A Possible Mechanism for the Inhibition of Ribosomal RNA Gene Transcription during Mitosis

Dieter Weisenberger and Ulrich Scheer

Department of Cell and Developmental Biology, Theodor-Boveri-Institute, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

Abstract. When cells enter mitosis, RNA synthesis ceases. Yet the RNA polymerase I (pol I) transcription machinery involved in the production of pre-rRNA remains bound to the nucleolus organizing region (NOR), the chromosome site harboring the tandemly repeated rRNA genes. Here we examine whether rDNA transcription units are transiently blocked or "frozen" during mitosis. By using fluorescent in situ hybridization we were unable to detect nascent pre-rRNA chains on the NORs of mouse 3T3 and rat kangaroo PtK2 cells. Appropriate controls showed that our approach was sensitive enough to visualize, at the light microscopic level, individual transcriptionally active rRNA genes both in situ after experimental unfolding of nucleoli and in chromatin spreads ("Miller spreads"). Analysis of the cell cycle–dependent redistribution of transcript-associated components also revealed that most transcripts are released from the rDNA at mitosis. Upon disintegration of the nucleolus during mitosis, U3 small nucleolar RNA (snoRNA) and the nucleolar proteins fibrillarin and nucleolin became dispersed throughout the cytoplasm and were excluded from the NORs. Together, our data rule out the presence of "frozen Christmas-trees" at the mitotic NORs but are compatible with the view that inactive pol I remains on the rDNA. We propose that expression of the rRNA genes is regulated during mitosis at the level of transcription elongation, similarly to what is known for a number of genes transcribed by pol II. Such a mechanism may explain the decondensed state of the NOR chromatin and the immediate transcriptional reactivation of the rRNA genes following mitosis.

C ELL division of higher eukaryotic cells is accompanied by a series of profound reversible structural changes such as breakdown of the nuclear envelope, disintegration of the nucleolus, condensation of chromatin, collapse of the cytoskeleton, formation of the mitotic spindle, and fragmentation of the membranes of the Golgi apparatus and endoplasmic reticulum into numerous vesicles (McIntosh, 1991). Concomitantly with the disassembly of the cell nucleus at prometaphase and repackaging of the interphase chromatin into the mitotic chromosomes, nuclear RNA synthesis ceases until telophase (Prescott, 1964; Johnson and Holland, 1965). There are a number of potential mechanisms that could mediate repression of RNA synthesis at mitosis. For instance, the physical condensation of the chromosomes into a form that is incompatible with transcription may lead to a global downregulation of RNA synthesis (Johnson and Holland, 1965). In fact, it is well established that gene activity is affected by the specific structural

organization of chromatin (for recent reviews see Grunstein, 1990; Wolffe, 1991; Kornberg and Lorch, 1992; Zlatanova and van Holde, 1992). Alternatively, inhibition of transcription and release of the nascent transcripts from the DNA template may be the primary step that allows chromatin to condense (Moreno and Nurse, 1990). Evidence supporting this view comes from studies using *Xenopus* egg extract which reproduces mitotic repression of RNA polymerase (pol)[1] III–dependent transcription in vitro (Hartl et al., 1993; Gottesfeld et al., 1994). In this model system mitotic repression is mediated, independent of chromatin condensation, by an inhibitory phosphorylation of the transcription initiation factor TFIIIB. Thus, at least in the case of pol III transcription, the mitotic block seems to be set in motion by mitosis-specific protein kinases that inhibit transcription initiation rather than by changes of the chromatin structure per se. Similar mechanisms have been implicated in the inactivation of genes transcribed by pol II. Based on a detailed in

---

Address all correspondence to Dr. Ulrich Scheer, Department of Cell and Developmental Biology, Biocenter, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany. Ph.: (0)931/8884250 or 8884251. Fax: (0)931/8884252.

---

1. *Abbreviations used in this paper:* DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; ETS, external transcribed spacer; ITS, internal transcribed spacer; NOR, nucleolus organizing region; pol, polymerase; nt, nucleotides; NTS, nontranscribed spacer; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA.

situ hybridization study, Shermoen and O'Farrell (1991) concluded that nascent pol II transcripts are destabilized and prematurely released from the template as cells progress through mitosis. Their data suggest that during each mitotic event the chromatin is stripped of elongating pol II transcripts. Whether the polymerases are also released has not been investigated by these authors (for earlier data indicating that pol II enzymes persist as structural components of mitotic chromosomes see Gariglio et al., 1974; Matsui et al., 1979).

