

# The rDNA Transcription Machinery Is Assembled during Mitosis in Active NORs and Absent in Inactive NORs

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**Abstract.** In cycling cells, the rDNAs are expressed from telophase to the end of G<sub>2</sub> phase. The early resumption of rDNA transcription at telophase raises the question of the fate of the rDNA transcription machinery during mitosis. At the beginning of mitosis, rDNA transcription is arrested, and the rDNAs are clustered in specific chromosomal sites, the nucleolar organizer regions (NOR). In human cells, we demonstrate that the rDNA transcription machinery, as defined *in vitro*, is colocalized in some NORs and absent from others whatever the mitotic phase: RNA polymerase I and the RNA polymerase I transcription factors, upstream binding factor and promoter selectivity factor (as verified for TATA-binding protein and TATA-binding protein-associated factor for RNA polymerase I [110]), were colocalized in the same NORs. The RNA polymerase I complex was localized using two different antibodies recognizing the two largest subunits or only the third largest subunit, respectively. These two antibodies immunoprecipitated the RNA polymerase I complex in

interphase cells as well as in mitotic cells. These results clearly indicated that the RNA polymerase I complex remained assembled during mitosis. In addition, RNA polymerase I and the transcription factors varied in the same proportions in the positive NORs, suggesting stoichiometric association of these components. The fact that the rDNA transcription machinery is not equally distributed among NORs most likely reflects the implication of the different NORs during the subsequent interphase. Indeed, we demonstrate that only positive NORs exhibit transcription activity at telophase and that the level of transcription activity is related to the amount of rDNA transcription machinery present in the NOR. We propose that assembly of rDNA transcription machinery preceding mitosis determines expression of the rDNAs at the beginning of the next cell cycle. Consequently, the association of rDNAs with the rDNA transcription machinery defines the "active" NORs and the level of activity at the transition telophase/interphase.

**I**N eukaryotic cells during interphase, nucleoli are the sites of rDNA transcription, rRNA processing, and the assembly of ribosomes (19, 50). In human cells, the rDNA transcription machinery, as defined *in vitro*, is composed of RNA polymerase I (RNA pol I)<sup>1</sup> in association with the RNA pol I transcription factors upstream binding factor (UBF) and promoter selectivity factor (SL1) (2, 3, 24). The latter transcription factor or the corresponding murine factor, transcription initiation factor

(TIF) IB, is a complex composed of the TATA-binding protein (TBP) and three TBP-associated factors (TAF) (7, 8, 11, 39, 54). UBF and SL1 most likely represent the minimal set of factors needed in addition to RNA pol I to direct rDNA transcription in human cells. Indeed, in addition to UBF and SL1 (designated TIF-IB in mice), two transcription factors designated TIF-IA and TIF-IC were identified in mouse cells. TIF-IA is a growth-dependent transcription initiation factor. It interacts physically with RNA pol I and converts it into a transcriptionally active RNA pol I (41). TIF-IA was purified from exponentially growing mouse cells and identified in HeLa cells (42). TIF-IC, implicated in both transcription initiation and elongation of RNA pol I (44), has not yet been identified in human cells.

However, even if the partners necessary to promote transcription of rDNAs have been recently well characterized *in vitro*, this characterization is not sufficient to predict how the rDNA transcription machinery proceeds in

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1. *Abbreviations used in this paper:* BrUTP, bromo-uridine 5'-triphosphate; DAPI, 4',6-diamidino-2-phenylindole; NOR, nucleolus organizer region; RNA pol I, RNA polymerase I; RT, room temperature; SL1, promoter selectivity factor; TAF, TBP-associated factor; TAF<sub>1</sub>, TBP-associated factor for RNA polymerase I; TBP, TATA-binding protein; TIF, transcription initiation factor; UBF, upstream binding factor.

vivo. In particular, it would be essential to know the fate of the rDNA transcription machinery when ribosomal transcription is repressed. To address these questions, we took advantage of the physiological conditions provided by the cell cycle during which there are alternate phases of expression and repression of the rDNAs. In cycling cells, the rDNAs are expressed from telophase to the end of interphase and are repressed during mitosis (34). In prophase while rRNA synthesis is down-regulated, nucleoli disappear. The reformation of nucleoli occurs in telophase at specific chromosomal sites designated nucleolar organizer regions (NOR) (25, 33) and requires rDNA transcription (4, 5, 13). Thus, NORs are the sites where the rDNA transcription machinery is activated in telophase and consequently, the sites where the mitotic inactive state should be investigated to understand the transition between mitosis and interphase.

NORs are characterized by the presence of rDNA clusters and of several nonhistone proteins. In humans, NORs are located in the secondary constrictions of the 10 acrocentric chromosomes (chromosome pairs 13, 14, 15, 21, and 22) (references 1, 12, 20, 47). The 400 copies of rDNAs found in humans are distributed in a nonuniform manner on these different chromosomes (26). In addition "active" and "inactive" mitotic NORs were defined by the presence of a class of proteins designated Ag-NOR proteins. These proteins were found associated only with the NORs in which the rDNAs were to be efficiently expressed during interphase (30, 31). These observations indicated that even during mitosis, in the absence of any transcription (34, 51), there are differences in the nonhistone proteins associated to NORs depending on their function during interphase. Nonhistone proteins associated with NORs during mitosis have already been identified as components of the rDNA transcription machinery, such as subunits of RNA pol I (28, 29, 40, 51) and the RNA pol I transcription factor UBF (6, 38, 53), or as being essential for rRNA synthesis, such as DNA topoisomerase I (16). However, the characteristics of the mitotic rDNA transcription machinery are still poorly understood. For example, even if it was clearly established that some subunits of the RNA pol I are localized in NORs, it has never been proven that the RNA pol I complex is maintained during mitosis and associated with NORs. Similarly, the localization of SL1 has not yet been investigated either during mitosis or during interphase. Moreover, as for RNA pol I, the presence of the SL1 complex during mitosis has never been reported.

The aim of the present study was to investigate if the rDNA transcription machinery, as it was defined by *in vitro* reconstituted transcription systems, remains associated with NORs during mitosis. Other questions concern the respective distribution of RNA pol I and transcription factors in the different NORs that could explain the variability of NOR activity during interphase. In addition, the stability of this association during mitosis and the partition between the daughter cells would shed light on the transmission of the rDNA transcription machinery through cell cycle. Finally, these results will help to clarify the definition of "active" contrary to "inactive" NORs at least for the proteins involved in the rDNA transcription. Since the human NORs differ with respect to the number of rDNA copies and potential activities (9, 10), these are ideal con-

ditions to compare the rDNA machinery associated with the different types of NORs. In this communication, we demonstrate that the rDNA transcription machinery, as defined *in vitro*, is colocalized in some NORs and absent from others whatever the mitotic phase. The results show that RNA pol I and SL1 complexes are maintained during mitosis. In addition, RNA pol I and the transcription factors varied in the same proportions in the positive NORs, suggesting stoichiometric association of these components in complexes.

## Materials and Methods

### Purified Proteins, Antisera, and Antibodies

The mouse and yeast RNA pol I were provided respectively by I. Grummt (German Cancer Research Center, Heidelberg, Germany) and A. Senenac and M. Riva (CEA, Saclay, France). The mouse anti-TBP mAb (16E8) was obtained from R. Tjian and R. Weinzierl (University of California, Berkeley, CA), and the rabbit polyclonal anti-TBP antibodies prepared against the full-length recombinant human protein were from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-bromodeoxyuridine mAb was from Boehringer Mannheim France S.A. (Meylan, France). The polyclonal rabbit anti-TAF<sub>110</sub> (TBP-associated factor for RNA poly I [110]) antibodies, kindly provided by H. Beckmann (University of California, Berkeley, CA), were described previously (54). The human autoimmune serum with specificity against UBF (A17) was described (38). Two other autoimmune sera (A18 and V11) were characterized in the present study. These autoimmune sera contained only autoantibodies characterized as IgGs and directed against nucleolar proteins. The antibodies were affinity purified using nucleolar proteins electrophoresed in 10% SDS-polyacrylamide gels and electrotransferred. Antibodies were eluted by incubation in 100 mM glycine, pH 3, at room temperature (RT) for 10 min. The eluted fractions were neutralized by adding 10% (vol/vol) of 1 M Tris-HCl, pH 8. FITC- and Texas red-conjugated secondary antibodies, with specificity for rabbit or mouse IgGs and for human IgGs, respectively, were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Peroxidase-conjugated secondary antibodies with specificity for human, rabbit, or mouse IgGs were obtained from Amer-sham France (Les Ulis, France).

### Preparation of Chromosome-associated Protein Extracts for SDS-PAGE

HeLa cells were blocked in mitosis by colchicine treatment (0.1 µg/ml) for 14 h. Mitotic cells were harvested by mechanical shock. The procedure used to isolate chromosomes was adapted from Young et al. (52). The cells (at least 98% mitotic cells) were centrifuged at 100 g for 10 min, suspended at 10<sup>7</sup> cells per ml in 75 mM KCl, and incubated at 37°C for 15 min. The cells were centrifuged (100 g, 10 min) and suspended in polyamine buffer (15 mM Tris-HCl, pH 8, 2 mM EDTA, 0.5 mM EGTA, 80 mM KCl, 20 mM NaCl, 0.2 mM spermine, 0.5 mM spermidine, and 3 mM dithiothreitol). Cell membranes were then mechanically disrupted by addition of 0.25% (vol/vol) Triton X-100 and passage through a G22 needle until most of the chromosomes appeared scattered as assessed by phase microscopy. To discard nonlyzed mitotic cells and contaminating nuclei, the chromosome suspension was centrifuged on a 0.25 M sucrose cushion in polyamine buffer at 600 g for 5 min, and the supernatant recentrifuged on a similar sucrose cushion at 3,000 g for 30 min. SDS-PAGE sample buffer (27) was added to the pellet containing the chromosomes. These chromosome extracts were sonicated, boiled for 5 min, and centrifuged. The proteins in the supernatant corresponding to cytoplasm were precipitated by 5 vol of cold acetone and kept at -20°C for 1 h. The precipitated proteins were collected by centrifugation and solubilized in SDS-PAGE sample buffer. The cytoplasmic extracts were sonicated, boiled for 5 min, and centrifuged.

