription reaction mixtures contained 200 ng of $pHrP_2$, either naked or nucleosome assembled by RSF; 150 ng of purified RNA polymerase I; 30 ng of SL-1; 20 ng of FLAG-UBF-1; 3 mM each ATP, CTP, UTP, and GTP; 12 U of RNasin (Promega); 10 mM HEPES-KOH, pH 7.9; 50 mM KCl; 6 mM $MgCl_2$; 1.25% (wt/vol) PEG 8000; and FACT (1.5 μg) or nucleolin (1.2 μg) as indicated. Reaction mixtures were incubated for 1 h at 30°C and treated with 20 U of RNase-free DNase I (Roche Diagnostics) for 1 h at 37°C, and reactions were stopped with 20 mM EDTA, pH 7.5, containing 0.2 M NaCl, 1% SDS (wt/vol), and 25 μg of glycogen. The RNA was deproteinized by sequential extractions with phenol and Trizol (Invitrogen), precipitated with 2-propanol, washed with 70% ethanol, and resuspended in 20 μl of H_2O. The RNA transcripts (1 μl) were amplified and labeled by One-Step reverse transcription (RT)-PCR (Roche Diagnostics) (16 cycles) in reaction mixtures containing [α-^{32}P]dATP at 3 μCi/reaction mixture (3,000 Ci/mmol; Perkin-Elmer) by following the manufacturer's procedures. The 5′ primer corresponded to positions +1 to +22 of the rRNA gene (GCTGACACGCTGTC

CTCTGGCG), and the 3′ primer was complementary to positions +251 to +270 (CGCCCCGGGCGCCCGCAGCG). The PCR products (one-fifth of the final reaction mixture) were separated by electrophoresis in 6% polyacrylamide gels cast and run in 0.25× TBE and visualized by autoradiography.

Immunoprecipitation of nucleolin-associated histones. Nuclei were purified from 293 cells synthesizing nucleolin carrying an N-terminal FLAG tag as described in a previous section. They were then resuspended in 10 mM Tris-HCl, pH 7.5, containing 10 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.1 mM PMSF, and chromatin was released from the nuclei by limited digestion with micrococcal nuclease (Sigma) at 37°C for 10 min (83). The digestion was stopped by addition of EDTA to 25 mM. Debris was removed by centrifugation, and the soluble chromatin was subjected to immunoprecipitation with M2 FLAG beads (Sigma) as described above. The eluted proteins were resolved by electrophoresis in 17% polyacrylamide gels containing 1% (wt/vol) SDS and visualized by Coomassie blue staining. Nucleophosmin, another nucleolar protein, was identified by mass spectrometry as described in a previous section.

Chromatin immunoprecipitation. Proteins were cross-linked to the DNA in HeLa cells in situ by the addition of formaldehyde to 1% (vol/vol) to the culture medium, with gentle rocking for 10 min at 25°C. Glycine was added to 0.125 M, and the cells were rocked for 5 min to stop the reaction. Cells were then washed with phosphate-buffered saline, and the nuclei were isolated by NP-40 extraction as described previously (22, 31). Nuclei were resuspended and subjected to limiting micrococcal nuclease digestion as described above. Six packed cell volumes of 10 mM Tris-HCl, pH 7.05, containing 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 μM $ZnCl_2$, 10% (vol/vol) glycerol, 0.1 mM PMSF, and 0.1% (vol/vol) Triton X-100 were added to ensure release of DNA. Following sonication for 20 s to produce DNA fragments of 500 to 2,000 bp, the insoluble debris was removed by centrifugation. The lysates were precleared by rotation with protein A/G beads that had been blocked by incubation with 1 μg/ml bovine serum albumin and 1 μg/ml sonicated salmon sperm DNA at 4°C overnight. The cross-linked DNA was immunoprecipitated by addition of 2 μg of antinucleolin monoclonal antibody (Stressgen) and preblocked protein A/G beads or preblocked protein A/G beads alone. Cross-links were reversed as described previously (35). The immunoprecipitated DNA was purified by using QIAGEN PCR purification spin columns and analyzed by limiting PCR in the presence of [α-^{32}P]dATP at 3 μCi/reaction mixture (3,000 Ci/mmol; Perkin-Elmer) with primer pairs specific for human genes encoding rRNA (positions +948 to +1028 of the 18S rRNA coding sequence), the intergenic rRNA gene (positions +35176 to +35346), geminin (positions +68 to +347), β-globin (positions +1460 to +1685), Rpl7 (positions +200 to +440), Rps28 (positions +254 to +440), $tRNA^{Ser}$ (positions −12 to 6 bp beyond the end of the gene), and $tRNA^{Val}$ (positions −9 to 4 bp beyond the end of the gene). Human β-actin DNA was examined with primers for a commercially available amplicon (Applied Biosystems). The number of PCR cycles and the concentration of input DNA were optimized for exponential amplification. The PCR products were resolved by electrophoresis in 6% polyacrylamide gels cast and run in 0.25× TBE, and signals were quantified with Image J software.

Nucleolin RNA interference (RNAi). Nucleolin Stealth RNAi Select (NCL-HSS106985; Invitrogen) and control Stealth RNAi Negative Control (catalog no. 12935-200; Invitrogen) were introduced into HeLa cells with TransFectin (Bio-Rad) by following the manufacturer's protocols. After 24 h, the medium was replaced and cells were harvested at 72 h post RNAi introduction. Total protein was extracted in 10 mM Tris-HCl, pH 7.05, containing 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 μM $ZnCl_2$, 10% (vol/vol) glycerol, 0.1 mM PMSF, and 0.5% (vol/vol) Triton X-100 and analyzed by immunoblotting. Total RNA was prepared by Trizol (Invitrogen) extraction, precipitated with 2-propanol, washed with 70% ethanol, resuspended, and digested with 20 U of RNase-free DNase I (Roche Diagnostics) for 1 h at 37°C. RNA was prepared by phenol extraction, precipitated with 2-propanol, washed with 70% ethanol, and resuspended in distilled H_2O. Target RNA was amplified as described for RNA polymerase I transcription assays. The primers used for PCR were as described in the RNA polymerase I transcription and chromatin immunoprecipitation sections.