Pol I–driven transcription of the rRNA genes also ceases during mitosis (Fan and Penman, 1971; Morcillo et al., 1976), concomitantly with the disintegration and disappearance of the nucleolus. Little information is currently available on the mechanisms involved in mitotic downregulation of rRNA gene expression. The tandemly repeated rRNA genes are clustered at specific chromosomal sites, the nucleolus organizing regions (NORs; Howell, 1982). It is remarkable that in spite of the mitotic repression of the rRNA genes, major components of the pol I–dependent transcription machinery remain stably associated with the NORs and are probably never completely disengaged from the rDNA template. Among them are pol I (Scheer and Rose, 1984; Haaf et al., 1988), the transcription initiation factor UBF (Chan et al., 1991; Rendon et al., 1992; Roussel et al., 1993; Zatsepina et al., 1993), and DNA topoisomerase I, an enzyme which plays an important role in transcriptional processes (Guldner et al., 1986; for involvement of topoisomerase I in rDNA transcription see Zhang et al., 1988). Though quiescent in RNA synthesis, the NOR-bound pol I molecules seem to be present as engaged transcriptional complexes since they can be reactivated in vitro by Sarkosyl or heparin, i.e., agents that remove histones and other proteins from DNA while blocking new initiation events (Gariglio et al., 1974; Matsui et al., 1979; Matsui and Sandberg, 1985). Together, all these data raise the possibility that transcriptional complexes with their nascent pre-rRNA chains may become transiently arrested or "frozen" during mitosis and resume elongation without interruption once the mitotic block is relieved (Scheer and Rose, 1984; Zatsepina et al., 1993). Here we examine this hypothetical model by using fluorescent in situ hybridization. Further, since nascent pre-rRNA transcripts assemble at their leading 5' end a processing complex containing U3 small nucleolar RNA (snoRNA) and the nucleolar protein fibrillarin (Kass et al., 1990; Scheer and Benavente, 1990; Mougey et al., 1993a, b) and are thought to bind nucleolin (Olson, 1990), we have also examined the distribution of these three nucleolar components during mitosis as an alternative approach to detect nascent transcripts.

Materials and Methods

Biological Materials

Cell lines derived from rat (RVF-SMC; Franke et al., 1980), mouse (3T3), and rat kangaroo (PtK2) tissues were cultured in DME (GIBCO BRL, Eggenstein, Germany) supplemented with 10% FCS at 37°C in a 5% CO_2 incubator. Xenopus A6 cells were grown as described (Smith and Tata, 1991). In some experiments cells were treated with the adenosine analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma, Deisenhofen, Germany) as described (Scheer et al., 1984). Salamanders of the species Pleurodeles waltl were kindly provided by Peter Eichhorn (German Cancer Research Center, Heidelberg).

Antibodies

Antibodies against the antigens listed below were used: pol I (human autoimmune serum S18; Reimer et al., 1987a); fibrillarin (mAb 72B9; Reimer et al., 1987b); nucleolin (affinity-purified rabbit antibodies against mouse and calf thymus nucleolin; Pfeifle et al., 1986; Sapp et al., 1986; mAb b6-6E7 against Xenopus nucleolin; Messmer and Dreyer, 1993); the NOR protein ppl35 (affinity-purified rabbit antibodies against mouse ppl35; Pfeifle et al., 1986); DNA topoisomerase I (affinity-purified rabbit antibodies against calf thymus DNA topoisomerase I; Rose et al., 1988), UBF (guinea pig antibodies against murine recombinant UBF; Voit et al., 1992), and the trimethyl-guanosine cap of small nuclear RNAs (rabbit antibodies, Reuter et al., 1984).

Hybridization Probes

Hybridization probes were prepared by in vitro transcription of cloned mouse rDNA restriction fragments inserted into Bluescript vector (Stratagene, Heidelberg, Germany). The original mouse rDNA containing plasmids pMr974 (Grummt et al., 1979) and pMr100 (Tiemeier et al., 1977) were kindly provided by Ingrid Grummt (German Cancer Research Center, Heidelberg). In vitro transcription with T3 and T7 RNA polymerase (SP6/T7 RNA labeling kit; Boehringer Mannheim, Mannheim, Germany) yielded digoxigenin-tagged riboprobes in sense and antisense orientation. The sense probes served as controls for the in situ hybridization. Incorporation of digoxigenin-11-UTP was monitored by one-dimensional formaldehyde-agarose gel electrophoresis of the probes followed by transfer onto nitrocellulose filters and incubation with alkaline phosphatase-coupled anti-digoxigenin antibodies (Boehringer Mannheim). The probes were then visualized by staining the filters with nitroblue tetrazolium and X-phosphate. The probes were derived from various regions of mouse rDNA as indicated in Fig. 1. Probe "974b," a 11.35-kb EcoRI-EcoRI fragment, comprised parts of the nontranscribed spacer (NTS), the 5' external transcribed spacer (ETS), and the majority of the 18S sequence (residues −5,715 to +5,635 relative to the transcription start site). In some experiments this probe was cleaved by alkaline hydrolysis into ∼200–400 nucleotides (nt) long fragments (Cox et al., 1984). Probe "5' ETSa," a 3,259-nt SalI-SalI fragment, comprised a short NTS sequence and a large part of the 5' ETS (residues −175 to +3,084). Probe "ITS1," a 228-nt AccI-KpnI fragment, was derived from internal transcribed spacer sequences (ITS1, residues +6,147 to +6,375). Probe "28S," a 1,516-nt BamHI-EcoRI fragment, was derived from 28S rDNA (residues +10,653 to +12,170). Xenopus U3 cDNA (Jeppesen et al., 1988) inserted into Bluescript vector was kindly provided by Susan Gerbi (Brown University, Providence, RI).