### Preparation of Cell, Nuclear, and Nucleolar Protein Extracts for SDS-PAGE

All extracts were prepared from exponentially growing HeLa cells. For

whole cell extracts, cells were washed in culture medium without serum and were lysed in SDS-PAGE sample buffer.

For nuclear and nucleolar protein extracts, cells were lysed at 4°C in TKM buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl and 3 mM MgCl<sub>2</sub>). Lysis was stopped when the nuclei appeared free of cytoplasmic components as assessed by phase microscopy. The nuclear and nucleolar proteins were prepared as previously described (37). The nucleoli were isolated by sonication of nuclei suspended in TKM buffer and purified by centrifugation on a 0.88 M sucrose cushion in TKM buffer at 1,300 g for 15 min. All steps were performed at 4°C, and all of the solutions contained an antiprotease cocktail: 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. The nucleolar proteins were solubilized in SDS-PAGE sample buffer.

### Immunoblotting

The proteins were separated by 10% SDS-PAGE using a Protean II cell (Bio-Rad Laboratories, Richmond, CA). Size standards from 200 to 14 kD (Bio-Rad Laboratories) were included in each gel. The polypeptides were electrotransferred to reinforced cellulose nitrate membranes (BA-S 83; Schleicher & Schuell, Dassel, Germany), that were then cut into strips. The strips were blocked by incubation for 1 h in PBS containing 5% (wt/vol) dried milk and 0.05% (vol/vol) Tween-20 and incubated with the sera for 2 h in the same buffer. They were then washed three times with PBS containing 5% (wt/vol) dried milk and 0.5% (vol/vol) Tween-20 and incubated for 1 h in the presence of HRP-labeled second antibodies. After several rounds of washing, the HRP activity was detected using the enhanced chemiluminescence kit (Amersham France) and recorded on x-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

### Immunoprecipitation

HeLa cells were metabolically labeled with [<sup>35</sup>S]methionine (Tran<sup>35</sup>S-label; ICN Biomedicals, Orsay, France). Interphase cells were scraped off in ice-cold PBS after eliminating mitotic cells by mechanical shock. Mitotic cells, obtained by colchicine treatment (0.1 µg/ml) overnight, were harvested by mechanical shock and washed in PBS. The interphase or mitotic cells were extracted in 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM EDTA, 1 mM iodoacetamide, and 1% (vol/vol) Triton X-100. Extracts were sonicated and centrifuged at 100,000 g for 1 h. The supernatants were adjusted to 50 mM Tris-HCl, pH 8, 2 mg/ml BSA, 150 mM NaCl, 5 mM EDTA, 1 mM iodoacetamide, and 0.2% (vol/vol) Triton X-100 and were used in the immunoprecipitation assays.

Before immunoprecipitation, antibodies were cross-linked to protein A covalently linked to agarose using the ImmunoPure IgG Orientation Kit (Pierce Chemical Co., Rockford, IL). Protein A-antibody complexes were incubated with extracts at 4°C for 2 h. Immunoprecipitates were washed twice in 50 mM Tris-HCl, pH 8, 2 mg/ml BSA, 150 mM NaCl, 5 mM EDTA, 1 mM iodoacetamide, and 1% (vol/vol) Triton X-100, once in 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM EDTA, 1 mM iodoacetamide, and 1% (vol/vol) Triton X-100, and finally twice in 100 mM Tris-HCl, pH 6.8. Each immunoprecipitation reaction was carried out using extracts from 500,000 cells. Immunoprecipitates obtained using human autoimmune sera (A18 and V11) and human nonautoimmune serum (C) were analyzed by 10% SDS-PAGE followed by autoradiography.

### Cell Culture and Immunofluorescence Labeling

HeLa cells were cultured in Eagle's minimum essential medium (Flow Laboratories, Paris, France) supplemented with 10% (vol/vol) FCS. Cells were seeded three times a week, and extracts were prepared 24 h after seeding from exponentially growing cells.

For immunolocalization, HeLa cells were grown as monolayers on glass slides. The glass slides were previously treated with 5% (wt/vol) KOH in methanol for 12 h, rinsed in water, treated with 0.01 N HCl for 1 h, washed in distilled water and ethanol, and sterilized. Cell monolayers were rinsed in PBS and fixed with 80% ethanol at -20°C for 8 min or with 2% (wt/vol) formaldehyde in PBS at RT for 20 min followed by washing in PBS and permeabilization with acetone at -20°C for 3 min.

HeLa chromosome spreading for immunolocalization was carried out as follows: HeLa cells were blocked in mitosis by incubation in 0.1 µg/ml of colcemid for 2 h. Mitotic cells harvested by mechanical shock were centrifuged at 100 g for 10 min, suspended at 10<sup>7</sup> cells per ml in FCS diluted 1:5, and incubated at RT for 25 min. Cells were then cytocentrifuged on glass slides and fixed in 2% (wt/vol) formaldehyde in PBS at RT for 20

min. After washing in PBS, cells were permeabilized with acetone at -20°C for 3 min.

For immunofluorescence labeling, cells grown as monolayers or chromosome spreads were incubated with sera at RT for 45 min, and the antibodies were revealed by Texas red-conjugated goat anti-human and/or FITC-conjugated goat anti-rabbit antibodies. DNA was visualized with 4',6-diamidino-2-phenylindole (DAPI). All preparations were mounted with an antifading solution (Citifluor, Canterbury, UK).

Fluorescent microscopy was performed using a microscope (DMRB; E. Leitz, Inc., Rockleigh, NJ). Images were photographed using a microscope camera system (DMRD; E. Leitz, Inc.). The superimposition of images was obtained by scanning micrographs using Ofoto 1.0.1 on Onescanner (Apple Computer, Inc., Cupertino, CA). Images were then assembled and printed using Canvas (Deneba Systems Inc., Miami, FL) and Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). All computer manipulations were done with a Macintosh Quadra 700 (Apple Computer Inc.). Images were printed directly from the computer on a printer (ColorEase PS Printer; Eastman-Kodak Co., Rochester, NY).

### Assay of RNA Polymerase Activity In Situ

The assay was performed on HeLa cells grown as monolayers essentially as described (32). Cells were incubated in the assay solution (100 mM Tris-HCl, pH 7.9, 12 mM 2-mercaptoethanol, 150 mM sucrose, 0.6 mM ATP, CTP, and GTP, 0.12 mM bromo-uridine 5'-triphosphate (BrUTP), and 12 mM MgCl<sub>2</sub>) at 37°C for 15 min. The reaction was terminated by rinsing the slides in PBS and postfixing with 2% (wt/vol) formaldehyde in PBS at RT for 15 min followed by permeabilization with acetone at -20°C for 3 min. BrUTP incorporation was then detected as previously described (49) by immunofluorescence labeling using a mouse anti-bromodeoxyuridine mAb revealed by FITC-conjugated goat anti-mouse antibodies. In some experiments, UBF was simultaneously detected using serum A17 revealed by Texas red-conjugated goat anti-human antibodies.

### Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was performed using an MRC-600 (Bio-Rad Laboratories), mounted on a microscope (Optiphot II; Nikon Inc., Garden City, NY) equipped with a ×60 objective (plan apo; NA 1.4). An argon ion laser adjusted to 488 nm was used for the fluorescein signal, and a helium-neon ion laser adjusted to 543 nm for Texas red. The emitted light was separated by a dichroic mirror (DR565; Bio-Rad Laboratories), and a (540DF30; Bio-Rad Laboratories) long pass filter was placed in front of the photomultiplier collecting the fluorescein emission. For each optical section, double fluorescent images were acquired in two passes: fluorescein first, Texas red second. The pinhole of the confocal system was adjusted to allow a field depth of ~0.5 µm, corresponding to the increment between two adjacent sections. A focal series including the green and red signals was collected for each specimen and then processed to produce single composite images (extended focus). For colocalization, each of the optical section or extended focus images were merged. In the latter case, stereo-pairs with a tilt of -0.6/+0.6 were generated, combining a high spatial resolution with the observation of the colocalization in the cell volume.

## Results

### Antibodies against the rDNA Transcription Machinery

The characterization of sera directed against the transcription machinery was carried out using nucleolar extracts prepared from actively growing HeLa cells and partially purified RNA pol I complex. The human autoimmune serum A18 recognized two polypeptides in HeLa cell nucleolar extracts (Fig. 1 A). The molecular masses of these two polypeptides were 200 and 120 kD. Using partially purified mouse RNA pol I complex (43, 44), these two polypeptides were identified as the two largest subunits of RNA polymerase I (Fig. 1 B). A18 also recognized the two largest subunits of the purified yeast RNA pol I (data not shown). Similarly, serum V11 that recognized a 60-kD polypeptide in HeLa cell nucleolar extracts (Fig. 1 A) rec-

ognized the 62-kD polypeptide corresponding to the third largest subunit of the partially purified mouse RNA pol I complex (Fig. 1 B). Serum A17, previously characterized as containing anti-UBF antibodies (38) and anti-TBP antibodies, were tested on these nucleolar extracts (Fig. 1 A). UBF, appearing as a doublet of 97–94 kD, and TBP were present in the nucleolar extracts. Except for TBP mainly found in nuclear extracts, UBF and the antigens revealed by A18 and V11 are concentrated in nucleolar extracts as illustrated for V11 (Fig. 2).