RESULTS

Analysis of RNA polymerase II transcription from chromatin templates. In order to identify proteins that enhance elongation in a reconstituted RNA polymerase II transcription system, we used purified chromatin templates. Such templates allow efficient initiation by RNA polymerase II and GTFs. However, engaged polymerase molecules stall upon encountering a nucleosome and hence cannot elongate transcripts. The DNA template used for assembly contained a 390-bp G-less cassette under the control of a synthetic promoter containing five GAL4 DNA-binding sites upstream of the basal adenovirus major late promoter. Chromatin was assembled onto this DNA by using purified core histones and the ATP-dependent chromatin assembly factor RSF (43–45). The GAL4-AD activator protein (described in Materials and Methods) was included in assembly reaction mixtures to induce the formation of a nucleosome-free promoter region (44). These assembly components are shown in Fig. 1A. As anticipated (43–45), micrococcal nuclease digestion of the chromatin template assembled in this way indicated the presence of regularly spaced nucleosomes (Fig. 1B). As previously reported, RNA polymerase II and the GTFs (TFIIB, TFIIE, TFIIF, TFIIH, and TBP) directed efficient transcription of the naked DNA template (Fig. 1C, left side) but failed to support transcription through the nucleosome array. As shown in Fig. 1C, such nucleosome-induced inhibition can be overcome by addition of FACT to the reaction mixtures.

Identification of proteins that stimulate transcription of chromatin by RNA polymerase II. We used reaction mixtures containing a limiting concentration of FACT to identify additional human proteins that stimulate RNA polymerase II transcription from chromatin templates. The concentration of FACT used in these assays precluded efficient transcription of the chromatin template (Fig. 2A, lanes 1 and 2). However, the addition of the 0.1 and 0.35 M P11 fractions derived from the second chromatographic step strongly stimulated transcription of the chromatin template (Fig. 2A, lanes 3 and 4). Immunoblotting of the P11 fractions for the human Spt16 protein confirmed that such stimulation was not mediated by endogenous FACT (Fig. 2A, bottom). This protein was not present in either the 0.1 M or the 0.35 M phosphocellulose fraction, and its concentration in the 0.65 M fraction was too low to stimulate transcription in this assay. The stimulatory activities present in both the 0.1 and 0.35 M phosphocellulose fractions were purified further (Fig. 2B). The 0.1 M fraction was fractionated through five additional chromatographic steps, culminating in gel filtration on Superdex 200, from which the stimulatory activity eluted in a single peak with an apparent molecular mass of ~125 kDa. Coomassie blue staining of the fractions separated by SDS-PAGE identified a single protein of ~125 kDa that coeluted with the stimulatory activity (Fig. 2C). The 0.35 M fraction was fractionated through three additional chromatographic steps, as summarized in Fig. 2B. Coomassie blue staining of the fractions from the final chromatographic step, a Superose 6 column, revealed a single protein of ~125 kDa that coeluted with the stimulatory activity (Fig. 2D). The 125-kDa proteins recovered from both the Superdex 200 and Superose 6 columns were identified by mass spectrometry as nucleolin, an abundant nucleolar protein that participates in ribosome biogenesis. We have not investigated the basis of the initial separation of nucleolin into two phosphocellulose fractions. However, nucleolin is subject to cell cycle-dependent phosphorylation at multiple sites, as well as to methylation and ADP ribosylation (78). It is therefore possible that the nucleolin recovered from the 0.1 and 0.35 M P11