Immunofluorescence Microscopy

Cultured cells grown on coverslips were fixed for 10 min in −20°C methanol, transferred for 1 min in −20°C acetone, air dried, and then incubated for 30 min at room temperature with the primary antibodies. After washing in PBS, appropriate FITC- or Texas red-conjugated secondary antibodies
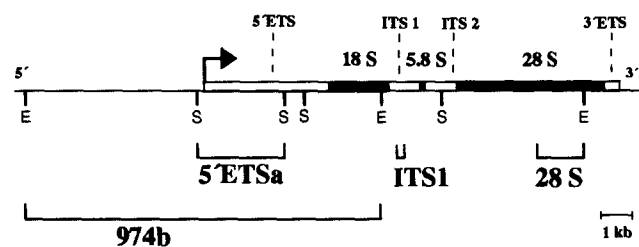

Figure 1. (Top) Map of the 13.25-kb mouse rDNA transcribed region (boxed) with the surrounding nontranscribed spacers (single lines). Regions corresponding to the mature 18S, 28S, and 5.8S rRNAs are black and transcribed spacers (ETS, external transcribed spacer; ITS, internal transcribed spacer) are white. The arrow indicates the transcription initiation site. (Bottom) Restriction fragments of rDNA were subcloned into Bluescript vector and used as templates for in vitro synthesis of digoxigenin-labeled sense and antisense RNA probes (probes 974b, 5'ETSa, ITS1, 28S). Some diagnostic restriction sites are indicated (E, EcoRI; S, SalI).

(Dianova, Hamburg, Germany) were added for another 30 min. Cells were then counterstained for DNA with Hoechst 33258 (5 µg/ml), washed in PBS, air dried from ethanol, and mounted in Mowiol (Hoechst, Frankfurt, Germany). Photographs were taken with a Zeiss Axiophot microscope equipped with epifluorescence optics (Zeiss, Oberkochen, Germany).

In Situ Hybridization

Cells grown on coverslips were fixed and air dried as described above. The cells were rehydrated for 30 min in 2× SSC at 70°C, and then acetylated with 0.5% acetic anhydride in 130 mM triethanolamin-hydrochloride, pH 8.0, for 10 min at room temperature (Wassef et al., 1979). After several wash steps in 2× SSC followed by equilibration in 5× SSC, 10 µl of the hybridization mix containing 5× SSC, 0.8 µg/ml tRNA, and 12 ng/µl probe were placed on a microscope slide, the coverslip placed cell side down on the hybridization mix and then sealed with rubber cement. After a 3-h incubation at 65°C, the specimens were washed sequentially with 5× SSC, 2× SSC, and PBS, and then incubated for 20 min at room temperature with anti–digoxigenin antibodies diluted 1:20 in TBS containing 0.5% blocking reagent (Boehringer Mannheim). After several rinses in PBS, TBS contain-

ing 0.1% Tween-20 and again PBS, Texas red–conjugated guinea pig anti–mouse Ig (Dianova; diluted 1:100 in PBS) was added for another 20 min. Specimens were then counterstained with Hoechst 33258, washed in PBS, TBS containing 0.1% Tween-20, PBS, and mounted as described above.

In some experiments cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature and extracted for 3 min at 4°C with the Triton X-100 containing "cytoskeleton buffer" of Fey et al. (1986) followed by in situ hybridization. Both protocols gave essentially the same results.