Immunoblotting seemed to indicate that serum A18 recognized the two largest subunits of RNA pol I, and serum V11 recognized the third largest subunit of RNA pol I. To confirm the specificity of these sera, immunoprecipitations were carried out anticipating that by using antibodies directed against one or two RNA pol I subunits, it would be possible to immunoprecipitate the entire RNA pol I complex and a similar complex with both sera. At least with respect to the major bands immunoprecipitated using both the A18 and V11 sera, the molecular masses correlated well with those of subunits of the RNA pol I complex typical of mammalian cells (43). The major bands immunoprecipitated from interphase cell extracts using A18 and V11 were 190-, 120-, 52-, 43-, 29-, and 17-kD polypeptides (Fig. 1 C). Minor bands were observed of 185, 170, 155, and 135 kD, corresponding most likely to proteolytic products of the 190-kD polypeptide. These cleavages were also observed in enriched mouse RNA pol I extracts (data not shown), in favor of the instability of the highest RNA pol I subunit. Serum V11 recognized a 60-kD polypeptide in human nucleolar extracts (Fig. 1 A) and immunoprecipitated a complex, as did A18 in which no 60 kD was found (Fig. 1 C). As the 52-kD polypeptide immunoprecipitated with both sera is more abundant with V11 than with A18, this

polypeptide could be a proteolytic product of the 60-kD subunit. However, the most striking point is the similarity of the complexes immunoprecipitated. This similarity argues in favor of the fact that both sera are directed against antigens of the same complex that are most likely different subunits of the RNA pol I complex.

### Association of the rDNA Transcription Machinery with Chromosomes during Mitosis

The antibodies were probed on chromosome-associated protein extracts and cytoplasmic extracts prepared from the same number of mitotic cells. The results obtained with serum V11, anti-UBF, and anti-TBP antibodies are presented (Fig. 3). The 60-kD polypeptide revealed by V11 (corresponding to the 60-kD subunit of the RNA pol I) was found only in the chromosome-associated protein extracts (Fig. 3, lane a), but not in the cytoplasmic extracts (Fig. 3, lane b). Likewise, UBF was observed in the chromosome-associated protein extracts (Fig. 3, lane c). A very weak amount of UBF, only detected when the film was overexposed, was present in the cytoplasmic extracts (not visible in lane d), most likely due to release during chromosome isolation. Similarly TBP (Fig. 3, lane e) was present in the chromosome-associated protein extracts.

The presence of one subunit of the RNA pol I that associated with chromosomes during mitosis did not prove the association of all the subunits of the complex. Therefore, immunoprecipitations were carried out to investigate the fate of the RNA pol I complex during mitosis. In mitotic cell extracts, similar results were obtained using sera A18 (Fig. 4 b) and V11 (Fig. 4 c). The same major bands were observed as in interphase but with two additional bands of 75 and 41 kD. The fact that similar patterns were obtained

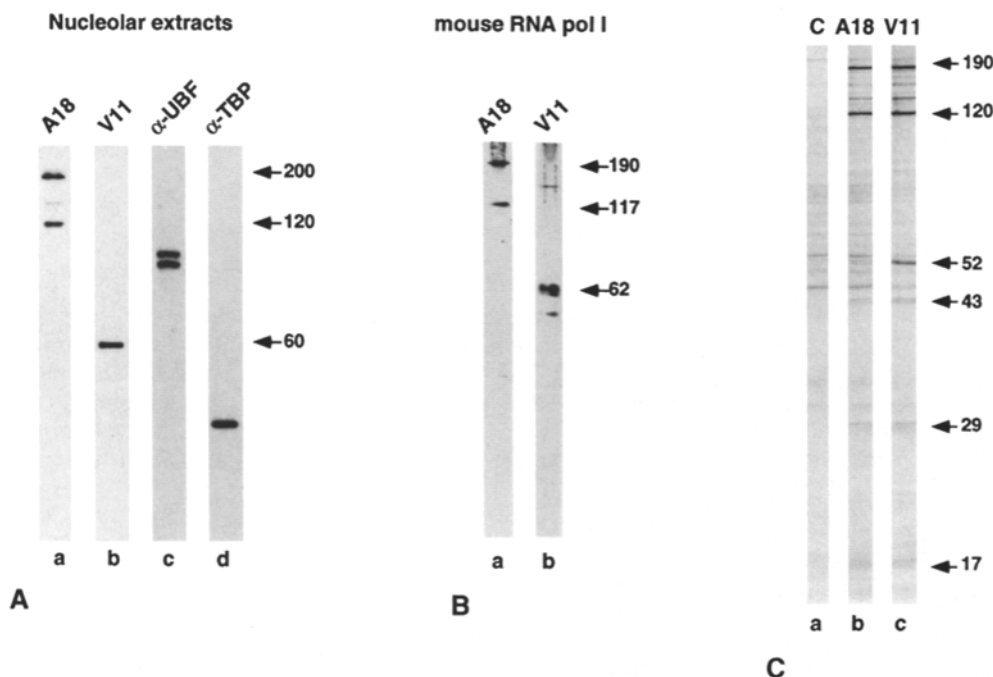
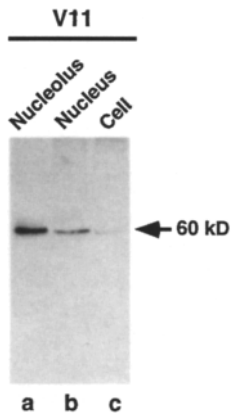


Figure 1. Identification of antigens by immunoblotting and immunoprecipitation. (A) HeLa nucleolar protein extracts revealed on Western blots by the sera A18 (lane a, revealing two polypeptides of 200 and 120 kD), V11 (lane b, revealing a 60-kD polypeptide), anti-UBF antibodies (lane c), and by anti-TBP antibodies (lane d). Numbers indicate the molecular masses in kD of proteins revealed with the sera A18 and V11. (B) Partially purified mouse RNA pol I revealed on Western blots by A18 (lane a) and V11 (lane b), recognizing the two largest subunits and the third largest subunit of mouse RNA pol I, respectively. (C) Immunoprecipitation assays carried out using control serum (C; lane a) and anti-RNA pol I antibodies (A18 and V11; lanes b and c) on interphase HeLa cell extracts.

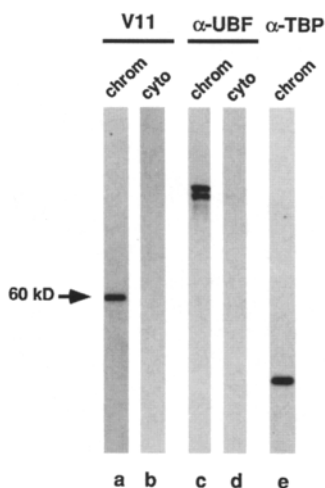


**Figure 2.** Localization of antigens during interphase by immunoblotting. The same amount of nucleolar (lane *a*), nuclear (lane *b*), and cell protein extracts (lane *c*) revealed on Western blots with V11. The position of the 60-kD polypeptide is indicated.

using mitotic cell extracts and interphase cell extracts proves that the RNA pol I complex present during interphase is also present during mitosis. We conclude that the subunits composing the RNA pol I complex remain associated during mitosis.

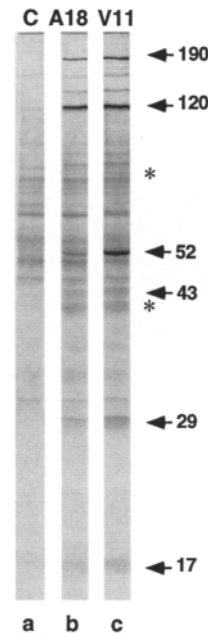
### Localization of the rDNA Transcription Machinery during Mitosis

In the chromosome-associated protein extracts of HeLa mitotic cells, we observed the presence of the RNA pol I subunits, TBP, and UBF by immunoblotting. This is compatible with the hypothesis that the rDNA transcription machinery remains associated to rDNAs during mitosis. To test this hypothesis, we proceeded to immunodetect in mitotic cells the major components of this machinery to define if they are colocalized. We first detected each component individually. As expected by the results of immunoblotting, RNA pol I, UBF, and TBP remained associated with the condensed chromosomes at all phases of the mitosis (data not shown). We also investigated the possible colocalization of the transcription factors SL1 and UBF that, in addition to RNA pol I, are necessary and sufficient to promote rDNA transcription in human cells. Since SL1 is composed of TBP, TAF<sub>110</sub>, TAF<sub>63</sub>, and TAF<sub>48</sub>, the localization of SL1 in mitotic HeLa cells was carried out using anti-TBP and anti-TAF<sub>110</sub> antibodies.



**Figure 3.** Localization of antigens during mitosis by immunoblotting. Comparable amounts of chromosome (lanes *a*, *c* and *e*) or cytoplasmic extracts (lanes *b* and *d*) revealed on Western blots with V11 (lanes *a* and *b*), anti-UBF antibodies (lanes *c* and *d*), and anti-TBP antibodies (lane *e*). The 60-kD polypeptide revealed by V11 (lane *a*) and UBF (lane *c*) is present in chromosome extracts and absent from cytoplasmic extracts (lanes *b* and *d*). TBP (lane *e*) is partially present in chromosome extracts.