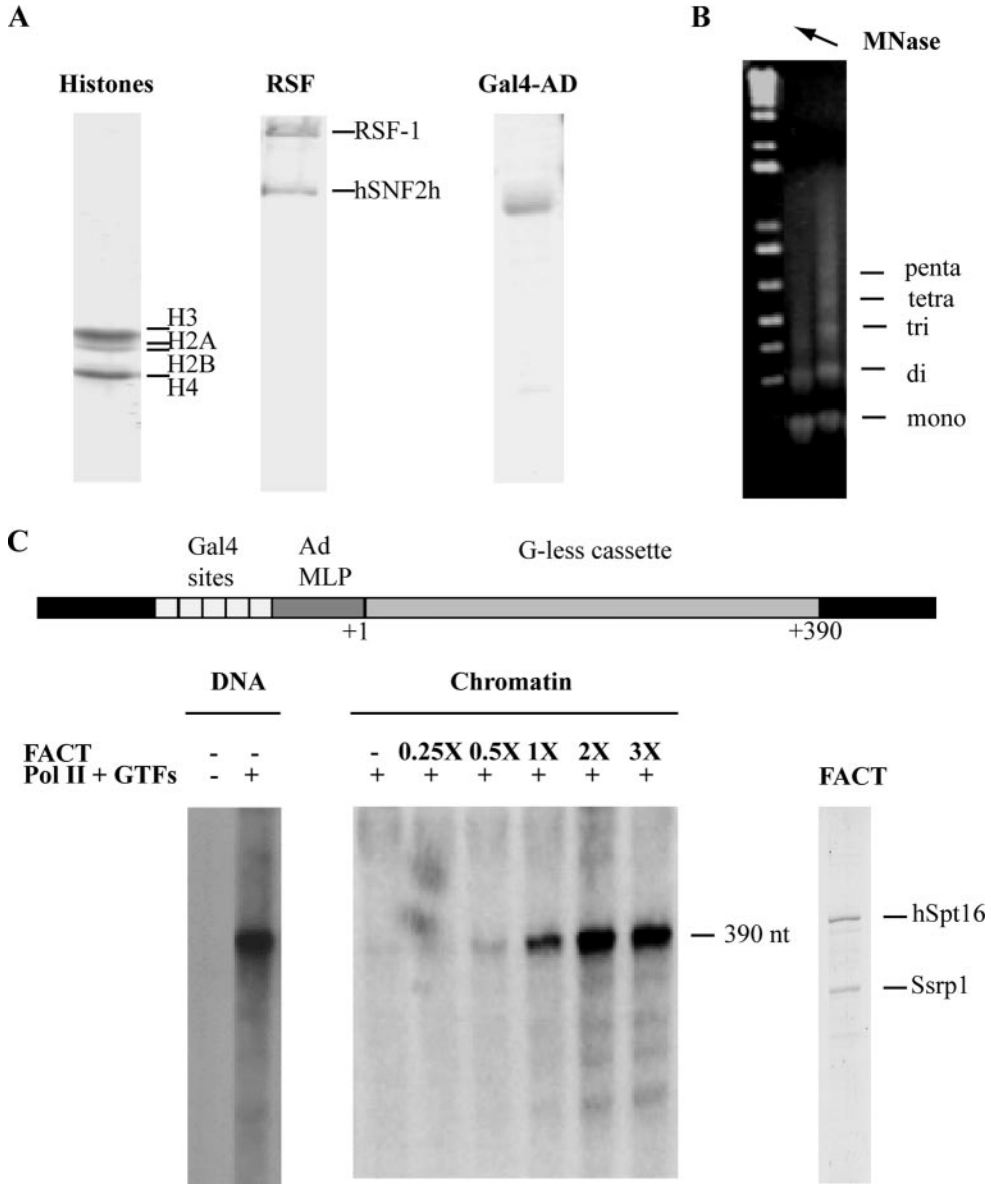

FIG. 1. FACT-dependent RNA polymerase II transcription of a chromatin template. (A) In vitro chromatin assembly. The proteins used for chromatin assembly are shown after electrophoresis in polyacrylamide gels containing SDS and Coomassie blue staining. (B) Chromatin assembled onto the pG5MLP plasmid was digested with two concentrations of micrococcal nuclease (MNase), resolved by electrophoresis in a 1.3% agarose gel, and visualized by ethidium bromide staining. The arrow indicates an increasing concentration of micrococcal nuclease. (C) Transcription reaction mixtures contained naked (left side) or chromatin-assembled (middle) pG5MLP and the transcriptional components and concentrations of FACT indicated. RNA labeled in vitro was purified and visualized as described in Materials and Methods. The right side shows purified FACT analyzed by SDS-PAGE and Coomassie blue staining. Ad MLP, adenovirus major late promoter.

fractions differed with respect to one or more of these modifications.

Nucleolin possesses FACT-like activity. In order to confirm that the stimulation of chromatin transcription was due to nucleolin and not some minor component(s) in the fractions, we performed immunodepletion experiments. The peak activity fractions from the Superdex 200 column were depleted with a monoclonal antibody against nucleolin coupled to protein A/G beads, with a control monoclonal antibody against an adenoviral protein coupled in the same fashion, or with A/G beads alone. The ability of purified nucleolin to stimulate transcription was inhibited only by the antinucleolin antibody (Fig. 3A, lane 5): neither beads alone nor the control antibody inhibited transcription (Fig. 3A, lanes 3 and 4) or decreased the concentration of nucleolin (Fig. 3B).

We next tested the activity of highly purified, FLAG-tagged nucleolin (Fig. 4). Nucleolin purified in this way also stimulated chromatin transcription (Fig. 4B, lane 4). Furthermore, it supported efficient chromatin transcription in the absence of FACT (Fig. 4B, lane 5), as did highly purified native nucleolin (data not shown). Moreover, nucleolin was as effective as a

