In Vivo Evidence That TATA-binding Protein/SL1 Colocalizes with UBF and RNA Polymerase I When rRNA Synthesis Is Either Active or Inactive

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Abstract. Here we show that the TATA-binding protein (TBP) is localized in the nucleoplasm and in the nucleolus of mammalian cells, consistent with its known involvement in transcription by RNA polymerase I, II, and III. In the nucleolus of actively growing cells, TBP colocalizes with upstream binding factor (UBF) and RNA polymerase I at the sites of rRNA transcription. During mitosis, when rRNA synthesis is down-regulated, TBP colocalizes with TBP-associated factors for RNA polymerase I (TAF_Is), UBF, and RNA polymerase I on the chromosomal regions containing the rRNA genes. Treatment of cells with a low concentration of actinomycin D inhibits rRNA synthesis and causes a redistribution of the rRNA genes that become concentrated in clusters at the periphery of the nucleolus. A similar redistribution was observed for the major components of the rRNA transcription machinery (i.e., TBP, TAF_Is, UBF, and RNA polymerase I), which still colocalized with each other. Furthermore, anti-TBP antibodies are shown to coimmunoprecipitate TBP and TAF_I63 in extracts prepared from untreated and actinomycin D–treated cells. Collectively, the data indicate that in vivo TBP/promoter selectivity factor, UBF, and RNA polymerase I remain associated with both active and inactive rRNA genes.

THE initiation of transcription by all three eukaryotic RNA polymerases depends on the assembly of specific transcription factors at the corresponding promoters, and substantial progress has been recently made in the definition of these molecules (for recent reviews see Reeder, 1990; Gabrielson and Sentenac, 1991; Drapkin et al., 1993). A particularly well-studied transcription factor is the TATA-binding protein (TBP)[1]. Although TBP was originally thought to be a specific component of the RNA polymerase II transcription machinery, recent evidence indicates that it is also required for transcription by RNA polymerases I and III (for review see Hernandez, 1993; Goodrich and Tjian, 1994). In the case of RNA polymerase II promoters containing a TATA sequence, TBP acts as a DNA-binding protein, whereas in the TATA-less promoters from RNA polymerases II, I, and III, TBP serves to direct protein–protein interactions with class-specific transcription factors (Struhl, 1994). Thus, the assembly of TBP with individual TBP-associated factors (TAF) generates distinct multiprotein complexes specific for each RNA polymerase.

In contrast with the substantial progress in dissecting the transcription machinery in vitro, little is known about how the different transcription factors are organized in vivo. Unlike RNA polymerase II and III, RNA polymerase I recognizes only one type of promoter, requires a small number of transcription factors, and transcribes the tandemly repeated rRNA genes that are compartmentalized in the nucleolus (Scheer and Weisenberger, 1994). Thus, it provides a good opportunity to study the spatial organization of a transcriptional apparatus within the cell nucleus.

A diploid human cell contains about 400 rRNA genes spread out in clusters on five different chromosome pairs (Long and Dawid, 1980). Each chromosomal cluster is called a nucleolus organizer region (NOR), and when the nucleolus is viewed with the electron microscope, the NORs are identified as pale-staining areas named fibrillar centers (for reviews see Scheer et al., 1993; Scheer and Weisenberger, 1994). Within each gene cluster, there are multiple rRNA transcription units lined up as tandem repeats separated by nontranscribed spacer sequences. Each

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1. *Abbreviations used in this paper*: NOR, nucleolus organizer region; SL1, promoter selectivity factor; TAF, TBP-associated factor; TAF_I, TBP-associated factor for RNA polymerase I; TBP, TATA-binding protein; TIF, transcription initiation factor; UBF, upstream binding factor; UTP, uridine 5′-triphosphate.

transcription unit contains a promoter that consists of two distinct control elements: the upstream control element, which spans the region from nucleotides −186 to −107, and the core promoter element, which extends from nucleotides −45 to +20. Accurate and specific transcription of rRNA genes by RNA polymerase I requires at least two transcription factors, upstream binding factor (UBF) and promoter selectivity factor (SL1) (Bell et al., 1988; Schnapp and Grummt, 1991). UBF is a DNA-binding protein that binds to the upstream control element, whereas SL1 has no DNA-binding activity but is involved in protein–protein interactions that confer promoter selectivity on RNA polymerase I (Bell et al., 1988). UBF consists of two alternatively spliced polypeptides of 97 and 94 kD (UBF1 and UBF2, respectively) and was found to be highly conserved among various vertebrates (O'Mahony and Rothblum, 1991; Hisatake et al., 1991). UBF interacts with DNA by way of multiple HMG-box domains and in doing so greatly enhances recruitment of SL1, which appears to mediate communication with RNA polymerase I (Jantzen et al., 1990; Bazett-Jones et al., 1994). SL1 is a multisubunit complex containing TBP and three TAFs of 110, 63, and 48 kD, which are essential to activate rRNA transcription (Comai et al., 1992; Eberhard et al., 1993; Rudloff et al., 1994).

In vitro reconstitution experiments have indicated that initiation of transcription by RNA polymerase I involves the stepwise association of transcription factors at the rDNA promoter (Bell et al., 1988; Schnapp and Grummt, 1991; Comai et al., 1994). As a first step, UBF is thought to bind to the promoter and to recruit SL1, thus forming a preinitiation complex. Then, RNA polymerase I can bind and initiate transcription. However, immunolocalization studies have revealed that both UBF and RNA polymerase I are present at the chromosomal NORs during mitosis, suggesting that in vivo the preinitiation complex may persist associated with the rDNA promoter when transcription is down-regulated (Scheer and Rose, 1984; Scheer et al., 1993; Roussel et al., 1993; Zatsepina et al., 1993). Here, we sought to investigate further this hypothesis by studying the intranuclear distribution of TBP, TBP-associated factor for RNA polymerase I (TAF_I), UBF, RNA polymerase I, and rRNA genes in human cells. We show that TBP colocalizes with TAF_Is, UBF, and RNA polymerase I at the sites of either active or inactive rRNA genes. This suggests that in vivo the association of TBP/SL1, UBF, and RNA polymerase I with rDNA persists when rRNA synthesis is down-regulated.

Materials and Methods

Cell Culture, Drug Treatment, and Transient Transfection

Human HeLa and mouse NIH 3T3 cells were grown as monolayers in DME supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD) and maintained mycoplasm free.

For inhibition of rRNA synthesis, actinomycin D (Sigma Chemical Co., St. Louis, MO) was added to the culture medium to a final concentration of 0.08 μg/ml and incubated for 60 min.