### Immunoprecipitation (mitosis)

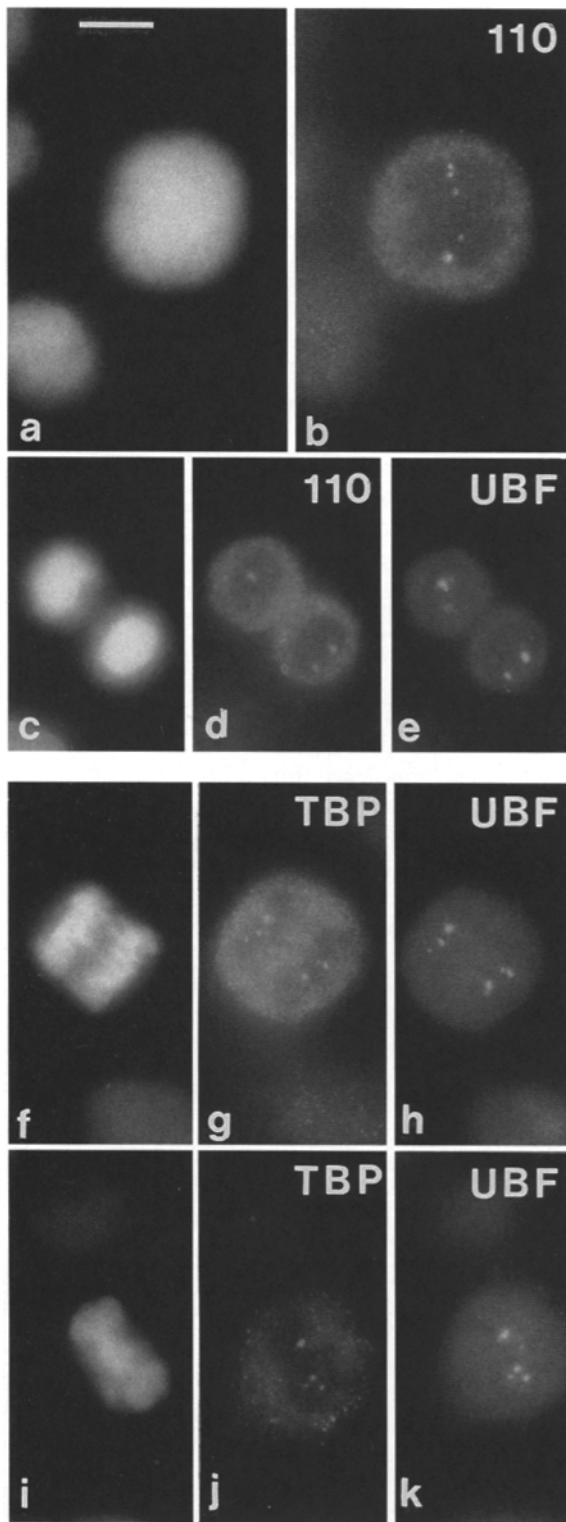


**Figure 4.** Immunoprecipitation assays carried out using control serum (*C*; lane *a*) and anti-RNA pol I antibodies (*A18* and *V11*; lanes *b* and *c*) on mitotic HeLa cell extracts. Proteins specifically immunoprecipitated are indicated by their molecular masses in kD. The asterisks point to specifically immunoprecipitated bands only observed with mitotic cell extracts.

As illustrated for early metaphase (Fig. 5 *b*), labeling corresponding to TAF<sub>110</sub> appeared as discrete spots of variable sizes in association with chromosomes (compare Fig. 5, *a* and *b*). The examination of UBF and TAF<sub>110</sub> in the same HeLa mitotic cells (illustrated for telophase, Fig. 5, *c-e*) showed a similar localization of TAF<sub>110</sub> and UBF and similar variations in the size of the spots. Indeed, similar patterns were obtained in both cases: the larger and smaller spots revealed with anti-TAF<sub>110</sub> antibodies (Fig. 5 *d*) corresponded respectively to the larger and smaller spots revealed with anti-UBF antibodies (Fig. 5 *e*). Similar results were obtained whatever the mitotic stage examined (data not shown).

The localization of TBP during mitosis in HeLa cells is illustrated for anaphase (Fig. 5 *g*) and for metaphase (Fig. 5 *j*). In both anaphase and metaphase, as was the case at all stages of mitosis (data not shown), part of TBP appeared in discrete spots in association with chromosomes. As expected, part of TBP was not associated with chromosomes but was dispersed in the cytoplasm (Fig. 5 *g*). The localization of both TBP and UBF in the same mitotic cells (Fig. 5, *f-h*, and *i-k*) showed that at all mitotic stages, the part of TBP appearing in spots in association with chromosomes was localized in the same position as UBF. As reported for TAF<sub>110</sub>, the spots varied in size, but these variations were similar for TBP and for UBF (compare Fig. 5, *g* and *h*, and *j* and *k*). The similar localization of TAF<sub>110</sub> and TBP indicates that the transcription factor SL1 is most probably maintained during mitosis and localized in NORs during mitosis as is UBF (38). The fact that the size of the spots varied in a correlated manner for UBF, TAF<sub>110</sub>, and TBP indicates that the amounts of UBF, TAF<sub>110</sub>, and TBP vary in the same proportions between different positive NORs.

To validate these observations, the colocalization of these factors in the volume of mitotic cells was established



**Figure 5.** Localization of UBF and TAF<sub>110</sub>, and of UBF and TBP, in the same mitotic cells. (a and b) Early metaphase HeLa cell labeled with anti-TAF<sub>110</sub> antibodies. The labeling appears in discrete spots (b) in association with chromosomes revealed by DAPI (a). (c–e) Telophase HeLa cell labeled both with anti-TAF<sub>110</sub> (d) and anti-UBF antibodies (e) and DNA stained by DAPI (c). The colocalization of UBF and TAF<sub>110</sub> is shown by identical labeling patterns obtained with both antibodies. The weak cytoplasmic labeling observed with anti-TAF<sub>110</sub> antibodies is homogeneous and visible in mitotic and interphase cells.

by confocal microscopy. The fluorescent patterns observed in the same mitotic HeLa cells for RNA pol I (using V11 and A18) and TBP (Fig. 6) or for UBF and TBP (data not shown) were identical from prophase to telophase. The same number of spots, and spots varying in size in the same proportions, were observed for both labelings in the two cases using extended focus images obtained after three-dimensional reconstruction of the serial optical sections (Fig. 6; illustrated for anaphase, metaphase, and interphase). The superimposition of RNA pol I and TBP labelings recorded in the same optical section indicated strict colocalization of RNA pol I and TBP in mitosis as well as in the nucleolus during interphase (Fig. 6, C, C', and C'', and D, D', and D''). In extended focus images six chromosomes were scored positive for RNA pol I colocalized with TBP (Fig. 6, A, A', and A'') and also for UBF colocalized with TBP (data not shown).

Because UBF is known to remain associated with only some NORs during mitosis (6, 38, 53), these results indicated that UBF, RNA pol I, and SL1 (verified for TBP and TAF<sub>110</sub>) were associated with the same NORs during mitosis in HeLa cells.

#### *Different Types of NORs*

In human cells there are 10 NOR-bearing chromosomes, but the rDNA transcription machinery remains associated in variable amounts with only six of them. We were interested in identifying the positive NOR-bearing chromosomes to determine if they constitute a characteristic in a given cell line.

Chromosome spreads prepared from HeLa cells blocked in mitosis made it possible to recognize acrocentric chromosomes (Fig. 7) that were previously proved to be the NOR-bearing chromosomes in human cells. Immunolabelings of UBF and RNA pol I (Fig. 7) obtained on HeLa chromosome spreads were similar. In both cases, six spots or double-spots were visible. These proteins were not equally distributed between NORs. Some NOR-bearing chromosomes scored negative, and the intensity of the labeling of the six positive NORs differed. Interestingly, UBF and RNA pol I varied in the same proportions in the six different positive NORs.

In this HeLa cell line, the labeling pattern was the same in the different chromosome spreads observed using antibodies directed against UBF or the RNA pol I subunits (Fig. 7). There were always six positive NOR-bearing chromosomes, three small acrocentric chromosomes (chro-

These images are reminiscent of background often associated using rabbit antibodies. (f–h) Anaphase HeLa cell labeled with anti-TBP (g) and anti-UBF antibodies (h) and DNA stained by DAPI (f). TBP labeling (g) appears as discrete spots in association with chromosomes (compare f and g) and as labeling dispersed in the cytoplasm. The discrete spots revealed by anti-TBP coincide exactly with the spots revealed by anti-UBF antibodies (h). (i–k) Metaphase HeLa cell labeled with anti-TBP (j) and with anti-UBF antibodies (k) and DNA stained by DAPI (i). A part of the TBP labeling appears as discrete spots (j) in association with chromosomes (i). The spots revealed with anti-TBP antibodies (j) coincide exactly with the spots revealed with anti-UBF antibodies (k). Bar, 10  $\mu$ m.

mosomes 21 or 22), and three large acrocentric chromosomes (chromosomes 13, 14, or 15). Thus, for a pair of homologous NOR-bearing chromosomes, the first chromosome could be positive, whereas the second one could be negative. Moreover, when the labeling of a positive chromosome appeared as a double-spot (i.e., when the two spots corresponding to the two chromatids were not coalesced), both spots composing the double-spot were of equal intensity. Therefore, the amount of protein associated with both chromatids of the same chromosome seemed to be equivalent. This explained the equal partition of UBF and RNA pol I and consequently of the rDNA transcription machinery during anaphase when the separation of chromatids occurs.