Chromatin Spread Preparations

Pieces of ovary were removed from anesthetized *Pleurodeles* females and placed in modified Barth medium (Peng, 1991). 10 nuclei were manually isolated from mid-sized oocytes in "3:1 medium" (75 mM KCl, 25 mM NaCl, 10 mM Tris-HCl, pH 7.2) and transferred into a droplet of low salt buffer (0.1 mM borate buffer, pH 9) on a siliconized glass slide at 4°C for 10 min. The dispersed nuclear contents were then centrifuged for 20 min at 3,000 g through a solution of 1% formaldehyde (freshly prepared from paraformaldehyde), 0.1 M sucrose, and 0.1 mM borate buffer (pH 8.5–9.0)
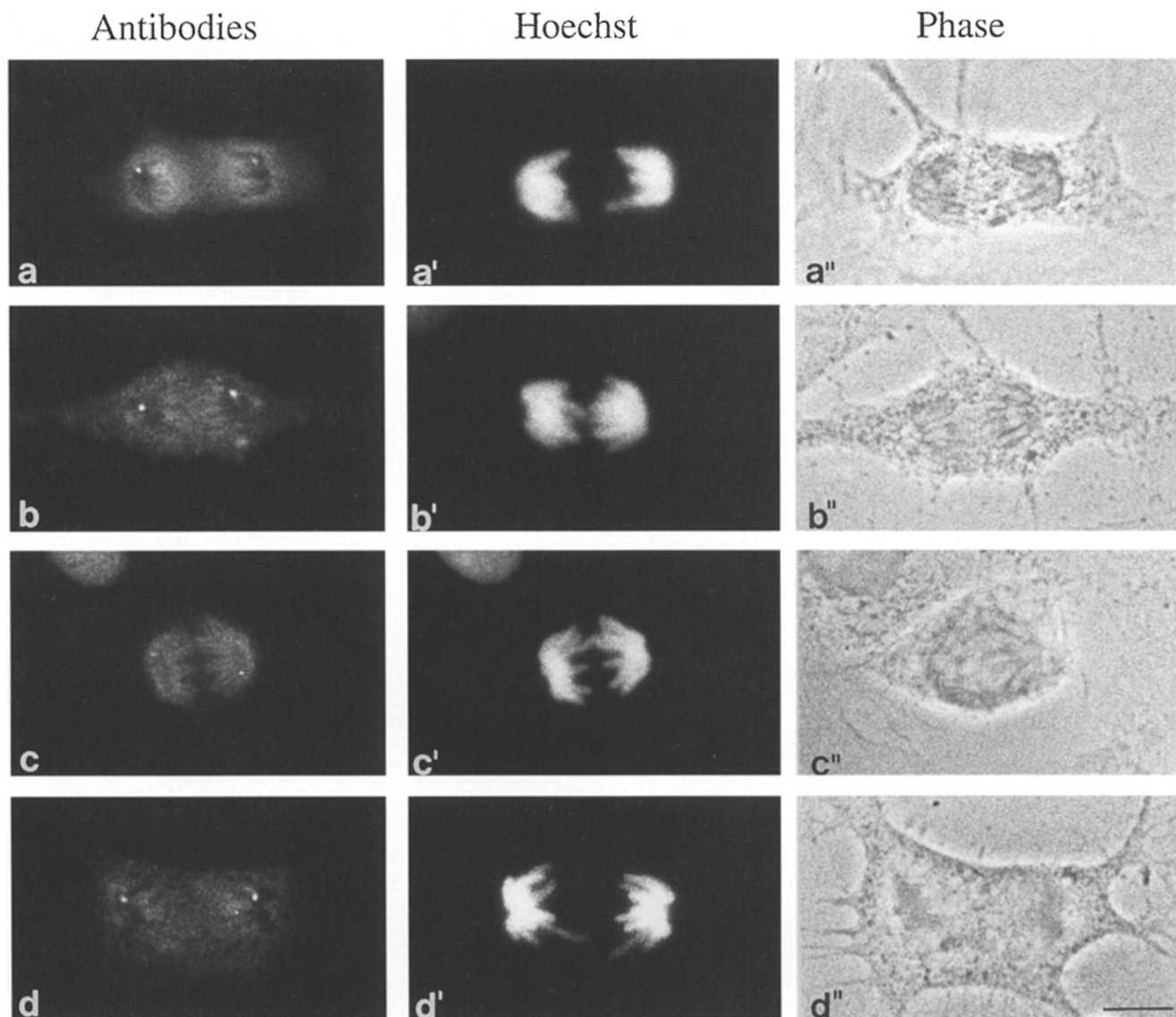

Figure 2. Visualization of chromosomal NORs of dividing PtK2 cells by immunofluorescence microscopy using antibodies to RNA pol I (*a*), the transcription initiation factor UBF (*b*), DNA topoisomerase I (*c*), and the phosphoprotein pp135 (*d*). The single NOR is visualized as a strongly fluorescent dot per chromosome complement. The corresponding DNA fluorescence with Hoechst dye 33258 (*a'–d'*) and phase contrast images (*a"–d"*) are shown. Bar, 10 µm.