HeLa cells were transiently transfected using the standard calcium phosphate precipitation method with plasmid pCMVheTBP. This was constructed by inserting the XbaI to NheI fragment of pETeHIID (human TBP containing a 12-amino acid influenza hemagglutinin epitope tag in its NH_2 terminus) (Lee et al., 1991) into the XbaI site of the CMV vector pSCT (Öhman et al., 1993). Cells were analyzed by immunofluorescence at 60-h posttransfection.

Immunofluorescence

For indirect immunofluorescence the cells were grown on 10 × 10 mm glass coverslips and harvested at 60–80% confluency. Coverslips with attached cells were washed twice in PBS and treated according to the following alternative protocols: (*a*) immediate fixation with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 10 min at room temperature, and subsequent permeabilization with 0.5% Triton X-100 in PBS for 15 min at room temperature; (*b*) permeabilization with 0.5% TritonX-100 in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1mM EGTA, pH 6.8) (Fey et al., 1986) containing 0.1 mM PMSF for 1 min on ice, and subsequent fixation with 3.7% formaldehyde in CSK for 10 min at room temperature; and (*c*) permeabilization with 0.2% Triton X-100 in PB buffer (100 mM CH_3-COOK, 30 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, pH 7.4) (Jackson et al., 1993) for 10 min on ice, and subsequent fixation with 3.7% formaldehyde in PB buffer for 10 min at room temperature.

After fixation and permeabilization the cells were rinsed in PBS containing 0.05% Tween-20, incubated for 1 h with primary antibodies diluted in PBS containing 0.05% Tween-20, washed, and incubated for 30 min with the appropriate secondary antibodies conjugated to either fluorescein or Texas red (Dianova GmbH, Hamburg, Germany). Finally, the coverslips were mounted in VectaShield (Vector Laboratories, Peterborough, UK) and sealed with nail polish.

The following antibodies were used: rabbit anti-hTBP KD 55/1 (kindly provided by Dr. R. Bravo, Bristol-Myers Squibb, Princeton, NJ) (Metz et al., 1994), mouse monoclonals anti-TBP 3G3 and 4C2 (Lescure et al., 1994), rabbit polyclonal antibodies C21 and E29 raised against human UBF (kindly provided by Dr. L. Rothblum, Weis Center, Danville, PA) (O'Mahony et al., 1992), autoimmune human anti-RNA polymerase I serum S18 (kindly provided by Dr. U. Scheer, University of Würzburg, Germany), rabbit anti-$hTAF_I$ sera (kindly provided by Drs. J. Zomerdijk and R. Tijan, HHMI, Berkeley, CA) (Comai et al., 1994), mAb 72B9 directed against fibrillarin (kindly provided by Dr. E. Tan, Scripps Clinic, La Jolla, CA) (Reimer et al., 1987), and mAb 12CA5-I, directed against the HA-epitope (obtained from BAbco, Berkeley, CA).

Visualization of Transcription Sites

Visualization of transcription sites was performed essentially according to Jackson et al. (1993). Briefly, cells grown as monolayers on coverslips were washed twice in PBS and incubated with 0.05% Triton X-100 in PB buffer for 2 min on ice to permeabilize selectively the plasma membrane. Then, the cells were incubated with a transcription mix containing bromo-uridine 5′-triphosphate (UTP) (Sigma Chemical Co.) for 20 min at 33°C. Subsequently, the cells were further incubated in 0.2% Triton X-100 for 10 min, fixed with 3.7% formaldehyde for 10 min, and the incorporated bromo-uridine detected using an mAb directed against bromodeoxyuridine (Boehringer Mannheim, GmBH, Mannheim, Germany).

To visualize the sites of transcription by distinct RNA polymerases, the transcription assay was performed in the presence of selective inhibitors. The drugs were added for 10 min before as well as during incubation with the modified nucleotide. In the presence of α-amanitin at a concentration of 100 μg/ml (to inhibit RNA polymerases II and III), the labeling was exclusively observed in the nucleolus. In the presence of actinomycin D at a concentration of 0.08 μg/ml (to inhibit transcription by polymerase I), the labeling was detected in the nucleoplasm but not in the nucleolus, and in the presence of 5 μg/ml actinomycin D (which inhibits transcription by all RNA polymerases), all labeling was abolished.

Visualization of rRNA Genes

HeLa cells grown on coverslips were washed in PBS, fixed in methanol/acetone (3:7) for 15 min at −20°C (Robert-Fortel et al., 1994), washed three times for 5 min each in PBS, and dehydrated in increasing concentrations of ethanol (70, 80, 90, and 100%, 3 min each). Samples were then digested with 0.5 μg/ml proteinase K in 20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$. After washing three times in PBS, the cells were fixed for 10 min in 1% formaldehyde in PBS containing 50 mM $MgCl_2$, washed three times in PBS, and dehydrated in increasing concentrations of ethanol. To hydrolyze all

cellular RNA, the 70% ethanol step was done for 4 min in the presence of 0.2 N NaOH (Carter et al., 1991). Cellular DNA was denatured for 5 min at 90°C in 70% deionized formamide, 2× SSC, 50 mM sodium phosphate, pH 7, and the cells were then rapidly chilled in ice-cold 70% ethanol and dehydrated. Subsequently, the coverslips were air dried and hybridized overnight with 5 ng/μl digoxigenin-labeled pUHrBES (Worton et al., 1984) and 1 μg/μl sonicated herring sperm DNA in hybridization buffer (50% deionized formamide, 2× SSC, 50 mM sodium phosphate, 10% dextran sulfate, pH 7). After hybridization, the samples were washed three times for 5 min each at 42°C in 50% deionized formamide, 2× SSC, 50 mM sodium phosphate, three times for 5 min each at 42°C in 2× SSC, and then for 5 min in 4× SSC, 0.1% Tween-20. To detect the hybridization sites, the samples were blocked with 3% BSA in 4× SSC for 30 min at 37°C and incubated with anti-digoxigenin mAbs using one amplification step (Boehringer Mannheim GmbH).

Fluorescence Microscopy

Samples were examined with a microscope (LSM 310; Zeiss, Oberkochen, Germany). Confocal microscopy was performed using argon ion (488 nm) and HeNe (543 nm) lasers to excite FITC and Texas red fluorescence, respectively. For double labeling experiments, images from the same confocal plane were sequentially recorded and superimposed. Alternatively, images were digitized using a SIT-camera and an ARGUS 10 image processor (Hamamatsu Phototonics, Hamamatsu City, Japan). The images were captured in the same focal plane using separate filters and then superimposed. To obtain a precise alignment of superimposed images, the equipment was calibrated using multicolor fluorescent beads (Molecular Probes, Eugene, OR) and a dual-band filter that allows simultaneous visualization of red and green fluorescence. The images were photographed on Fujichrome 100 (Fuji Photo Film Co., Ltd., Tokyo, Japan) or TMax100 film (Eastman-Kodak Co., Rochester, NY), using a Polaroid Freeze Frame Recorder (Cambridge, MA). Alternatively, data files were directly printed on a XLS 8300 Digital Printer (Eastman-Kodak Co.).