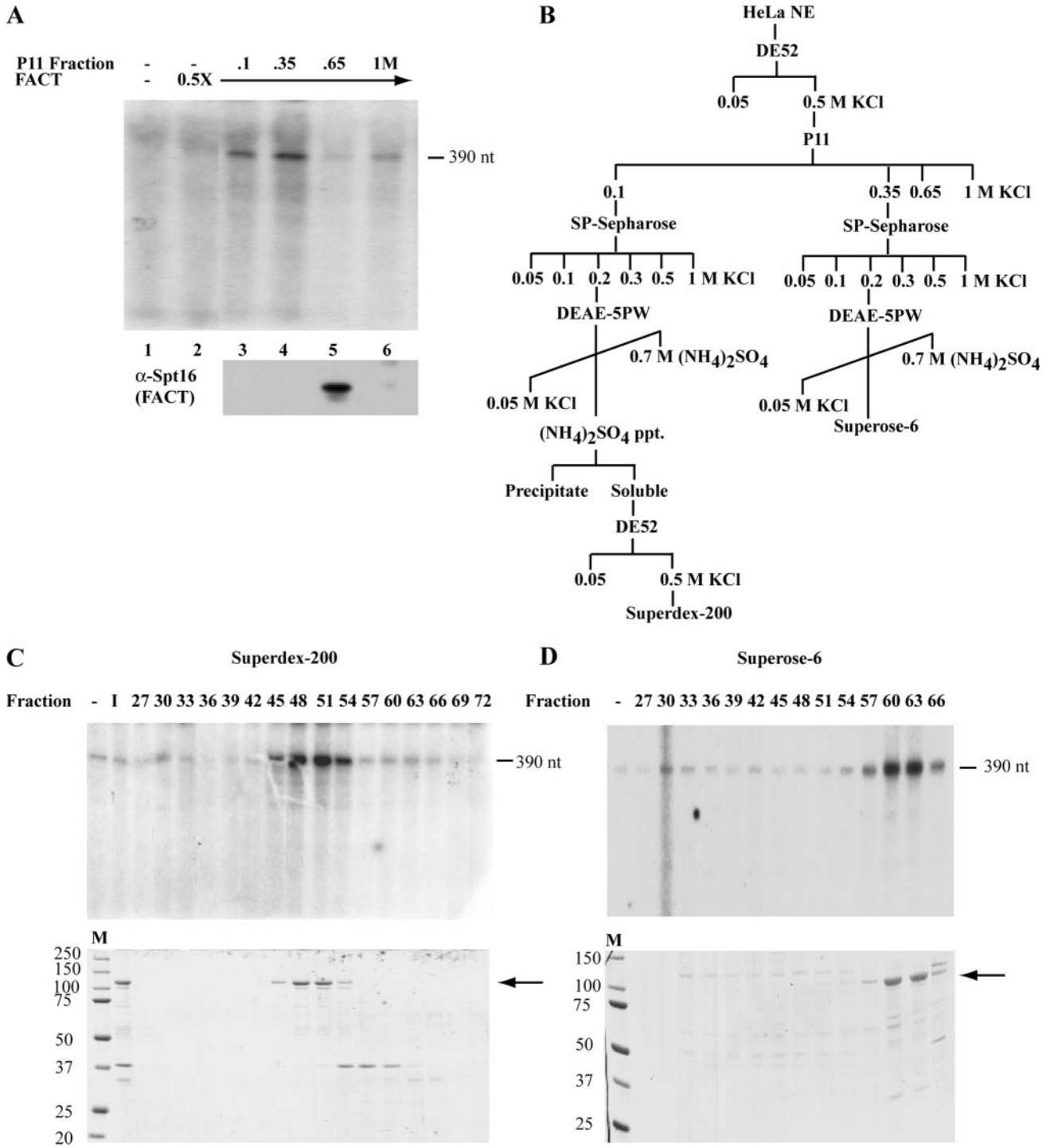

FIG. 2. Purification of proteins that stimulate transcription of chromatin templates by RNA polymerase II. (A) Transcription reaction mixtures contained the chromatin template described in the legend to Fig. 1B, the quantities of FACT indicated at the top, and equal volumes of the P11 fractions indicated. The bottom part shows the immunoblotting of these fractions for the hSpt16 subunit of FACT. (B) Summary of purification of stimulatory activities from the 0.1 and 0.35 M P11 fractions. NE, nuclear extract; ppt., precipitation. Panels C and D show the results of the final steps in purification of the activities detected in the 0.1 and 0.35 M P11 fractions. In each case, the top and bottom parts show the results of assaying the fractions indicated under the conditions described for panel A and of analysis of the proteins present in the fractions by SDS-PAGE and Coomassie blue staining, respectively. The proteins marked by the arrows were identified as nucleolin by mass spectroscopy. nt, nucleotides; M, markers.

saturating concentration of FACT (compare lanes 2 and 5, Fig. 4B). Importantly, nucleolin, like FACT, is chromatin specific because it failed to stimulate transcription from the naked DNA template (Fig. 4C).

Nucleolin and RNA polymerase I localize to nucleoli. To determine whether nucleolin exists outside nucleoli, where it might affect transcription by RNA polymerase II, we examined its subcellular distribution by immunofluorescence. In agree-

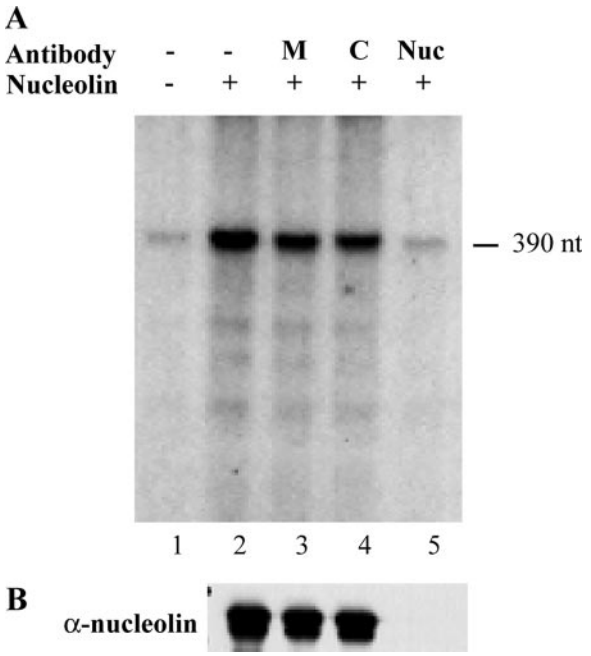

FIG. 3. Nucleolin is the protein responsible for chromatin transcription activity. (A) Chromatin templates were transcribed in the presence of a limiting concentration (0.5×) of FACT (all lanes) and purified nucleolin (lane 2) or purified nucleolin that had been mock depleted with beads alone (M) (lane 3) or incubated with control antibody bound to beads (C) (lane 4) or with antinucleolin antibody bound to beads (Nuc) (lane 5). nt, nucleotides. (B) Immunoblotting of these same nucleolin preparations with an antinucleolin antibody.

ment with previous studies (26, 73), we found that nucleolin is present primarily in the nucleoli (Fig. 5A). Also in agreement with previous reports, RNA polymerase I localized to nucleoli, the sites of rRNA gene transcription (Fig. 5B) (72). In contrast, TFIIF and RNA polymerase II were observed throughout the nucleus but were excluded from nucleoli (Fig. 5C and D). These results suggest that in vivo nucleolin most likely participates in RNA polymerase I transcription. However, as nucleolin is an abundant protein (5, 21), we cannot exclude the possibility that a small percentage is present at extranucleolar sites.

Nucleolin facilitates transcription of chromatin by RNA polymerase I. To asses the role of nucleolin in transcription by RNA polymerase I, we used an RNA polymerase I transcription system composed of highly purified components (Fig. 6A). Transcription was monitored by RT-PCR with primers corresponding to positions +1 to +270 of the human rRNA gene. In conjunction with the TBP-containing protein SL-1, RNA polymerase I initiated transcription from the rRNA gene promoter in a naked DNA template (Fig. 6B, lane 3). Such transcription was further enhanced by the site-specific upstream binding factor UBF (Fig. 6B, lane 4) (16). However, nucleolin did not stimulate transcription from this naked DNA template (Fig. 6C). To investigate whether nucleolin could allow RNA polymerase I to elongate transcription on a nucleosomal template, the plasmid containing the rRNA gene promoter was assembled into chromatin as described in a previous section. The reconstituted RNA polymerase I transcription system failed to transcribe the chromatin template (Fig. 6D, lane 1). However, in the presence of nucleolin (or FACT), robust transcription was observed (Fig. 6D, lanes 2 and 3).

Nucleolin is associated with chromatin containing the rRNA gene. To determine whether nucleolin associates with chromatin, we performed chromatin immunoprecipitation experiments. Nuclei were isolated from 293 cells synthesizing FLAG-nucleolin by hypotonic lysis and treated with micrococcal nuclease to release chromatin fragments ranging in size from 1 to 20 nucleosomes. The chromatin was immunoprecipitated with FLAG-antibody-beads, which were then washed and eluted by competition with FLAG peptide. The proteins present in the various fractions were separated by SDS-PAGE and visualized by staining with Coomassie blue. FLAG-nucleolin was found to be associated with all four core histones, as well as with nucleophosmin, another abundant nucleolar protein (Fig. 7A, lane 4). The observation that nucleolin can associate with both core histones and nucleophosmin suggested that endogenous nucleolin might be bound to nucleolar chromatin formed on rRNA genes.