Hybridization   Hoechst   Phase
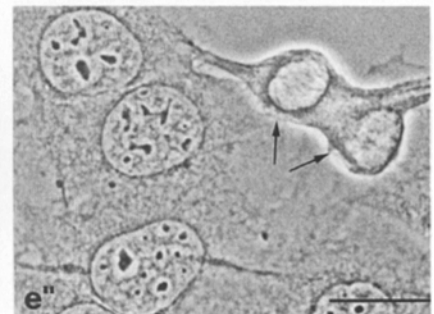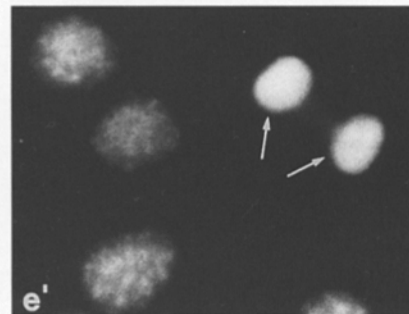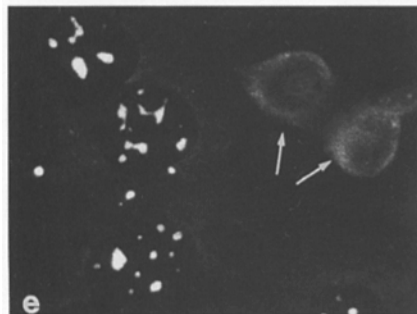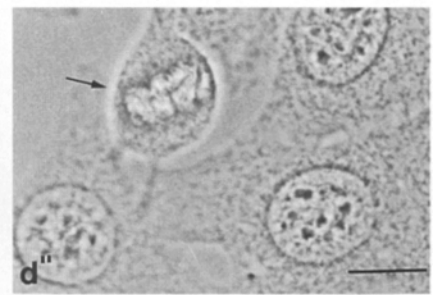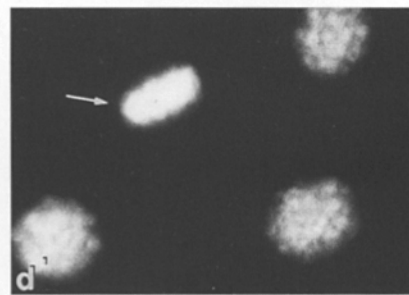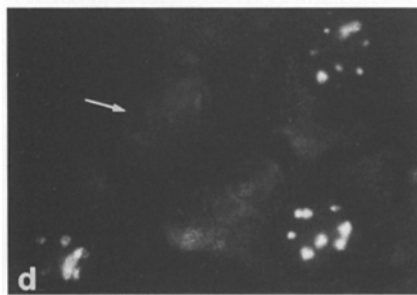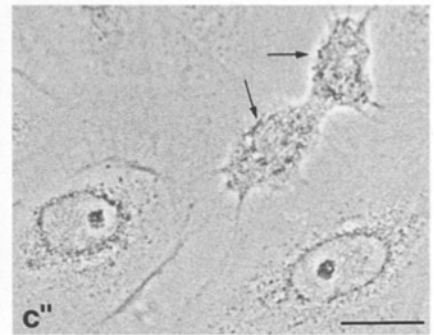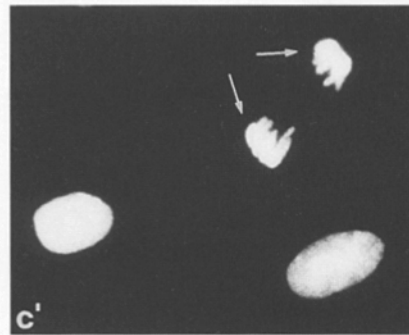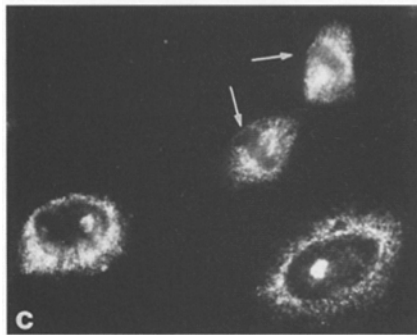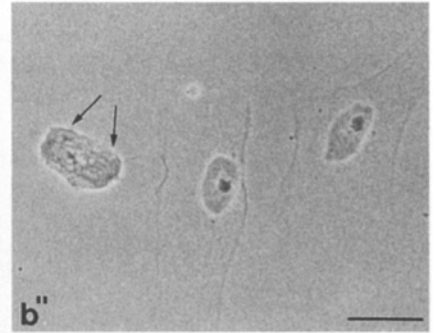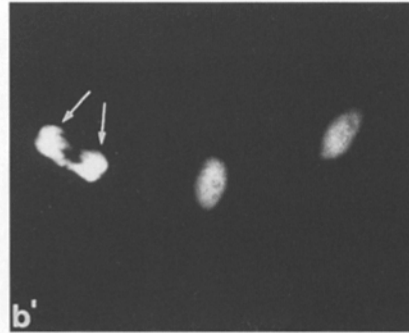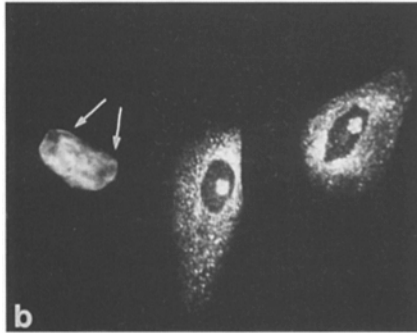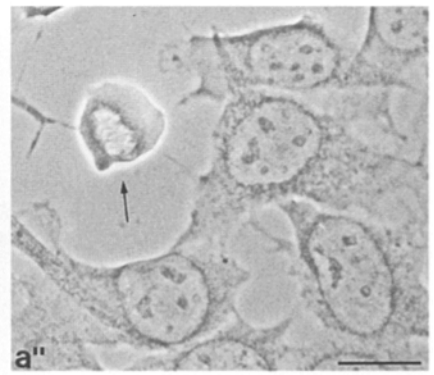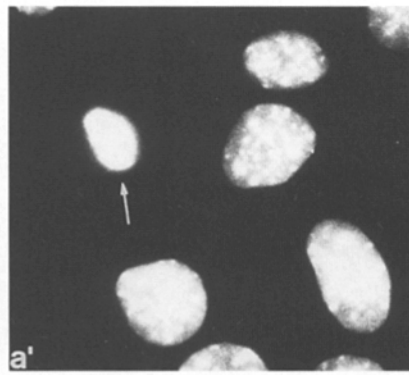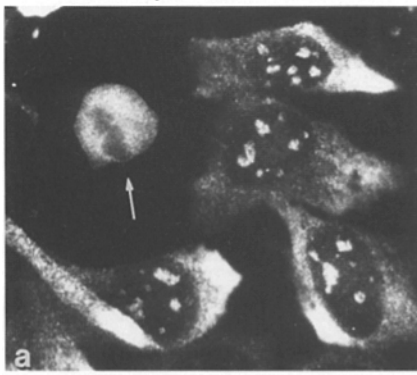
essentially as described by Miller and Bakken (1972), except that we used freshly glow-discharged carbon-coated coverslips rather than EM grids. After centrifugation, the coverslips were immersed for 5 min in 70% ethanol. For in situ hybridization, they were then transferred for 5 min in 5× SSC and incubated with the hybridization mix (see above). For immunofluorescence, coverslips were washed in PBS followed by incubation with the primary and secondary antibodies as outlined above.