Subcellular Fractionation, Immunoprecipitation, SDS-PAGE, and Immunoblotting

HeLa cells grown in 10-cm plates were washed twice in PBS, once in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$), and harvested with a rubber-policeman. Cells (10^7 per ml) were left for swelling 20 min on ice in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 1 mM DTT, 1 mM PMSF, 1 mM 1,10-phenanthroline, 1 mM EGTA, 10 μM E64, and 10 μg/ml of each aprotinin, leupeptin, and pepstatin A) (Sigma Chemical Co.). A crude nuclear pellet was isolated by forcing the cell suspension through a 25-gauge needle and centrifuging 5 min at 700 *g*. Nuclei (10^7 per ml) were resuspended in lysis buffer, disrupted by 15 brief sonication pulses (Tyc and Steitz, 1989), and centrifuged at 5,000 *g* for 5 min. The resulting supernatant was operationally defined as "nucleoplasm," whereas the pellet was highly enriched in nucleoli, as assessed by EM.

For immunoprecipitation, the nucleoli-enriched pellets were extracted in lysis buffer containing 300 mM KCl for 45 min at 4°C, and separated into supernatant and pellet by centrifugation at 16,000 *g* (5 min at 4°C). NP-40 was added to the supernatant to a final concentration of 0.65%. For immunoprecipitation, protein A–Sepharose beads (CL-4B; Pharmacia, Uppsala, Sweden) were washed in IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.65% NP-40) and coated for 1 h with rabbit anti-TAF_I63 or anti-UBF sera in IP buffer. In the case of the mAbs against TBP and fibrillarin, the beads were first incubated with rabbit anti–mouse IgG (Dako, Glostrup, Denmark). The antibody-coated beads were washed, added to the nucleolar extracts, and incubated for 90 min at 4°C in a rotating shaker. Beads were washed three times for 10 min each in IP buffer and boiled in SDS-PAGE sample buffer.

Proteins were separated by SDS-PAGE in 8% acrylamide minigels (Bio-Rad Laboratories, Richmond, CA) and electroblotted to a nitrocellulose membrane at 100 mA for 50 min in 48 mM Tris, 39 mM glycine, 0.04% SDS, and 10% methanol. The membranes were blocked and washed in PBS containing 0.1% Tween-20 and 2% low-fat milk powder. The blots were incubated for 1 h with primary antibodies, washed three times for 10 min each in the same buffer, incubated with the appropriate secondary antibodies conjugated with HRP (Bio-Rad Laboratories), and developed using a chemiluminescence reaction (ECL; Amersham Corp., Arlington Heights, IL).

Results

TBP Colocalizes with UBF at Sites of Active RNA Polymerase I Transcription

The cellular localization of TBP was studied by indirect immunofluorescence microscopy using both monoclonal and polyclonal antibodies. Since the distribution of nuclear antigens has been reported to vary depending on the methods used for fixation and permeabilization of the cells (Carmo-Fonseca et al., 1991), we decided to test different procedures. Fixation with formaldehyde is generally considered to produce the best structural preservation under isotonic conditions, but it requires to be combined with a detergent to allow penetration of the antibodies to the nucleus. When HeLa cells were first fixed in paraformaldehyde and then permeabilized with Triton X-100, the anti-TBP antibodies produced a diffuse staining of the nucleoplasm, and the nucleoli were usually not labeled (data not shown). When a brief extraction with Triton X-100 preceded the fixation, TBP foci became visible in the nucleoli besides the nucleoplasmic staining (Fig. 1 *B*). When the detergent extraction was extended to 10 min (Jackson et al., 1993), TBP was clearly labeled both in the nucleolus and in the nucleoplasm (Fig. 1, *A* and *C*). Similar results were observed with the three distinct antibodies tested.

Within the nucleolus, TBP was detected in discrete foci arranged in a necklace-like pattern (Fig. 1, *A–C*). Since both TBP and UBF are known to be required for transcription by RNA polymerase I, and UBF has been previously detected in similar necklace-like structures in the nucleolus (Roussel et al., 1993; Zatsepina et al., 1993), we asked whether these proteins colocalize. Double-labeling experiments demonstrated that anti-TBP and anti-UBF antibodies label the same intranucleolar foci (Fig. 1, *A* and *D*). In addition, these intranucleolar foci were double labeled by antibodies against TBP and RNA polymerase I (Fig. 1, *B* and *E*).

To determine whether the intranucleolar foci containing TBP, UBF, and RNA polymerase I correspond to sites of transcription by RNA polymerase I, we have made use of a recently developed method that allows to visualize nascent RNA chains in the fluorescence microscope (Jackson et al., 1993; Wansink et al., 1993). HeLa cells were mildly permeabilized with 0.05% Triton X-100 for 2 min and incubated with brominated UTP for 20 min. Subsequently, the incorporated bromo-UTP was visualized using anti-bromyl antibodies. The sites of transcription were observed as multiple discrete foci both in the nucleoplasm and in the nucleolus, as previously reported (Jackson et al., 1993; Wansink et al., 1993). To unambiguously identify the sites of RNA polymerase I transcription, the cells were incubated with bromo-UTP in the presence of 100 μg/ml α-amanitin to inhibit RNA polymerases II and III (Lindell, 1980). Under these conditions the labeling was exclusively detected in nucleolar foci (Fig. 1 *F*), and double-labeling experiments clearly demonstrate that these foci contain TBP (Fig. 1, *C* and *F*).