Keeping in mind that the different components of the rDNA transcription machinery are colocalized during mitosis, we can conclude that RNA pol I, UBF, and also SL1 are associated with the same NORs in a given cell and always with the same NORs in this HeLa cell line, and that they vary in the same proportions in the different positive NORs.

### *The Transcription Activity of Positive NORs*

Because the reformation of nucleoli occurs in telophase at NORs and requires rDNA transcription, we could predict that NORs are the sites where the rDNA transcription machinery is activated. To verify this point, we took advantage of the fact that RNA pol activities may be preserved in situ in cells (32). During interphase the RNA pol I activity (insensitive to  $\alpha$ -amanitin) occurring in nucleoli is particularly well detected in the conditions used (32). At the transition interphase/mitosis, the RNA pol I activity stopped rapidly before the destructuring of nucleoli (data not shown). During mitosis, no RNA pol activity was observed as illustrated for prometaphase HeLa cells (Fig. 8, C and C'), except for during telophase (Fig. 8, A, A', A'', B, B', C, and C'). During telophase RNA pol I activity was derepressed as evidenced by the incorporation of BrUTP appearing in spots in association with chromosomes (compare Fig. 8, A', A'', A, B', B, C', and C). The fluorescent patterns observed in the same telophase HeLa cells for BrUTP incorporation and for UBF (Fig. 8, B', B'', C', and C'') showed that both labelings were superimposable. It is noteworthy that the unusual background obtained using anti-UBF antibodies was most likely due to the procedure used to detect RNA pol activity. The localization of UBF, and consequently of the rDNA transcription machinery, and the detection of RNA pol I activity made it possible to observe that the rRNA transcription machinery was derepressed at telophase at NORs. Because up to six spots corresponding to sites of RNA pol I activity could be observed in telophase HeLa cells (Fig. 8, A' and A''), the rDNA transcription machinery associated with the six NORs seemed to be derepressed simultaneously. Moreover, since the intensity of the labelings corresponding to the sites of BrUTP incorporation varied in the same proportion as the size of the spots corresponding to UBF (compare Fig. 8, B' and B'', and C' and C''), the level of transcription activity seemed to be directly related to the amount of rDNA transcription machinery present in the NOR.

## **Discussion**

In vitro studies made it possible to define the rDNA transcription machinery necessary to obtain accurate in vitro rDNA transcription. The rDNA transcription machinery is composed in humans of the RNA pol I complex, the transcription factor UBF, and the transcription factor SL1, composed of TBP and three TAF<sub>1</sub>s: TAF<sub>110</sub>, TAF<sub>63</sub>, and TAF<sub>48</sub> (2, 3, 7, 24). It was recently proven that the three TAF<sub>1</sub>s together with TBP are necessary and sufficient to reconstitute a transcriptionally active SL1 complex (54). In vivo, expression of the rDNAs is dependent on cell cycle and cell growth and takes place in the nucleoli. During mitosis, rDNA transcription is arrested, but the early resumption of its transcription at telophase raises the question of the fate of the rDNA transcription machinery during mitosis. As the NORs are the sites where rDNA transcription is activated in telophase (4, 5, 13, 25, 33), we focused our attention on the proteins associated with the NORs during mitosis to investigate the transitions between mitosis and interphase.

### *Targeting of the rDNA Transcription Machinery during Mitosis*

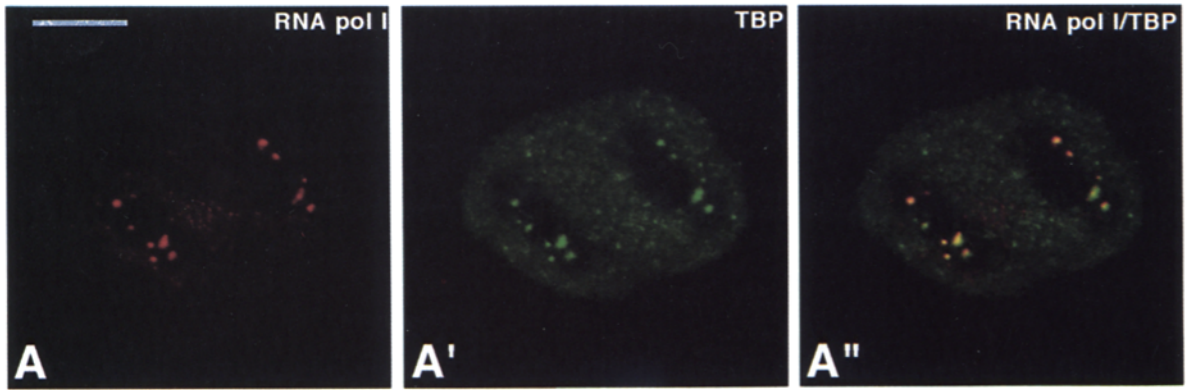
For accurate rDNA transcription, it seems reasonable to postulate that the same partners interact in vivo as in vitro. In this case, the minimal transcription machinery for in vivo transcription is active RNA pol I complex and the specific RNA pol I transcription factors UBF and SL1.

RNA pol I has already been localized into NORs during mitosis in different mammalian cells (14, 17, 18, 23, 40, 51). The mammalian subunits composing the RNA pol I complex are not as well characterized as the yeast subunits (45), but recent progress indicated a similar number of subunits in mouse and yeast RNA pol I complex (46). In the present study, we present evidence for the presence of the three largest RNA pol I subunits in the NORs by immunolocalization, and by immunoprecipitation we demonstrate that mitotic cell extracts contain RNA pol I complexes (see below for the discussion of the mitotic RNA pol I complex).

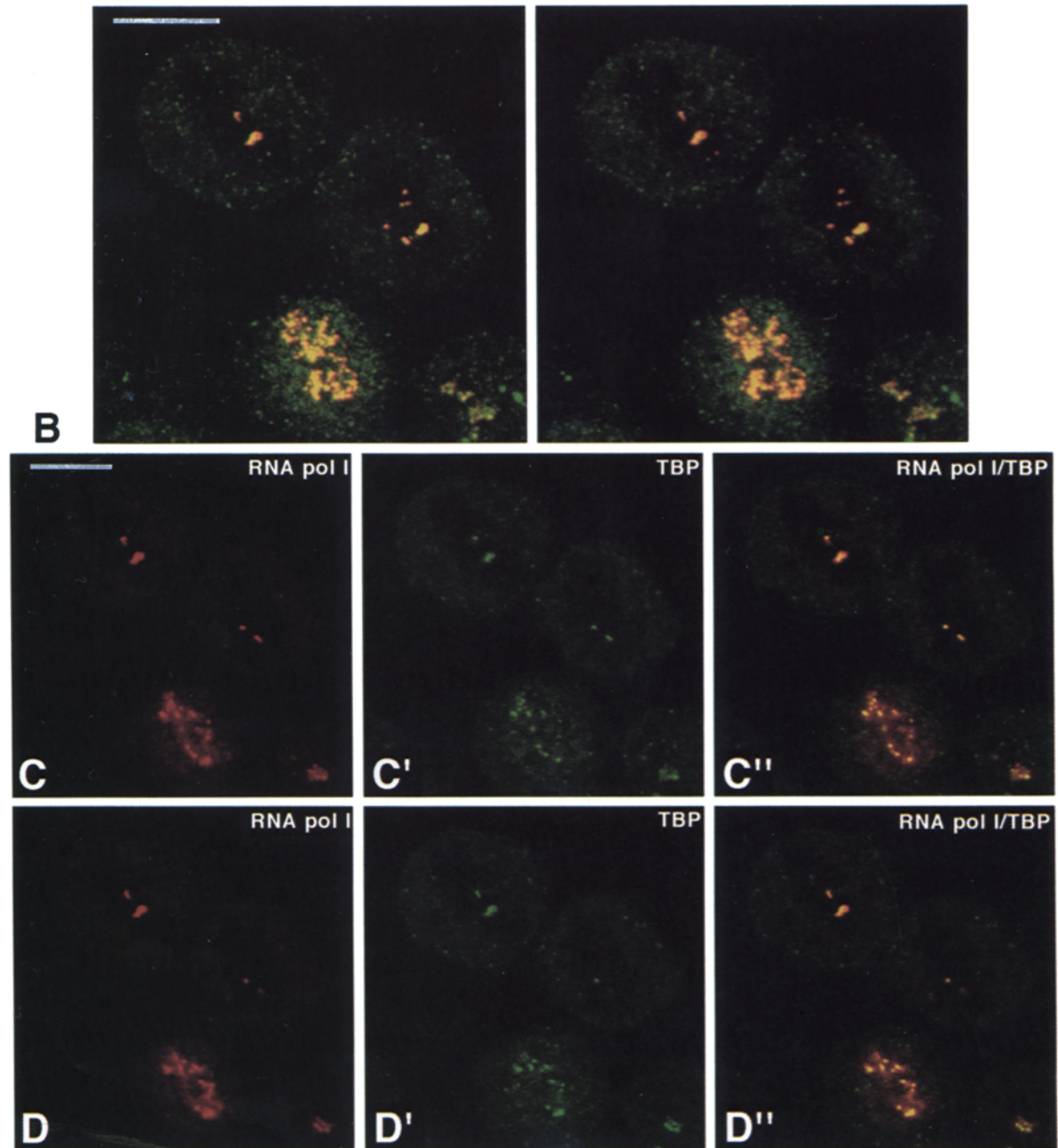
RNA pol I transcription factors were also associated with the mitotic chromosomes as already reported for the UBF (6, 35, 38, 53). In addition, we proved that SL1 (as verified for TBP and TAF<sub>110</sub>) is most likely maintained during mitosis and also remains associated with NORs. Moreover, all of the rDNA transcription machinery as defined in vitro is colocalized at NORs during mitosis: RNA pol I, UBF, and SL1 are associated with the same NORs whatever the mitotic stage investigated. We failed to obtain labeling with TAF<sub>48</sub> antibodies in mitosis or in interphase. Because we cannot suspect the absence of TAF<sub>48</sub> in active rDNA transcription machinery in interphase, this result is most likely due to inaccessibility of the epitopes or modifications induced by fixing the cells.