Results

Identification of NORs by Immunofluorescence Microscopy

Cells of the male rat kangaroo (line PtK2) have a single NOR located on the X chromosome (Hsu et al., 1975) in contrast to most other mammalian species where multiple NORs are distributed among several autosomal pairs (Howell, 1982). Whenever possible, we have chosen PtK2 cells for the present study because all copies of their rRNA genes are clustered at a single chromosomal site (Robert-Fortel et al., 1993), a situation that is likely to increase the sensitivity for detection of rDNA-associated proteins and potential nascent transcripts in mitotic cells. The chromosomal NOR of anaphase PtK2 cells was readily visualized as a single distinct fluorescent spot per chromosome complement following immunofluorescence microscopy with antibodies directed against pol I (Fig. 2 a), the rDNA transcription initiation factor UBF (Fig. 2 b), DNA topoisomerase I (Fig. 2 c) and the nucleolar phosphoprotein pp135 (Fig. 2 d). On spread metaphase plates of PtK2 cells, the pol I–dependent immunofluorescence has been shown to correspond to the secondary constriction of the X chromosome (Scheer and Rose, 1984). The same antibodies also labeled the NORs of other mammalian species with the difference that the multiple fluorescence dots appeared smaller and less intense (data not shown; see also Haaf et al., 1988).