TBP Colocalizes with UBF and RNA Polymerase I during Mitosis

Since UBF, SL1, and RNA polymerase I were proposed to

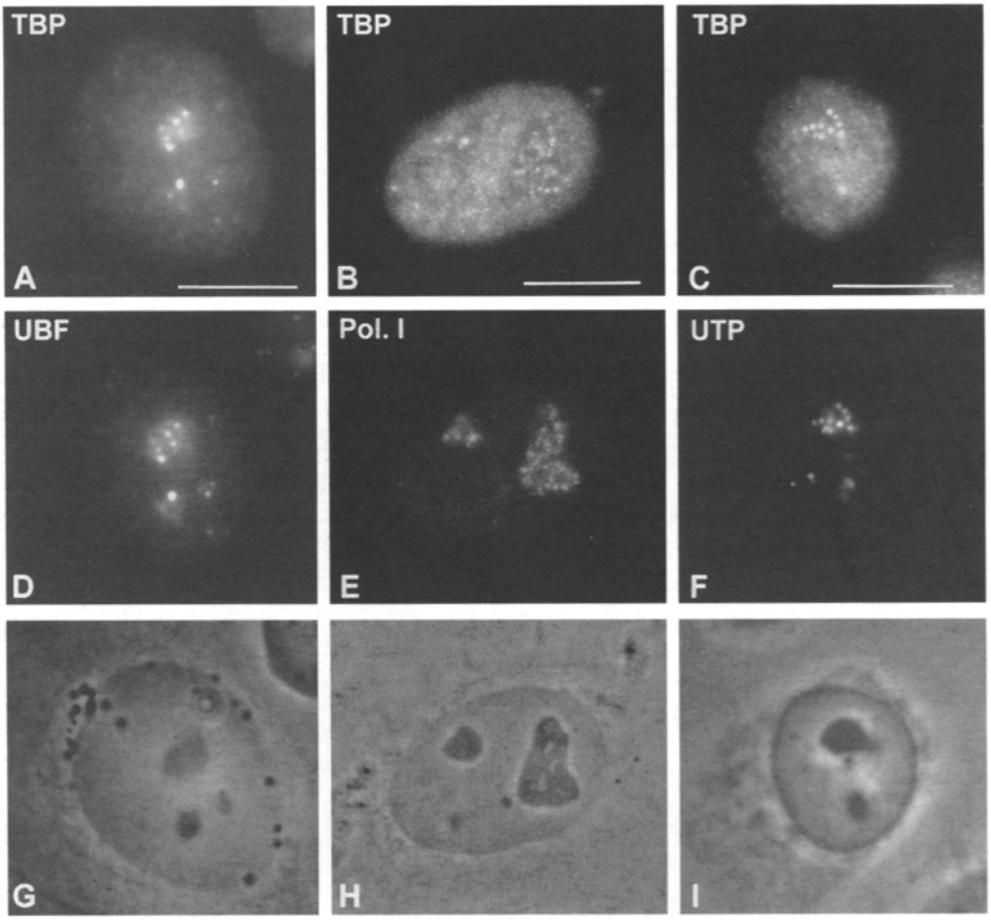

Figure 1. Colocalization of TBP with UBF and RNA polymerase I at the sites of rRNA synthesis. HeLa cells were permeabilized with Triton X-100 for either 1 min (*B* and *E*) or 10 min (*A* and *D*; *C* and *F*) and then fixed in formaldehyde. Immunofluorescence microscopy was performed with anti-TBP antibodies (3G3 or KD55-1, both diluted 1:100). The labeling of intranucleolar foci is particularly evident after the long detergent treatment (*A* and *C*). Double-labeling experiments using antibodies against either TBP and UBF (C21, diluted 1:1,000) (*A* and *D*) or TBP and RNA polymerase I (S18, diluted 1:300) (*B* and *E*) show colocalization of these proteins in intranucleolar foci. Incorporation of bromo-UTP in the presence of 100 μg/ml α-amanitin (to inhibit RNA polymerases II and III) is detected at discrete sites in the nucleolus (*F*), which colocalize with the foci labeled by anti-TBP antibody (see *C* and *F*). *G–I* depict the corresponding phase-contrast micrographs. *B*, *E*, and *H* were obtained with the confocal microscope, whereas the remaining panels were recorded using the SIT-camera. Bar, 10 μm.

assemble on the rDNA promoter in a stepwise fashion, one might expect this multiprotein complex to disintegrate whenever transcription becomes inactivated. Therefore, we analyzed the intracellular distribution of TBP, UBF, and RNA polymerase I during mitosis, when rRNA transcription is down-regulated. Other laboratories have previously described that during mitosis UBF associates with the chromosomal NORs, i.e., the regions containing the rRNA genes (Roussel et al., 1993; Zatsepina et al., 1993). Also, RNA polymerase I in mitotic cells has been localized to discrete chromosomal regions that are likely to correspond to the NORs (Scheer and Rose, 1984; Matsui and Sanberg, 1985; Scheer et al., 1993). When mitotic HeLa cells were labeled with anti-TBP antibodies, we observed a diffuse staining of the cytoplasm with additional labeling of discrete spots on the chromosomes. Identical results were obtained with the different anti-TBP antibodies irrespective of whether cells were first fixed with formaldehyde or first permeabilized with Triton X-100 (data not shown). Double-labeling experiments using either anti-TBP and anti-UBF antibodies or anti-TBP and anti-RNA polymerase I antibodies show that these three proteins colocalize in discrete chromosomal regions (Fig. 2). Thus, during mitosis TBP colocalizes with UBF and RNA polymerase I in the chromosomal NORs, i.e., at the rRNA genes.

TBP Colocalizes with UBF and RNA Polymerase I after Inhibition of rRNA Synthesis by Actinomycin D

To investigate further whether the RNA polymerase I preinitiation complex remains associated with rDNA when rRNA synthesis is inactivated, we treated HeLa cells with actinomycin D. At a concentration range of 0.001–0.1 μg/ml, actinomycin D has been reported to inhibit preferentially the synthesis of rRNA (Perry, 1962; Lindell, 1980). Accordingly, incubation of cells with bromo-UTP in the presence of 0.08 μg/ml actinomycin D abolished labeling of the nucleolus but apparently did not affect staining of the nucleoplasm (data not shown). We next proceeded to inhibit rRNA synthesis in vivo by exposing the cells to 0.08 μg/ml actinomycin D for 1 h. After this treatment the cells were double labeled using either anti-TBP and anti-UBF antibodies (Fig. 3, *A–C*) or anti-TBP and anti-RNA polymerase I antibodies (Fig. 3, *D–F*). The results show that