To test this hypothesis, we performed a conventional chromatin immunoprecipitation experiment with a monoclonal antibody that recognizes native nucleolin. HeLa cell chromatin was cross-linked in situ with formaldehyde, released, and immunoprecipitated with this antibody or protein with A/G beads alone, as described in Materials and Methods. The immunoprecipitated DNA was purified and analyzed by limiting PCR with primers for human genes transcribed by RNA polymerases I, II, and III. The rRNA gene that is actively transcribed was significantly enriched in the chromatin immunoprecipitated by the antinucleolin antibody compared to that precipitated by beads alone (Fig. 7B). Quantification of four independent experiments indicated that the enrichment of the rRNA gene was approximately 11-fold (Fig. 7B). In these experiments, nucleolin immunoprecipitation of chromatin did not result in significant enrichment of several genes transcribed by RNA polymerase II, including those encoding ribosomal proteins Rpl7 and Rps28, or of two tRNA genes transcribed by RNA polymerase III (Fig. 7B).

Nucleolin is essential for RNA polymerase I transcription. To investigate the role of nucleolin in transcription by RNA polymerase I in vivo, we used short interfering RNAs (siRNAs), which induce the degradation of mRNAs to which they are targeted (RNAi) (14). Introduction of an siRNA specific for nucleolin resulted in a decrease in the concentration of this protein, but not of β-actin, whereas a control siRNA had no effect (Fig. 8A). We therefore examined the concentration of 45S pre-rRNA in HeLa cells exposed to these siRNAs by RT-PCR. The reduction in the concentration of nucleolin induced by RNAi was accompanied by a decrease in the transcription of rRNA genes mediated by RNA polymerase I but not in transcription by RNA polymerase II (Fig. 8B). Furthermore, in several independent experiments, the extent of inhibition of RNA polymerase I transcription correlated with the degree to which nucleolin was knocked down (data not shown). These results indicate that nucleolin is required for transcription of rRNA genes by RNA polymerase I in vivo.

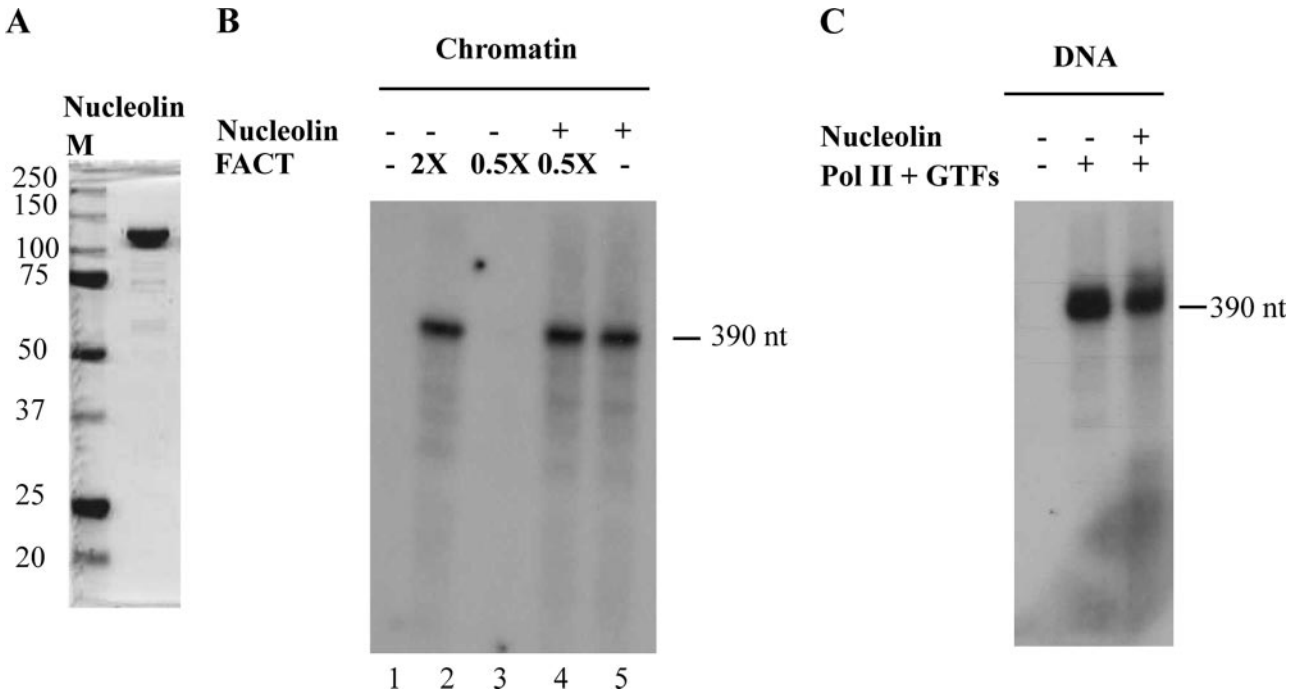

FIG. 4. Nucleolin specifically stimulates transcription of a chromatin template. (A) Coomassie blue staining of SDS-PAGE of purified FLAG-tagged nucleolin. (B) FLAG-nucleolin was assayed in chromatin transcription assays. (C) The naked DNA template was transcribed in the presence of FLAG-nucleolin as indicated. nt, nucleotides; M, markers.

DISCUSSION

Nucleolin is an abundant, conserved, RNA-binding protein that resides primarily in eukaryotic cell nucleoli. Numerous correlative studies have implicated this protein in various aspects of ribosome biogenesis, including pre-rRNA processing, assembly of ribosomal subunits, and movement of ribosomes or their components between the nuclear and cytoplasmic compartments of eukaryotic cells (21, 85). Nevertheless, few molecular functions have been unequivocally ascribed to nucleolin. Here we demonstrate for the first time that nucleolin is required for transcription of rRNA genes by RNA polymerase I in vivo: knockdown of nucleolin by RNAi specifically blocked synthesis of pre-rRNA in HeLa cells (Fig. 8B). Furthermore, the results of chromatin immunoprecipitation experiments established that nucleolin is associated in vivo with chromatin containing rRNA genes but not with any of several genes transcribed by RNA polymerase II or III (Fig. 7B).