Distribution of rRNAs and Their Precursors during the Cell Cycle

We examined the distribution of rRNA molecules during interphase and mitosis by hybridization of digoxigenin-labeled antisense riboprobes in situ to PtK2 and mouse 3T3 cells. The resulting RNA–RNA hybrids were visualized by immunofluorescence microscopy using Texas red–conjugated antibodies to digoxigenin. The corresponding sense probes served as controls. Hybridization of the 28S antisense probe produced a strong fluorescence of the multiple nucleoli of interphase mouse 3T3 cells and a finely granular fluorescence dispersed throughout the cytoplasm (Fig. 3 a). The cytoplasmic fluorescence reflected the distribution of ribosomes as previously shown by EM in situ hybridization (Fischer et al., 1991). Specific labeling was not observed when hybridization was performed with the corresponding sense probe (data not shown) indicating that the probes hybridized to rRNA only and not to rDNA. Hybridization of the mouse 28S antisense probe to PtK2 cells produced a similar pattern with

a strong nucleolar and cytoplasmic fluorescence (Fig. 3 b). This result indicates that sufficient sequence complementarity exists with the 28S rRNA sequences to permit detection of PtK2 rRNAs with the heterologous mouse probe (the same holds true for the 18S rRNA sequences, see below).

In dividing cells the mitotic chromosomes including their NORs were completely negative and appeared as dark structures embedded in the diffusely fluorescent cytoplasm (Figs. 3, a and b). The absence of rRNAs from mitotic chromosomes has also been noted in dividing HeLa and rat kidney cells after in situ hybridization with a biotinylated rDNA probe complementary to 18S, 28S, 5.8S, ITS1, and ITS2 sequences (Jiménez-Garcia et al., 1994). It is notable that the fluorescent signal was not enhanced at the periphery of the chromosomes. Since our probes hybridized to nucleoli of interphase cells, the absence of peripheral labeling of mitotic chromosomes is not due to inaccessibility of pre-rRNA by bound proteins or pre-rRNA folding. Thus, our in situ evidence does not support the concept based on earlier biochemical fractionation studies that considerable amounts of rRNA precursor molecules are stably associated with chromosomes during mitosis (Fan and Penman, 1971).

The 28S probe was complementary to sequences located towards the 3' end of the pre-rRNA and thus only recognized nearly complete transcripts. To find out whether early transcripts might be associated with the NORs, we used probe 974b complementary to the 5' ETS and most of the 18S sequences (see Fig. 1). The resulting fluorescence pattern closely resembled that obtained with the 28S probe (Fig. 3 c). Most importantly, mitotic chromosomes were also completely negative, both of PtK2 (Fig. 3 c) and mouse 3T3 cells (not shown). In order to exclude the possibility that we might have overlooked a potential weak NOR signal due to the surrounding cytoplasmic fluorescence, we also used probes complementary to the 5' ETS or ITS1 regions, i.e., sequences that are not included in the mature rRNAs. Due to limited interspecies homology of the transcribed spacer sequences we hybridized the probes (derived from mouse rDNA) to mouse 3T3 cells. As expected, the cytoplasmic fluorescence intensity was reduced to background levels and the interphase nucleoli stood out as strongly labeled structures (Figs. 3, d and e). Dividing cells occasionally revealed some cytoplasmic fluorescence that might have been caused by the presence of pre-rRNAs derived from the disintegrated maternal nucleolus. However, their chromosomes were completely negative and we failed to find any evidence for chromosomal NOR staining (Figs. 3, d and e).

NORs were negative during all stages of mitosis as revealed by in situ hybridization with the antisense 28S probe (Fig. 4). Cells in prophase still showed the typical interphase situation with a rather uniform cytoplasmic fluorescence, weak labeling of the nucleoplasm in a network-like arrangement and strong labeling of the nucleolus (Fig. 4 a). Concomitant with the breakdown of the nuclear envelope, the fluorescence pattern changed remarkably and only cytoplas-

---

Figure 3. Chromosomal NORs of mitotic PtK2 (b and c) and mouse 3T3 (a, d, and e) cells are negative following in situ hybridization with the digoxigenin-labeled antisense riboprobes 28S (a and b), 974b (c), 5'ETSa (d), and ITS1 (e). *Arrows* denote dividing cells. Nucleoli of adjacent interphase cells are strongly fluorescing. In addition, probes 28S and 974b label the cytoplasmic ribosomes (a–c). The corresponding DNA staining with Hoechst (a'–e') and phase contrast images (a"–e") are shown. Bars, 20 μm.