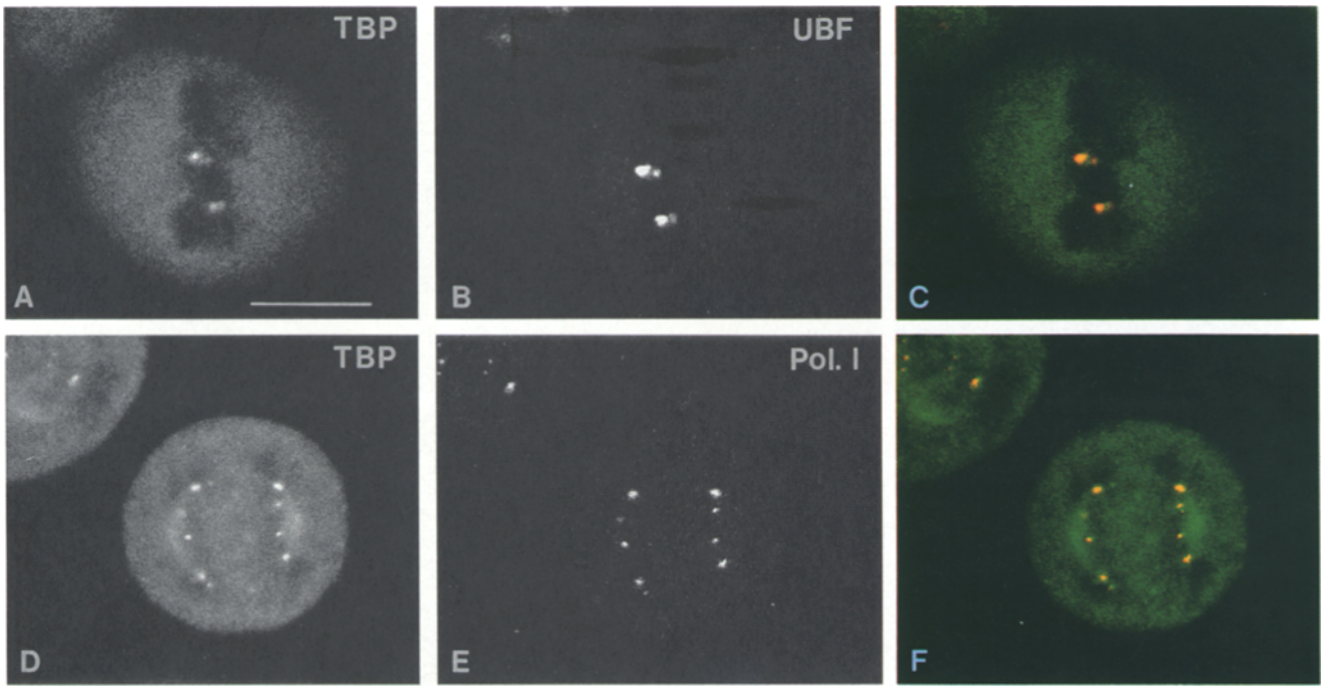

Figure 2. Colocalization of TBP with UBF and RNA polymerase I during mitosis. HeLa cells were first fixed with formaldehyde and then extracted with Triton X-100. In mitotic cells TBP was found in bright foci attached to the chromosomes, as well as diffuse in the cytoplasm (*A* and *D*). Antibodies against UBF (*B*) or RNA polymerase I (*E*) label similar chromosomal foci. Confocal images from each fluorochrome were recorded and superimposed (*C* and *F*) to demonstrate that TBP (*green staining*) and UBF or RNA polymerase I (*red staining*) colocalize in the same foci (i.e., *yellow staining*). Bar, 10 μm.

TBP still colocalizes with both UBF and RNA polymerase I in nucleolar foci. However, these foci are larger and less numerous than in untreated cells, and they no longer form necklaces within the nucleolus but rather localize at the nucleolar periphery (see Figs. 1 and 3). A similar redistribution of UBF has been previously observed by Zatsepina et al. (1993) in cells that were either nutritionally starved or treated with 5 μg/ml actinomycin D.

The presence of TBP in perinucleolar clusters was further confirmed by transient transfection of HeLa cells with epitope-tagged human TBP. In untreated cells, indirect immunofluorescence microscopy using anti-tag antibodies revealed a bright staining of the nucleoplasm (data not shown). However, when the transfected cells were treated with actinomycin D, the anti-tag antibody labeled distinct perinucleolar spots that colocalized with the spots labeled by anti-UBF antibodies (Fig. 3, *G–I*).

The requirements for in vitro transcription by RNA polymerase I appear to differ in humans and rodents because the SL1 analog transcription initiation factor (TIF) IB was capable of binding directly to the mouse rDNA promoter in the absence of UBF (Schnapp et al., 1990; Kuhn and Grummt, 1992). Therefore, we sought to extend our analysis to mouse cells. Indirect immunofluorescence microscopy was performed in 3T3 mouse fibroblasts using anti-UBF antibodies and mAb 3G3 anti-TBP, which recognize the corresponding mouse proteins (Rudloff et al., 1994). The results obtained in untreated and in actinomycin D–treated 3T3 cells were similar to those observed in HeLa cells (data not shown).

In conclusion, inhibition of rRNA synthesis by actinomycin D treatment induces a redistribution of TBP, UBF, and RNA polymerase I, which colocalize in large spots at the edge of the nucleolus. This redistribution could indicate that the transcription machinery separated from the rDNA promoters, but it could also reflect a relocalization of the rRNA genes. Therefore, we decided to compare the distribution of the rRNA genes in the nucleus of actinomycin D–treated and untreated cells. In situ hybridization was performed using a DNA probe that spans part of the promoter region, the transcription start, and the 5′ sequences of the external transcribed spacer (Fig. 4) (Worton et al., 1984). To specifically detect the rRNA genes, the cellular RNA was hydrolyzed by NaOH before hybridization. The results show that in untreated cells, the rRNA genes are visualized as multiple foci forming a necklace in the nucleolus, as previously observed (Fig. 4 *A*) (Robert-Fortel et al., 1993). After treatment with actinomycin D, the rRNA genes appear concentrated in a few large dots at the periphery of the nucleolus (Fig. 4 *B*). Thus, the observed redistribution of the transcription machinery is likely to mirror the relocalization of the rDNA templates. Interestingly, we were unable to visualize simultaneously the rRNA genes and the transcription factors because it was necessary to digest the cells with proteases to succeed in hybridizing the probe to the genes, both in untreated and in actinomycin D–treated cells. This suggests that the rDNA target sequences (which include the promoter region) are similarly protected by proteins when the genes are either active or inactive, thus providing further evidence that the preinitiation complex probably remains assembled on the rDNA promoter.