### *The rDNA Transcription Machinery Is Colocalized in Active NORs and Absent from Inactive NORs*

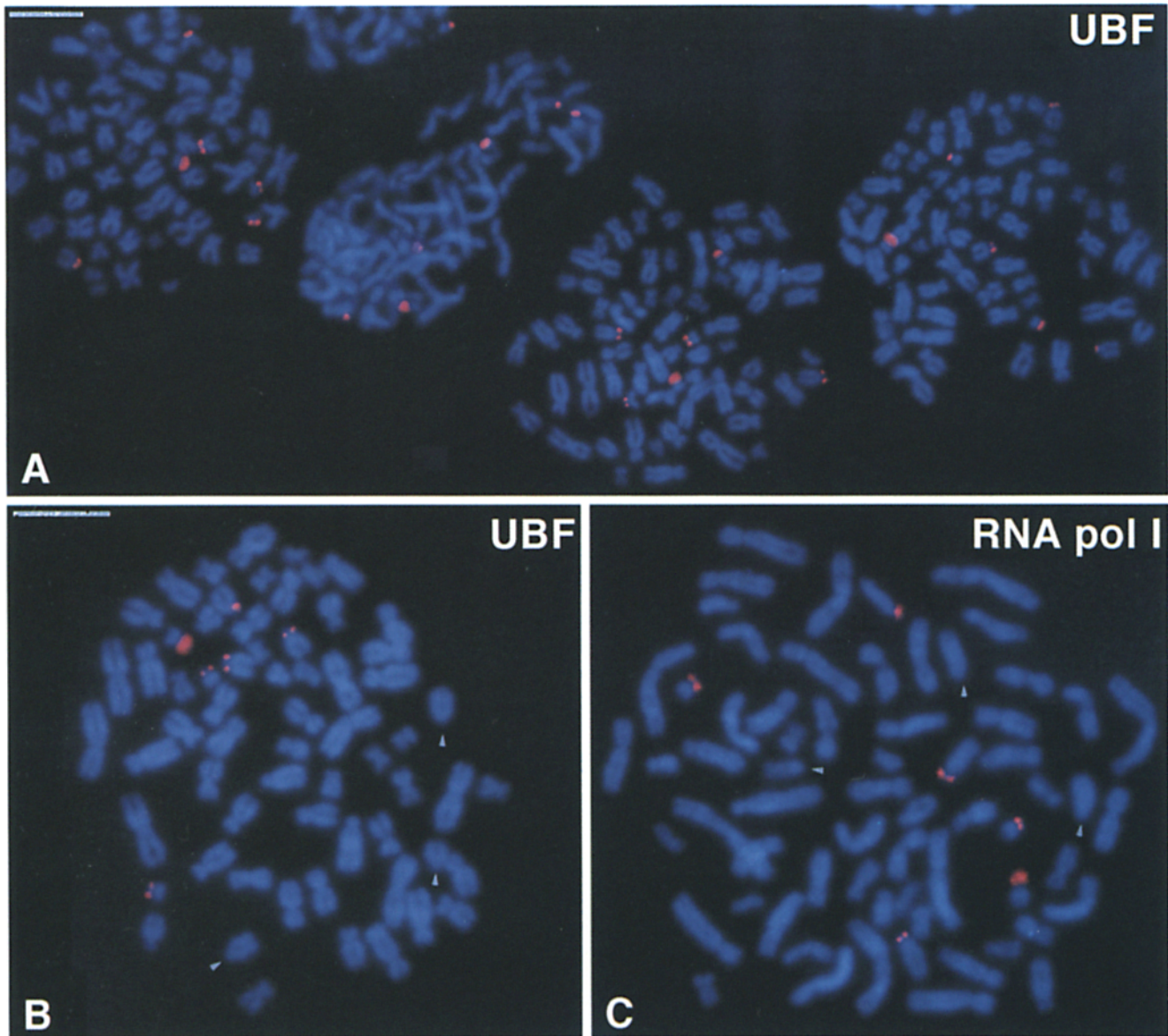
The active NORs were previously defined as NORs in which potentially active rDNAs are located. This implies that these genes can be transcribed during interphase as



3-D stereoscopic projections (RNA pol I/TBP)





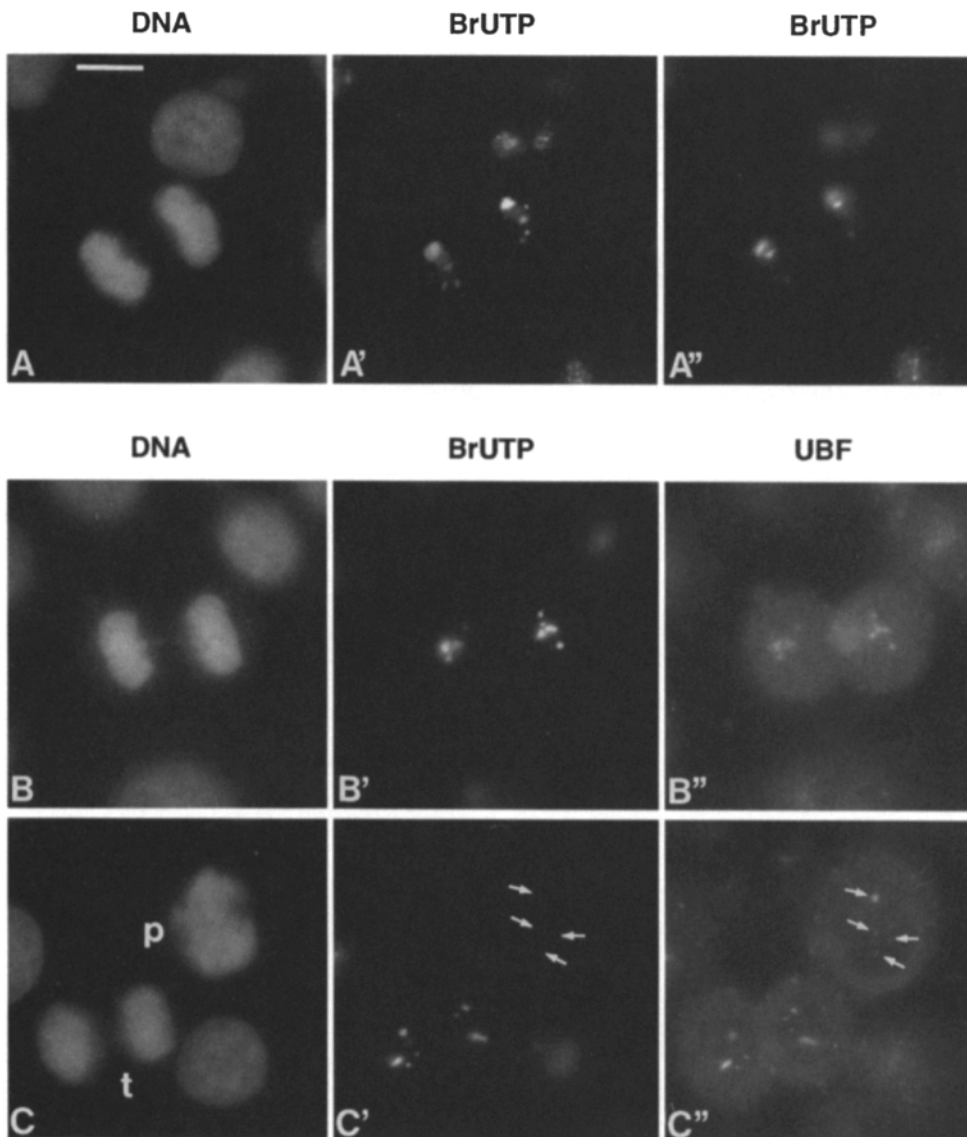


**Figure 7.** Localization of UBF and RNA pol I by immunofluorescence on HeLa chromosome spreads. HeLa chromosome spreads stained by DAPI and labeling obtained with anti-UBF antibodies (*A* and *B*) or with V11 (*C*) were superimposed. The overall picture (*A*) shows that the labeling pattern is the same when comparing one chromosome spread to another. The comparison between labeling obtained with anti-UBF (*B*) and anti-RNA pol I antibodies (*C*) demonstrates that the same six acrocentric chromosomes (NOR-bearing chromosomes in humans) are labeled. Both labelings vary in the same proportions in the six positive chromosomes. The acrocentric chromosomes devoid of either labeling are indicated by arrowheads (*B* and *C*). Bar, 5  $\mu\text{m}$ .

opposed to the other rDNA copies that cannot be transcribed. As demonstrated by experiments using interspecific somatic cell hybrids, NORs bearing potentially active rDNAs are characterized by their association with Ag-