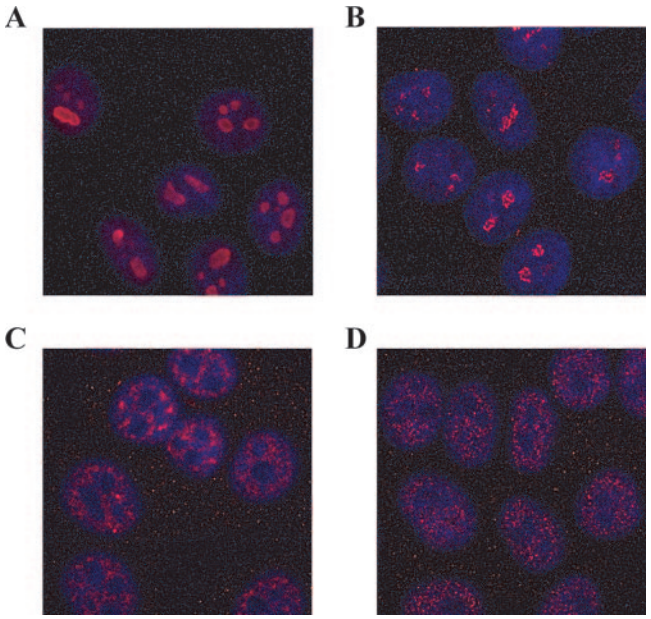

FIG. 5. Nucleolin and RNA polymerase I localize to nucleoli. HeLa cells were fixed and stained (red) with antibodies against nucleolin (A), RNA polymerase I (B), TFIIF (C), or RNA polymerase II (D). In all cases, DNA was stained with DAPI (blue).

The conclusion that nucleolin is necessary for efficient transcription of rRNA genes in vivo appears to contradict previous reports that this protein represses transcription by RNA polymerase I in hamster cells and *Xenopus* oocytes (6, 68). However, in both cases this function was ascribed to nucleolin on the basis of the effects of overproduction of the protein, as a result of either inhibition of proteolytic processing (6) or microinjection of highly purified, exogenous nucleolin (68). As it is clear from the results of siRNA-mediated knockdown that nucleolin is necessary for maximal rRNA gene transcription in vivo (Fig. 8), it is likely that these higher-than-physiological concentrations of nucleolin inhibited transcription indirectly. High concentrations of this protein could, for example, sequester components that are required for transcription by RNA polymerase I, such as UBF, with which fluorescently labeled, exogenous nucleolin colocalizes (68). As phosphorylation of nucleolin serine residues correlates with active transcription of the rRNA gene (see below), it is also possible that abnormally

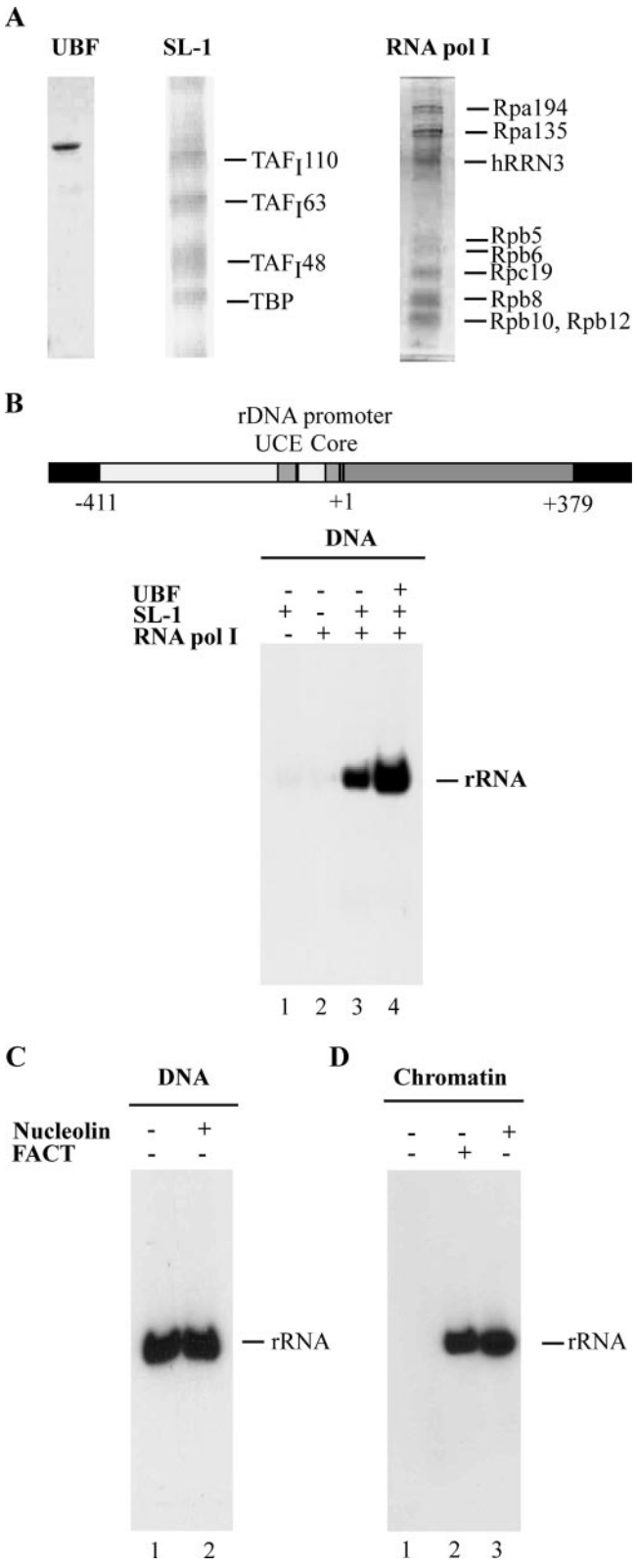

FIG. 6. Nucleolin facilitates RNA polymerase (pol) I transcription of chromatin templates. (A) Coomassie blue staining of SDS-polyacrylamide gels containing the purified proteins used to reconstitute RNA polymerase I transcription. (B) RNA polymerase I, SL-1, and UBF-1 were added to transcription reaction mixtures containing an rRNA gene template as indicated at the top of the panel. The transcripts were amplified by one-step RT-PCR as described in Materials and Methods. No products were observed in reaction mixtures that lacked SL1 (lane 2) or contained only nucleolin (data not shown), establishing the specificity for rRNA transcripts and the absence of rRNA bound to nucleolin, respectively. (C) Nucleolin does not stimulate RNA polymerase I transcription from a naked template. Proteins were added as indicated at the top of the panel. All reaction mixtures contained RNA polymerase I, SL-1, and UBF-1. (D) Transcription reaction mixtures contained the components shown in panel A, the rRNA gene template assembled into chromatin and FLAG-nucleolin, or FACT, as indicated at the top.

high nucleolin concentrations inhibited pre-rRNA synthesis by perturbing the balance among differentially phosphorylated forms of the protein.