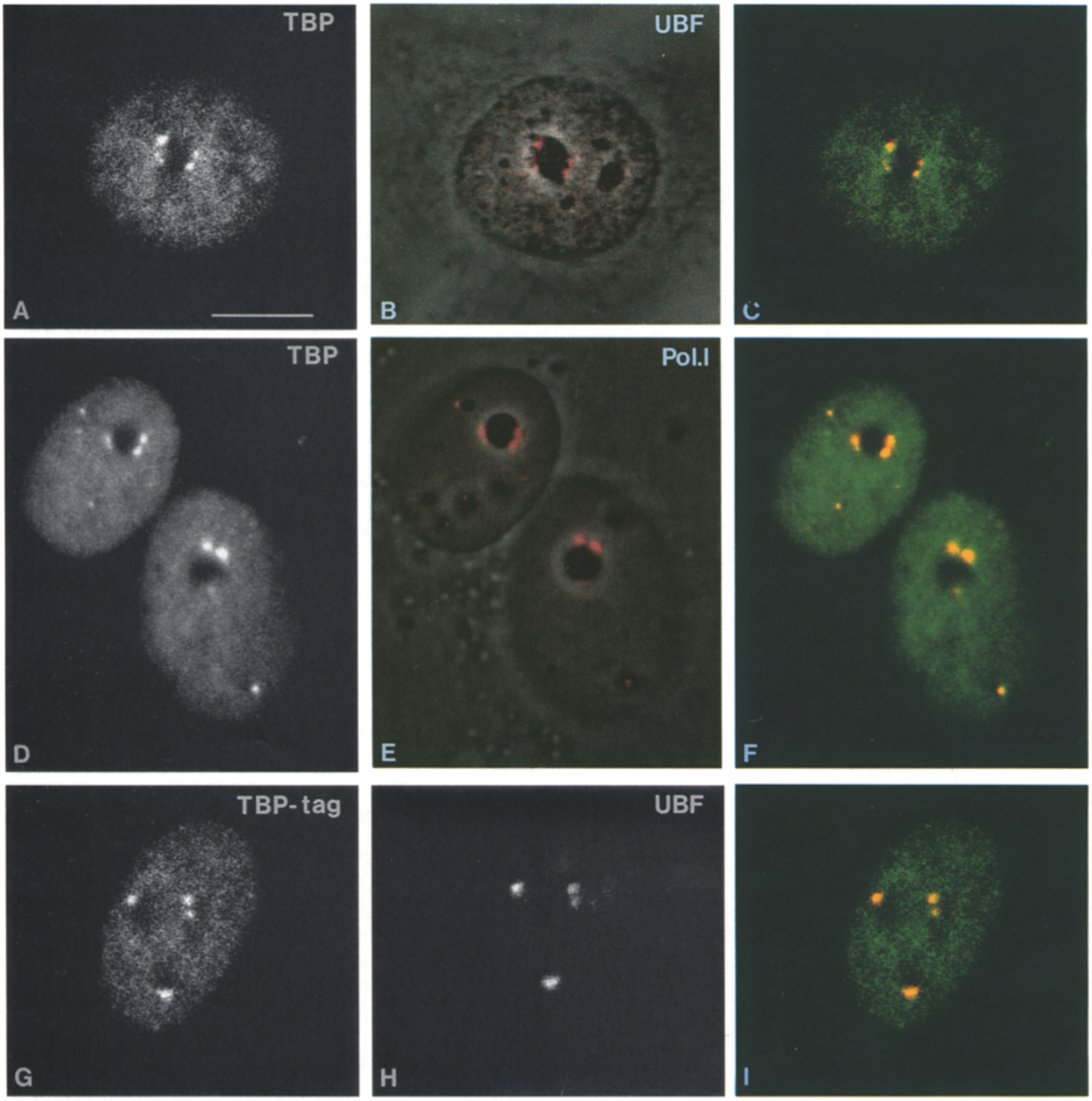

Figure 3. Effect of actinomycin D treatment on the localization of TBP, UBF, and RNA polymerase I. HeLa cells were incubated for 1 h with 0.08 μg/ml actinomycin D to inactivate nucleolar transcription. Cells were then briefly extracted with Triton X-100, fixed, and stained with antibodies against TBP (*A* and *D*). The widespread nucleoplasmic TBP staining was maintained, but nucleolar TBP appeared accumulated in a few large spots at the periphery of the nucleolus. Anti-UBF (*B*) and anti-RNA polymerase I antibodies (*E*) labeled the same perinucleolar foci. This is demonstrated for both factors using the immunofluorescent staining superimposed to the phase-contrast image of the same cell (*B* and *E*). Confocal pseudo-color images obtained using anti-TBP (*A*, *green staining*) and anti-UBF (*B*, *red*) or anti-TBP (*D*, *green*) and anti-RNA polymerase I (*E*, *red*) were recorded and superimposed (*C* and *F*) to demonstrate colocalization (i.e., *yellow staining*). In addition, HeLa cells were transiently transfected with a plasmid encoding human epitope-tagged TBP, treated with 0.08 μg/ml actinomycin D, and double labeled using anti-tag (*G*, *green staining*) and anti-UBF (*H*, *red staining*) antibodies. Superimposition of confocal images demonstrates colocalization in perinucleolar foci (*I*, *yellow staining*). Bar, 10 μm.

The TBP-associated Factors TAF_I63 and TAF_I110 Colocalize with RNA Polymerase I at Sites of Inactive rRNA Genes

The RNA polymerase I transcription factor SL1 is composed of TBP and three associated factors, TAF_I48, TAF_I63, and TAF_I110. To address the question of whether the detection of TBP on inactive rRNA genes would represent the complete SL1 complex, we performed immunofluorescence microscopy and immunoprecipitation studies using the recently developed antibodies against these TAF_Is

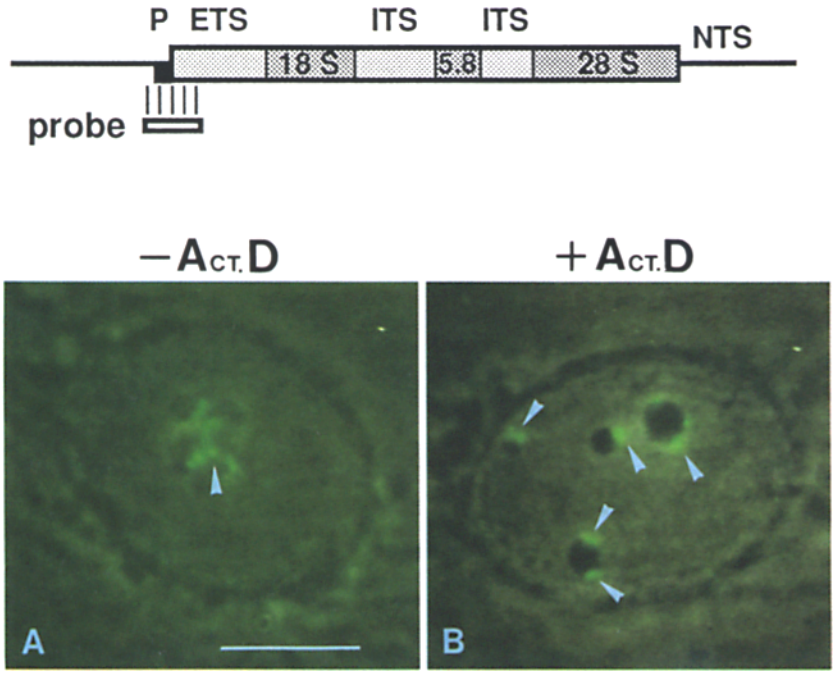

Figure 4. Effect of actinomycin D treatment on the localization of rRNA genes. The rRNA genes were visualized by fluorescent in situ hybridization and are depicted as green spots (*arrows*) superimposed on the corresponding phase-contrast image of the whole cell. In untreated cells rRNA genes are detected as multiple foci forming a necklace inside the nucleolus (*A*). After treatment with 0.08 μg/ml actinomycin D, the rDNA becomes clustered at the periphery of the nucleolus (*B*). The diagram depicts the probe used for in situ hybridization: *NTS*, nontranscribed spacer sequence; *ETS*, external transcribed spacer; *ITS*, internal transcribed spacer; *18S*, *5.8S*, and *28S*, coding regions for the 18S-, 5.8S-, and 28S-rRNA, respectively; *P*, promotor region. Bar, 10 μm.