NOR proteins (30, 31). The Ag-NOR proteins are easily visualized in situ by cytological silver staining (15, 22). We previously reported that in mitotic HeLa cells, UBF is only localized in active NORs where Ag-NOR proteins are also

**Figure 6.** Confocal laser scanning microscopy demonstrating colocalization of RNA pol I and TBP during mitosis. (*A*, *A'*, and *A''*) The anaphase HeLa cell was labeled with V11 and anti-TBP antibodies. (*A*) Extended focus of labeling obtained with V11. (*A'*) Extended focus of labeling obtained with anti-TBP antibodies. (*A''*) Merged image of both labelings. Yellow color indicates colocalization of RNA pol I and TBP present in six spots in association with chromosomes of each daughter cell. (*B–D*) Metaphase and interphase HeLa cells were labeled with V11 and anti-TBP antibodies. (*B*) Three-dimensional stereoscopic projections of optical sections of both labelings showing the colocalization of RNA pol I and TBP in the cell volume. *C*, *C'*, and *C''*, and *D*, *D'*, and *D''* show the labeling obtained with V11 (*C* and *D*) and anti-TBP antibodies (*C'* and *D'*) in two successive optical sections. (*C''*) corresponds to the merged image of both labelings (shown in *C* and *C'*) recorded in the first optical section, and *D''* corresponds to the merged image of both labelings (shown in *D* and *D'*) recorded in the second optical section. Yellow color indicates strict colocalization of both labelings. Bar, 10  $\mu\text{m}$ .



**Figure 8.** Detection of RNA pol I activity in mitotic HeLa cells. (A, A', A'') The telophase HeLa cell was processed to detect the sites of BrUTP incorporation. (A) DNA staining. (A' and A'') Two differently focused images of the six sites of BrUTP incorporation in association with the two groups of chromosomes revealed by DAPI (A). (B, B', B'') The telophase HeLa cell was processed to detect simultaneously BrUTP incorporation and UBF. (B) DNA staining. (B') BrUTP incorporation. (B'') UBF labeling. (C, C', C'') Telophase and prometaphase HeLa cells were processed to detect simultaneously BrUTP incorporation and UBF. (C) DNA staining allowing recognition of the telophase (t) and the prometaphase cell (p). (C') BrUTP incorporation. (C'') UBF labeling. The arrows point to the labeling observed for UBF (C'') and the absence of BrUTP incorporation (C') in the prometaphase cell. Bar, 10  $\mu$ m.

localized (38). These results are corroborated by the identification of the largest subunit of RNA pol I and UBF as Ag-NOR proteins (36). Considering the published results (9, 10, 30, 31) and the results obtained in this study concerning the colocalization of RNA pol I, UBF, and SL1, we may conclude that the rDNA transcription machinery remains associated only with active NORs during mitosis. In the inactive NORs, the rDNAs are not associated with RNA pol I, UBF, or SL1. We do not know what prevents such associations. However, the level of methylation of the rDNAs could at least be partly responsible, since high levels of rDNA methylation are correlated with inactive rDNAs (9, 10). The presence of a repressor inhibiting such associations can also be proposed, but this hypothesis is not presently supported by biological evidence.

#### ***The rDNA Transcription Machinery Is Not Randomly Distributed in Different Active NORs***

The rDNA transcription machinery remains associated

only with the active NORs during mitosis (i.e., six active NORs in the HeLa cell line used). In mitotic cells and chromosome spreads, the number of positive chromosomes are always the same in a given cell line. This characteristic has also been reported for Ag-NOR proteins (47). In addition, the amount of NOR-associated proteins varies between different active NORs in human cells, and this variation correlates with NOR activity during interphase as proposed for Ag-NOR proteins (1) and for UBF (38).

As shown for UBF (38, 53), the rDNA transcription machinery is not equally distributed between active NORs. Indeed the intensity of the labeling of positive NORs differed. Double immunofluorescence labeling experiments showed that the different components of the rDNA transcription machinery vary in the same proportions in different positive NORs, suggesting a stoichiometric association of these different components. By analogy with Ag-NOR proteins for which the amount in a given NOR is related to the number of potentially active rDNA copies in this NOR (10, 48), we propose that the amount of each component

of the rDNA transcription machinery in an active NOR reflects the number of potentially active rDNA copies (i.e., that will be transcribed during the following interphase).

### **Components of the rDNA Transcription Machinery Are Not Released into the Cytoplasm in Mitotic Cells**

By an immunocytochemical approach, RNA pol I, UBF, and TAF<sub>110</sub> were colocalized into NORs, whereas no labeling was found in the cytoplasm of mitotic cells. Because of the detection limits of immunofluorescent labeling when the antigens are diffusely distributed, it was necessary to confirm these results by another approach. This was performed by immunoblotting using cytoplasmic and chromosome extracts prepared from mitotic cells. The targeting of RNA pol I and UBF on the chromosomes was confirmed since they were not detectable or very weakly detectable in cytoplasmic extracts. In this latter case, the signal of the cytoplasmic extracts compared to the chromosome extracts most likely indicates a release during cell lysis and chromosome isolation from the cytoplasm. This finding is in agreement with results of biochemical studies on isolated metaphase chromosomes, demonstrating that RNA pol I is primarily associated with NOR-bearing chromosomes (28). Similarly, UBF is more difficult to solubilize from mitotic than from interphase cells (23), and this can be due to a more efficient association with the rDNA promoter during mitosis than during interphase. Concerning TBP, labeling was observed only partially in NORs as expected, because TBP is involved in RNA pol I, II, and III transcription (21). Therefore, only the part of TBP implicated in rDNA transcription appears associated with rDNAs in NORs during mitosis. The selectivity of the association of TBP with rDNAs is determined by its exclusive association with TAF<sub>s</sub> (8). This implies that TAF<sub>s</sub> are associated to TBP in NORs and that the presence of TAF<sub>s</sub> determines the localization of TBP at NORs during mitosis.

### **What Is an Active NOR Compared to an Inactive NOR?**

By definition, during mitosis, active NORs are the NORs in which the rDNAs are associated with Ag-NOR proteins. It has been demonstrated that the presence of these NOR proteins indicates the potential of ribosomal genes to be transcribed during interphase as opposed to the ribosomal genes that are not. However, as long as the NOR proteins were not characterized, the potential to be transcribed remained indefinite. In the present work, we established the link between the original definition of active NORs and rDNA transcription. Therefore during mitosis an active NOR is a NOR in which some rDNAs associated with their specific transcription machinery are gathered and in which rDNA transcription is derepressed at telophase. In this study, equal partition of the rDNA transcription machinery between daughter cells was observed, indicating that in cycling cells, the level of the rDNA transcription machinery remaining associated with NORs during mitosis determines rDNA transcription activity at the following interphase. We propose that active NORs are the chromosomal domains around which nucleoli will be organized at the end of mitosis when rDNA transcription

will be derepressed, and that conversely, the inactive NORs are excluded from nucleogenesis.

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### **References**

1. Babu, K.A., and R.S. Verma. 1985. Structural and functional aspects of nucleolar organizer regions (NORs) of human chromosomes. *Int. Rev. Cytol.* 94:151-176.
2. Bell, S.P., R.M. Learned, H.-M. Jantzen, and R. Tjian. 1988. Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. *Science (Wash. DC)*. 241:1192-1197.
3. Bell, S.P., C.S. Pikaard, R.H. Reeder, and R. Tjian. 1989. Molecular mechanisms governing species-specific transcription of ribosomal RNA. *Cell*. 59:489-497.
4. Benavente, R., K.M. Rose, G. Reimer, B. Hügler-Dörr, and U. Scheer. 1987. Inhibition of nucleolar reformation after microinjection of antibodies to RNA polymerase I into mitotic cells. *J. Cell Biol.* 105:1483-1491.
5. Benavente, R., M.S. Schmidt-Zachmann, B. Hügler-Dörr, G. Reimer, K.M. Rose, and U. Scheer. 1988. Identification and definition of nucleolus-related fibrillar bodies in micronucleated cells. *Exp. Cell Res.* 178:518-523.
6. Chan, E.K.L., H. Imai, J.C. Hamel, and E.M. Tan. 1991. Human autoantibody to RNA polymerase I transcription factor hUBF. Molecular identity of nucleolus organizer region autoantigen NOR-90 and ribosomal RNA transcription upstream binding factor. *J. Exp. Med.* 174:1239-1244.
7. Comai, L., N. Tanese, and R. Tjian. 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell*. 68:965-976.
8. Comai, L., J.C.B.M. Zomerdijk, H. Beckmann, S. Zhou, A. Admon, and R. Tjian. 1994. Reconstitution of transcription factor SL1: exclusive binding of TBP by SL1 or TFIID subunits. *Science (Wash. DC)*. 266:1966-1972.
9. de Capoa, A., P. Marlekaj, A. Baldini, M. Rocchi, and N. Archidiacono. 1985. Cytologic demonstration of differential activity of rRNA gene clusters in different human tissues. *Hum. Genet.* 69:212-217.
10. de Capoa, A., C. Alexandre, M.P. Felli, L. Ravenna, M.A. Costantino, P. Giancotti, O. Vicenti, I. Poggesi, C. Grappelli, and D.A. Miller. 1991. Inheritance of ribosomal gene activity and level of DNA methylation of individual gene clusters in a three generation family. *Hum. Genet.* 88:146-152.
11. Eberhard, D., L. Tora, J.-M. Egly, and I. Grummt. 1993. A TBP-containing multiprotein complex (TIF-IB) mediates transcription specificity of murine RNA polymerase I. *Nucleic Acids Res.* 21:4180-4186.
12. Evans, H.J., R.A. Buckland, and M.L. Pardue. 1974. Location of the genes coding for 18S and 28S ribosomal RNA in the human genome. *Chromosoma (Berl.)*. 48:405-426.
13. Géraud, G., F. Laquerrière, C. Masson, J. Arnoult, B. Labidi, and D. Hernandez-Verdun. 1989. Three-dimensional organization of micronuclei induced by colchicine in PtK1 cells. *Exp. Cell Res.* 181:27-39.
14. Gilbert, N., L. Lucas, C. Klein, M. Menager, N. Bonnet, and D. Ploton. 1995. Three-dimensional co-location of RNA polymerase I and DNA during interphase and mitosis by confocal microscopy. *J. Cell Sci.* 108:115-125.
15. Goodpasture, C., and S.E. Bloom. 1975. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma (Berl.)*. 53:37-50.
16. Guldner, H.-H., C. Szosteki, H.-P. Vosberg, H.-J. Lakomek, E. Penner, and F.A. Bautz. 1986. Scl 70 autoantibodies from scleroderma patients recognize a 95 kDa protein identified as DNA topoisomerase I. *Chromosoma (Berl.)*. 94:132-138.
17. Haaf, T., G. Reimer, and M. Schmid. 1988. Immunocytogenetics: localization of transcriptionally active rRNA genes in nucleoli and nucleolus organizer regions by use of human autoantibodies to RNA polymerase I. *Cytogenet. Cell Genet.* 48:35-42.
18. Haaf, T., D.L. Hayman, and M. Schmid. 1991. Quantitative determination of rDNA transcription units in vertebrate cells. *Exp. Cell Res.* 193:78-86.
19. Hadjiolov, A.A. 1985. The nucleolus and ribosome biogenesis. In *Cell Biology Monographs*. Vol. 12. M. Alfert, W. Beermann, L. Goldstein, K.R. Porter, and P. Sitte, editors. Springer-Verlag, Wien/New York. 1-268.
20. Henderson, A.S., D. Warburton, and K.C. Atwood. 1972. Localization of ribosomal RNA in the human chromosome complement. *Proc. Natl.*