Previous studies have indicated that nucleolin participates in the earliest reaction in pre-rRNA processing, endonucleolytic cleavage at a conserved site within the 5′ external transcribed spacer. Binding of nucleolin to a conserved sequence in the pre-rRNA substrate is required for cleavage at this site in vitro (19, 20). It has been proposed that the interaction of nucleolin with pre-RNA initiates assembly of a pre-RNA processing complex by recruitment of processing components such as U3 small nucleolar ribonucleoprotein (19). The association of nucleolin with nascent pre-rRNA in vivo (30) is consistent with this model. It therefore seems likely that nucleolin coordinates transcription of rRNA genes by RNA polymerase I with pre-rRNA processing and in this respect resembles proteins that both facilitate elongation by RNA polymerase II and participate in subsequent reactions in mRNA biogenesis (see references 34, 48, 69, and 77 for reviews).

Like RNA polymerase II, RNA polymerase I is incapable of transcribing DNA packaged by nucleosomes in the presence of its initiation proteins in vitro (Fig. 6) (25). The robust transcription observed upon the addition of nucleolin to such reaction mixtures (Fig. 6) indicates that nucleolin facilitates elongation by RNA polymerase I during the transcription of chromatin templates. Whether it also facilitates initiation by this enzyme has not yet been examined. In the reconstituted transcription systems used in these studies, nucleolin and FACT both efficiently stimulated chromatin transcription by RNA polymerases I and II (Fig. 3, 4, and 6). Such reciprocity is consistent with our current understanding of the biochemical properties of these proteins. FACT, which interacts specifically with histones H2A and H2B (4, 58), has been reported to act as a histone chaperone and is believed to destabilize the histone octamer (1, 4). Similarly, nucleolin has been shown to stimulate remodeling and mobilization of nucleosomes by Swi/Snf and Acf and, alone, to induce transfer of H2A-H2B dimers to H3-H4 tetramers (1). As the N-terminal 300 amino acids of nucleolin comprise an H1-like domain (13), it is probable that nucleolin also binds directly to nucleosomal histones. Indeed, the N-terminal domain has been reported to decondense chromatin in vitro (13), and nucleolin associates with core histones in vivo (Fig. 7A).

It is well established that the chromatin in which vertebrate rRNA genes reside regulates rRNA gene transcription and that entire arrays, or individual genes within rRNA gene repeats, can be repressed by deacetylation and methylation of histones (25). How such inhibitory histone modifications are