(Comai et al., 1994). During interphase, the anti-TAF_Is antibodies failed to produce a specific staining, suggesting that within active nucleoli, these proteins are inaccessible for antibody binding. However, during mitosis the anti-TAF_I63 antibodies labeled discrete spots in chromosomes (Fig. 5 *A*). Double-labeling experiments using anti–RNA polymerase I antibodies reveal that these spots correspond to the chromosomal regions containing the rRNA genes (Fig. 5, *A–C*). When rRNA transcription was blocked by treating the cells for 1 h with 0.08 μg/ml actinomycin D, both anti-TAF_I63 and anti-TAF_I110 antibodies stained a few large dots localized at the periphery of the nucleolus, which were also labeled by anti–polymerase I antibodies (Fig. 5, *D* and *E*, and data not shown). The anti-TAF_I48 serum failed to label the cells in all immunofluorescence experiments tested. It is interesting to note that, like the TAF_Is, TBP was also more readily detectable upon inactivation of rRNA transcription, suggesting that some conformational change occurs in the complex that renders these proteins more amenable for antibody binding.

For immunoprecipitation, a nucleoli-enriched fraction was prepared from HeLa cells and extracted in the presence of 300 mM KCl. This nucleolar extract was incubated under nondenaturing conditions with anti-TBP, anti-TAF_I63, and anti-UBF (Fig. 6). Controls were performed using anti-fibrillarin antibodies. Each antibody immunoprecipitated its cognate protein and, in addition, the anti-TBP antibody was able to coimmunoprecipitate TAF_I63 in extracts prepared from either untreated cells (data not shown) or cells treated with 0.08 μg/ml actinomycin D for 1 h (Fig. 6, lane *3*), suggesting that the interaction of TBP with TAF_Is persists upon inhibition of rRNA synthesis by actinomycin D. In our experiments, the anti-TBP antibody also coimmunoprecipitated a small amount of UBF (data not shown). However, since it has been recently shown that UBF may interact in vitro with the TBP-containing pol II transcription factor TFIID (Kwon and Green, 1994), it is not possible to conclude from this observation that UBF is specifically associated with SL1.

Discussion

This study demonstrates that TBP/SL1, UBF, and RNA polymerase I colocalize with rDNA when rRNA synthesis is either active or inactive. Our immunofluorescence microscopy data using antibodies directed against TBP show that this universal transcription factor is localized both in the nucleoplasm and in the nucleolus, a finding consistent with its well-known involvement in transcription by RNA polymerase I, II, and III (Hernandez, 1993; Goodrich and Tjian, 1994). While TBP is present throughout the nucleus, the RNA polymerase I specific factor UBF is predominantly detected in the nucleolus, and within this compartment TBP and UBF colocalize with RNA polymerase I at the sites of active rRNA synthesis.

During mitosis the transcription of rRNA is down-regulated, and the nucleolus breaks down. Yet, surprisingly, both RNA polymerase I and UBF have been shown to persist associated with the NORs that harbor the rRNA genes (Scheer and Rose, 1984; Roussel et al., 1993; Scheer et al., 1993; Zatsepina et al., 1993). Here we extend these observations by demonstrating that throughout mitosis TBP colocalizes with TAF_Is, UBF, and RNA polymerase I at the chromosomal NORs.

In the interphase nucleus of actively growing cells, the synthesis of rRNA can be blocked by treating the cells with low concentrations of actinomycin D (Perry, 1962; Lindell, 1980). Here we show that actinomycin D at a concentration of 0.08 μg/ml affects the in situ incorporation of brominated UTP in the nucleolus, thus confirming an inhibitory effect upon rRNA synthesis. Furthermore we show that treatment of living cells with 0.08 μg/ml actinomycin D for 1 h causes a drastic redistribution of the rRNA genes, which become localized at the periphery of