- Acad. Sci. USA.* 69:3394–3398.
21. Hernandez, N. 1993. TBP, a universal eukaryotic transcription factor? *Genes & Dev.* 7:1291–1308.
  22. Howell, W.M., and D.A. Black. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia (Basel).* 36:1014–1015.
  23. Imai, H., M.J. Fritzler, R. Neri, S. Bombardieri, E.M. Tan, and E.K.L. Chan. 1994. Immunocytochemical characterization of human NOR-90 (upstream binding factor) and associated antigens reactive with autoimmune sera. *Mol. Biol. Rep.* 19:115–124.
  24. Jantzen, H.-M., A. Admon, S.P. Bell, and R. Tjian. 1990. Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature (Lond.).* 344:830–836.
  25. Jiménez-García, L.F., L.I. Rothblum, H. Busch, and R.L. Ochs. 1989. Nucleologenesis: use of non-isotopic in situ hybridization and immunocytochemistry to compare the localization of rDNA and nucleolar proteins during mitosis. *Biol. Cell.* 65:239–246.
  26. Krystal, M., P. D'Eustachio, F.H. Ruddle, and N. Arnheim. 1981. Human nucleolus organizers on nonhomologous chromosomes can share the same ribosomal gene variants. *Proc. Natl. Acad. Sci. USA.* 78:5744–5748.
  27. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
  28. Matsui, S., and A.A. Sandberg. 1985. Intranuclear compartmentalization of DNA-dependent RNA polymerases: association of RNA polymerase I with nucleolar organizing chromosomes. *Chromosoma (Berl.).* 92:1–6.
  29. Matsui, S.-I., H. Weinfeld, and A.A. Sandberg. 1979. Quantitative conservation of chromatin-bound RNA polymerases I and II in mitosis. *J. Cell Biol.* 80:451–464.
  30. Miller, D.A., V.G. Dev, R. Tantravahi, and O.J. Miller. 1976. Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. *Exp. Cell Res.* 101:235–243.
  31. Miller, O.J., D.A. Miller, V.G. Dev, R. Tantravahi, and C.M. Croce. 1976. Expression of human and suppression of mouse nucleolus organizer activity in mouse-human somatic cells hybrids. *Proc. Natl. Acad. Sci. USA.* 73:4531–4535.
  32. Moore, G.P.M., and N.R. Ringertz. 1973. Localization of DNA-dependent RNA polymerase activities in fixed human fibroblasts by autoradiography. *Exp. Cell Res.* 76:223–228.
  33. Morcillo, G., and C. de la Torre. 1979. Mapping nucleologenesis in relation to transcription. *Biol. Cell.* 36:1–6.
  34. Prescott, D.M. 1964. Cellular sites of RNA synthesis. *Prog. Nucleic Acid Res.* 3:33–57.
  35. Rendon, M.C., R.M. Rodrigo, L.G. Goenechea, G. Garcia-Herdugo, M.M. Valdivia, and F.J. Moreno. 1992. Characterization and immunolocalization of nucleolar antigen with anti-NOR serum in HeLa cells. *Exp. Cell Res.* 200:393–403.
  36. Roussel, P., and D. Hernandez-Verdun. 1994. Identification of Ag-NOR proteins, markers of proliferation related to ribosomal gene activity. *Exp. Cell Res.* 214:465–472.
  37. Roussel, P., P. Bélenguer, F. Amalric, and D. Hernandez-Verdun. 1992. Nucleolin is an Ag-NOR protein; this property is determined by its amino-terminal domain independently of its phosphorylation state. *Exp. Cell Res.* 203:259–269.
  38. Roussel, P., C. André, C. Masson, G. Géraud, and D. Hernandez-Verdun. 1993. Localization of the RNA polymerase I transcription factor hUBF during the cell cycle. *J. Cell Sci.* 104:327–337.
  39. Rudloff, U., D. Eberhard, L. Tora, H. Stunnenberg, and I. Grummt. 1994. TBP-associated factors interact with DNA and govern species specificity of RNA polymerase I transcription. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2611–2616.
  40. Scheer, U., and K.M. Rose. 1984. Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunocytochemistry. *Proc. Natl. Acad. Sci. USA.* 81:1431–1435.
  41. Schnapp, A., C. Pfeleiderer, H. Rosenbauer, and I. Grummt. 1990. A growth-dependent transcription initiation factor (TIF-1A) interacting with RNA polymerase I regulates mouse ribosomal RNA synthesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2857–2863.
  42. Schnapp, A., G. Schnapp, B. Erny, and I. Grummt. 1993. Function of the growth-regulated transcription initiation factor TIF-1A in initiation complex formation at the murine ribosomal gene promoter. *Mol. Cell Biol.* 13:6723–6732.
  43. Schnapp, G., F. Santori, C. Carles, M. Riva, and I. Grummt. 1994. The HMG box-containing nucleolar transcription factor UBF interacts with a specific subunit of RNA polymerase I. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:190–199.
  44. Schnapp, G., A. Schnapp, H. Rosenbauer, and I. Grummt. 1994. TIF-1C, a factor involved in both transcription initiation and elongation of RNA polymerase I. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4028–4035.
  45. Sentenac, A. 1985. Eukaryotic RNA polymerases. *CRC Crit. Rev. Biochem.* 18:31–90.
  46. Song, C.Z., K. Hanada, K. Yano, Y. Maeda, K. Yamamoto, and M. Muramatsu. 1994. High conservation of subunit composition of RNA polymerase I(A) between yeast and mouse and the molecular cloning of mouse RNA polymerase I 40-kDa subunit RPA40. *J. Biol. Chem.* 269:26976–26981.
  47. Tantravahi, R., D.A. Miller, V.G. Dev, and O.J. Miller. 1976. Detection of nucleolus organizer regions in chromosomes of human, chimpanzee, gorilla, orangutan and gibbon. *Chromosoma (Berl.).* 56:15–27.
  48. Tantravahi, U., R.V. Guntaka, B.F. Erlanger, and O.J. Miller. 1981. Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methylcytosine. *Proc. Natl. Acad. Sci. USA.* 78:489–493.
  49. Wansink, D.G., W. Schul, I. van der Kraan, B. van Steensel, R. van Driel, and L. de Jong. 1993. Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J. Cell Biol.* 122:283–293.
  50. Warner, J.R. 1990. The nucleolus and ribosome formation. *Curr. Opin. Cell Biol.* 2:521–527.
  51. Weisenberger, D., and U. Scheer. 1995. A possible mechanism for the inhibition of ribosomal RNA gene transcription during mitosis. *J. Cell Biol.* 129:561–575.
  52. Young, B., M. Fergusson-Smith, R. Sillar, and E. Boyd. 1981. High-resolution analysis of human peripheral lymphocyte chromosomes by flow cytometry. *Proc. Natl. Acad. Sci. USA.* 78:7727–7731.
  53. Zatssepina, O.V., R. Voit, I. Grummt, H. Spring, M.V. Semenov, and M.F. Trendelenburg. 1993. The RNA polymerase I-specific transcription initiation factor UBF is associated with transcriptionally active and inactive ribosomal genes. *Chromosoma (Berl.).* 102:599–611.
  54. Zomerdijk, J.C.B.M., H. Beckmann, L. Comai, and R. Tjian. 1994. Assembly of transcriptionally active RNA polymerase I initiation factor SL1 from recombinant subunits. *Science (Wash. DC).* 266:2015–2018.