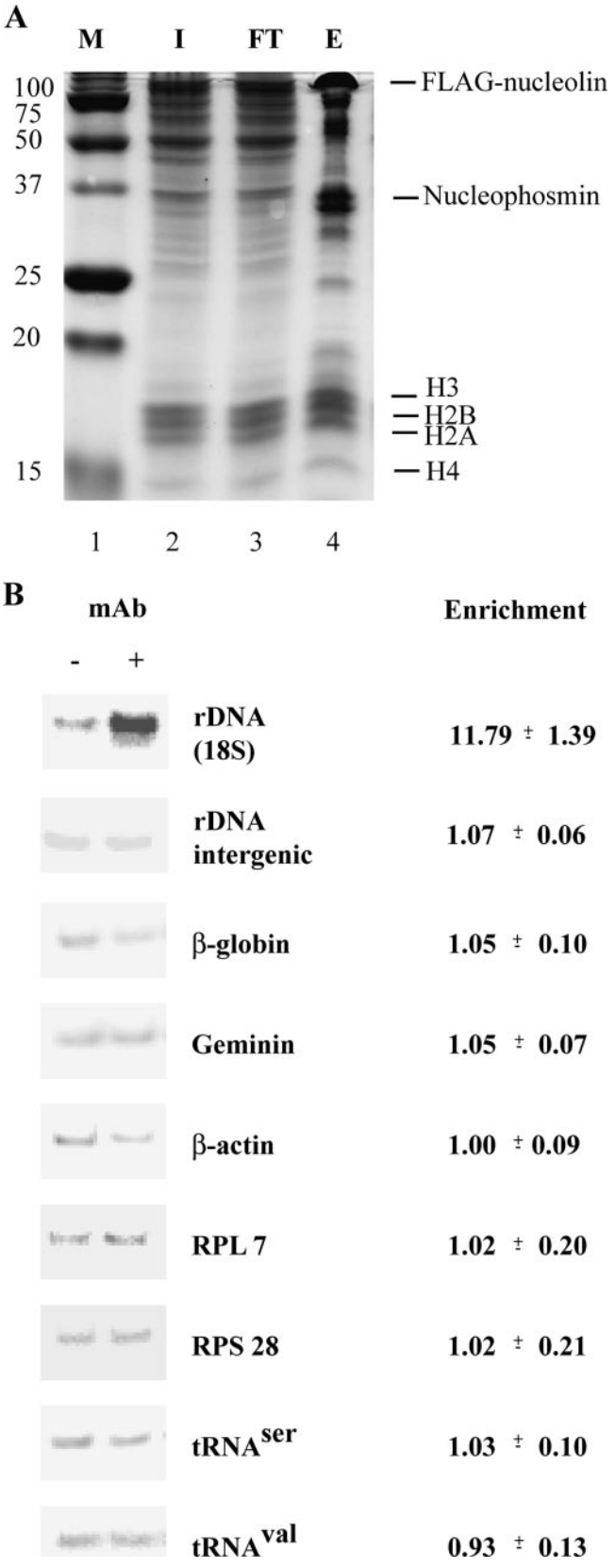

FIG. 7. Nucleolin binds to chromatin containing rRNA. (A) The association of FLAG-nucleolin with chromosomal histones was examined by immunoprecipitation. The protein present in input chromatin (I) and the flowthrough (FT) and peptide-eluted (E) fractions was examined by SDS-PAGE and Coomassie blue staining. Nucleophosphin (fraction E) was identified by mass spectrometry. M, markers (the values on the left are sizes in kilodaltons). (B) Chromatin immunoprecipitations of cross-linked HeLa cell chromatin were performed with control beads (lanes 1) or with 2 μg of nucleolin antibody conjugated to beads (lanes 2). DNA isolated from the immunoprecipitates was analyzed by PCR for the genes indicated. Signals were quantified with Image J software and used to calculate the DNA concentration in the nucleolin immunoprecipitate relative to that of the control. The values shown are the means of four independent experiments. mAb, monoclonal antibody.

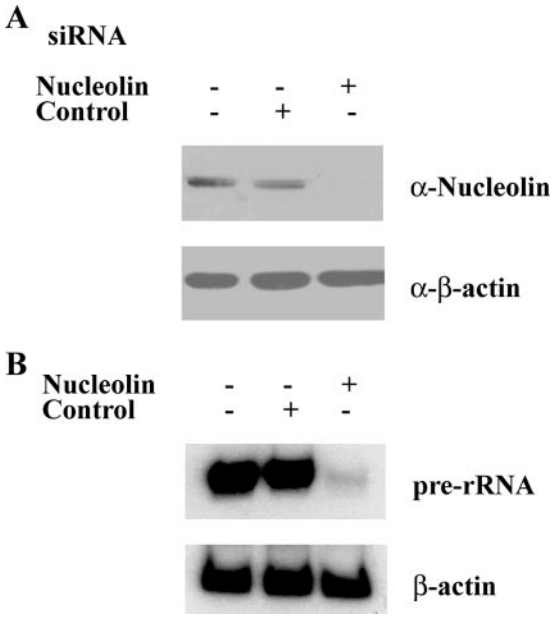

FIG. 8. Nucleolin is required for RNA polymerase I transcription. (A) Immunoblotting of proteins isolated from cells treated with siRNA for nucleolin, as indicated. (B) RNA isolated from cells treated with siRNA for nucleolin, as indicated, was amplified by one-step RT-PCR as described in Materials and Methods.

reversed when transcription is activated and the definitive features of chromatin associated with actively transcribed rRNA genes are not understood. However, it is reasonable to suppose that nucleolin might mark nucleolar chromatin that is being transcribed or that can be transcribed. This hypothesis is consistent with our observation that the association of nucleolin with chromatin containing the rRNA gene showed no decrease when HeLa cells were subjected to heat shock at 42°C for 2 h (data not shown), a stress that inhibits transcription of RNA genes by some 95% (18). Interestingly, nucleophosmin, which is also associated with the rRNA gene, has been shown to stimulate transcription preferentially from a chromatin template containing acetylated histones (82). As nucleophosmin does not possess FACT-like activity (58), a reasonable hypothesis is that the histone chaperone activity of nucleolin and the binding of nucleophosmin to acetylated histones function in concert to permit transcription of active rRNA genes in vivo.

Nucleolin is subject to various posttranslational modifications, notably cell cycle-dependent phosphorylation of specific

residues (78). During interphase, numerous serines, many of which are located in the N-terminal H1-like domain, are phosphorylated by casein kinase II, whereas during mitosis threonine residues in the same domain are modified by Cdc2 (3, 7, 61). Serine phosphorylation correlates with active transcription of rRNA genes. When cells become contact inhibited, transcription of these genes is reduced by 95%, casein kinase II leaves nucleoli, and serine phosphorylation is lost. Furthermore, during mitosis, when pre-rRNA is not made (86), the N-terminal domain is heavily phosphorylated by Cdc2 (3, 61). It is therefore important to determine whether these or other modifications of nucleolin regulate its ability to associate with chromatin and allow transcription by RNA polymerase I.

The effect of nucleolin on transcriptional elongation by RNA polymerase II was also examined in a recent study using elongation complexes stalled on a mononucleosomal template (1). In this system, nucleolin induced a modest (1.3-fold) increase in transcription through the nucleosome to the end of the template (1). Our observation that, in a fully defined transcription system, nucleolin stimulated transcription of chromatin templates by at least a factor of 10 (Fig. 4 and 5) provides compelling support for the conclusion that this protein can stimulate transcription by RNA polymerase II. Furthermore, these data establish that nucleolin is not only necessary but also sufficient to allow such transcription. Whether it functions in the same way in vivo is less clear, as is the contribution of FACT to transcription by RNA polymerase I. The lack of specificity exhibited by these proteins in vitro can be attributed, at least in part, to their similar biochemical properties. However, the highly simplified nature of the chromatin template used in these experiments is likely to be of even greater importance. These templates were built with nucleosomal histones (Fig. 1A) and consequently lacked both non-histone protein components and the higher-order structure characteristic of native chromatin. Furthermore, no in vitro system can reproduce the spatial and functional compartmentalization of chromatin within the nucleus. Consequently, we believe that the reciprocity exhibited by nucleolin and FACT in vitro does not reflect the physiological functions of these proteins and propose that the primary role of nucleolin in vivo is to permit chromatin transcription by RNA polymerase I. This view is consistent with our observations that nucleolin was associated with chromatin containing the rRNA gene in HeLa cells but not with any of several genes transcribed by RNA polymerases II and III (Fig. 7B) and that knockdown of nucleolin resulted in inhibition of transcription by RNA polymerase I but not by RNA polymerase II (Fig. 8B). Nevertheless, we cannot exclude the possibility that nucleolin also contributes to transcription by RNA polymerase II, as suggested by previous studies. For example, nucleolin has been reported to alter the structure of the chromatin in which human papillomavirus type 18 oncogenes reside in cells derived from a cervical carcinoma (24) and to interact in vitro with several proteins that regulate transcription by RNA polymerase II (76, 88, 89). Global analyses of the genes with which nucleolin associates in cells that are proliferating or arrested (2) and of the effects of RNAi against nucleolin are required to address this issue.

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