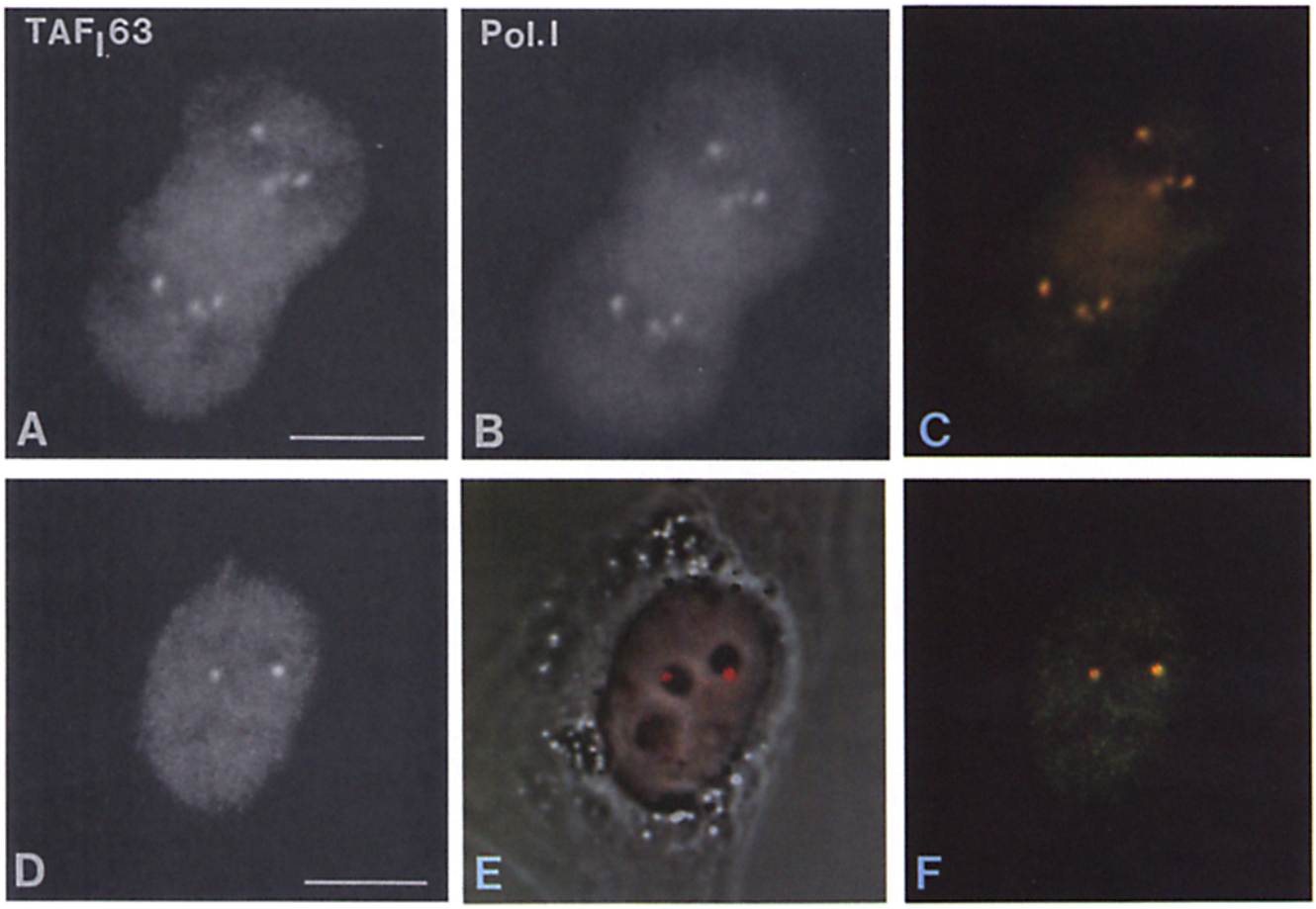

Figure 5. TAF$_I$63 colocalizes with RNA polymerase I during mitosis and in actinomycin D–treated cells. During mitosis, the anti-TAF$_I$63 antibodies (1:100) label bright foci associated with chromosomes (*A*); similar foci are labeled by anti-RNA polymerase I antibodies (*B*). After actinomycin D treatment, both anti-TAF$_I$63 (*D*) and anti-RNA polymerase I (*E*) antibodies label perinucleolar foci. Confocal images from each fluorochrome were recorded and superimposed (*C* and *F*) to demonstrate that TAF$_I$63 (*green color*) and RNA polymerase I (*red color*) colocalize in the same foci (i.e., *yellow staining*). Bar, 10 μm.

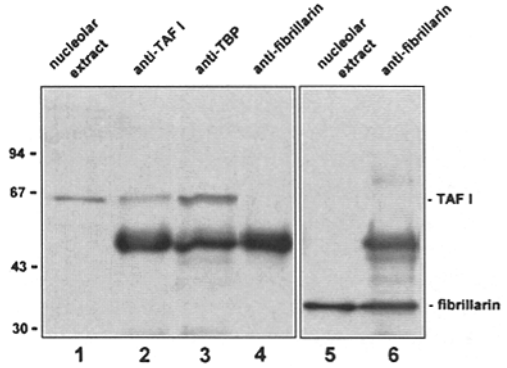

Figure 6. TAF$_I$63 is coimmunoprecipitated by anti-TBP antibodies after inhibition of rRNA synthesis. Nucleoli-enriched fractions were prepared from cells treated for 1 h with 0.08 μg/ml actinomycin D. The nucleolar pellets were extracted with 300 mM KCl, and the resulting supernatant used for immunoprecipitation. In lanes *1* and *5*, the soluble nucleolar proteins were probed by Western blotting using anti-TAF$_I$63 (lane *1*) or anti-fibrillarin (lane *5*) antibodies. For immunoprecipitation, the extracts were mixed with protein A–Sepharose beads coated with antibodies against TAF$_I$63 (lane *2*), TBP (3G3; lane *3*), and the mAb 72B9 against fibrillarin (lane *4*) as a control. The immunoprecipitates were analyzed by immunoblotting using either anti-TAF$_I$63 (lanes *2–4*) or anti-fibrillarin (lanes *5* and *6*) antibodies. Molecular weight markers are indicated in kD.

the nucleolus (Fig. 4). Interestingly, five of the known components of the rRNA transcription machinery (i.e., UBF, TBP, TAF$_I$63, TAF$_I$110, and RNA polymerase I) become similarly redistributed and still colocalize with each other (Figs. 3 and 5). It has been recently shown that the three TAF$_I$s interact with each other and bind individually to TBP, forming a stable TBP–TAF complex that is responsible for promoter and RNA polymerase specificity (Comai et al., 1994). Consistent with these in vitro data, we have observed that TBP and TAF$_I$63 coimmunoprecipitate in extracts prepared from nucleoli-enriched fractions. Furthermore, our results indicate that such interaction persists when rRNA transcription is inhibited by actinomycin D. Taken together, our immunofluorescence and immunoprecipitation studies suggest that the entire SL1 complex remains associated with UBF and RNA polymerase I at the sites of rDNA when rRNA synthesis is down-regulated during mitosis or inhibited by actinomycin

D. This strongly supports the previous idea that assembly of the preinitiation complex at the rDNA promoter is unlikely to represent a major mechanism for regulating rRNA synthesis (for review see Reeder, 1990; Scheer et al., 1993). One possibility is that regulation of rRNA synthesis involves additional factors, and important candidates are the murine factors TIF-IA and TIF-IC (Schnapp and Grummt, 1991). TIF-IA has been described to be a regulatory protein whose activity correlates with cell proliferation (Schnapp et al., 1990, 1993), and TIF-IC was found to stimulate transcription elongation and to suppress pausing of RNA polymerase I (Schnapp et al., 1994). In this respect it is interesting to note that the expression of several genes transcribed by RNA polymerase II appears to be regulated by a postinitiation mechanism that involves pausing of the polymerase at an early stage of RNA elongation (Bentley and Groudine, 1986; Chinsky et al., 1989; Giardina et al., 1992). Therefore, proteins involved in elongation of RNA polymerase I (Schnapp et al., 1994; Weisenberger and Scheer, 1995) might also play a role in regulation of class I gene transcription. Additional hypotheses for the regulation of rRNA transcription include subtle reversible modifications of some components of the transcriptional machinery (e.g., phosphorylation) (Voit et al., 1995) or the existence of inhibitory factors. In support of the latter possibility, Kermekchiev and Muramatsu (1993) have described a polymerase I inhibitory activity present in extracts from growth-arrested cells and proposed that it corresponds to a new type of repressor factor.

In conclusion, our data strengthen the view that regulation of rRNA synthesis in vivo is likely to involve more than the simple stepwise assembly of UBF, TBP/SL1, and RNA polymerase I on the rDNA.

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