28 S   Hoechst   Phase
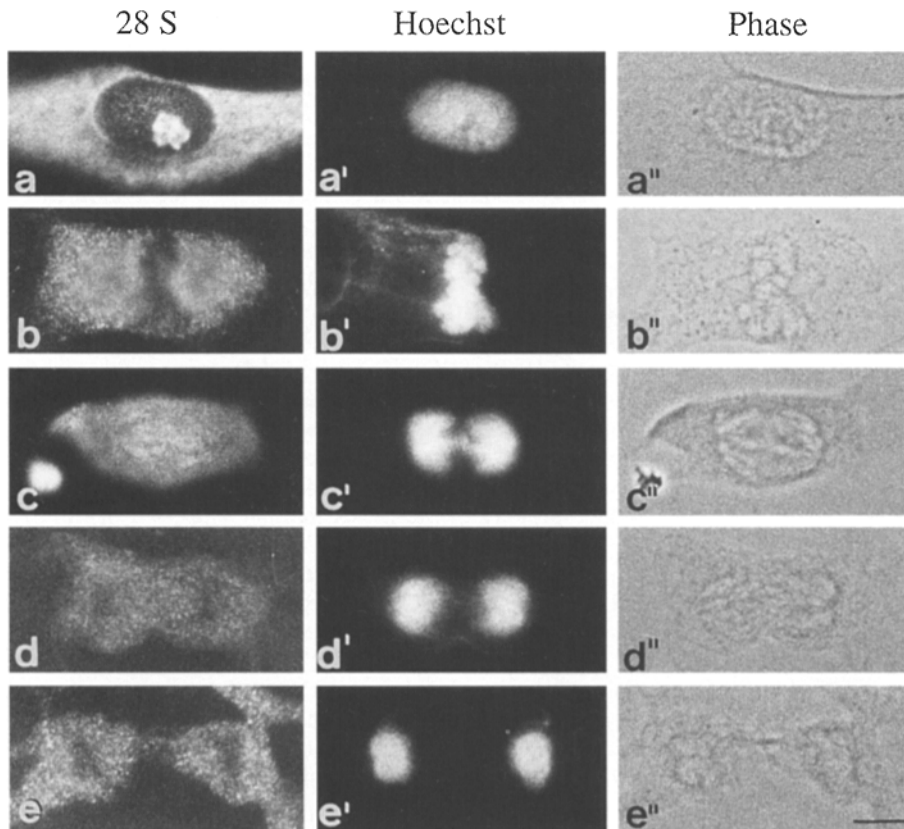
Figure 4. Distribution of rRNAs and their precursors in PtK2 cells during mitosis as visualized by fluorescent in situ hybridization with the antisense 28S probe (*a–e*). The corresponding DNA fluorescence (*a'–e'*) and phase contrast images (*a"–e"*) are shown. In early mitotic prophase (*a*), the characteristic interphase pattern is seen with a strongly stained nucleolus, faint nucleoplasmic labeling in a network-like fashion, and uniform cytoplasmic fluorescence reflecting the presence of ribosomes. In metaphase (*b*), anaphase (*c*), early (*d*), and late (*e*) telophase, chromosomes appear as unstained structures embedded into the fluorescent cell plasm. Note the absence of fluorescent NORs. Bar, 10 μm.

mic fluorescence was seen surrounding the completely negative chromosomes (Fig. 4, *b–e*) until nucleoli reformed at late telophase.

As an alternative approach for the cytological detection of rRNAs and their precursors we have used mAb mRF7 directed against 18S and 28S rRNAs (Reimer, G., M. C. Totoritis, R. Rubin, A. N. Theofilopoulos, and E. M. Tan. 1986. *Arthritis Rheum.* 29:72a). The fluorescence patterns of cells in interphase and mitosis were essentially identical to those obtained by in situ hybridization with riboprobes 28S and 974b. Again, mitotic chromosomes including the NORs were completely negative (data not shown).

Light Microscopic Visualization of Individual Transcription Units of rRNA Genes

For a meaningful interpretation of the results described above we needed to know the sensitivity of our in situ hybridization method. Therefore we treated cells with DRB, a drug which is known to induce a reversible unfolding of the interphase nucleolus into extended beaded strands without inhibiting transcription of the rRNA genes (Scheer et al., 1984). Each of the beads are thought to contain a single rDNA transcription unit (Scheer et al., 1984; Haaf et al., 1991). When probed with antibodies to pol I, the nucleolar "necklaces" appeared as linear arrays of fluorescent entities (Fig. 5, *a* and *c*). Following in situ hybridization with the 28S probe, they were labeled in the same fashion (Fig. 5, *b* and *d*).

Since we could not categorically rule out that a fluorescent dot contained, in addition to the nascent transcripts, also re-

cently terminated pre-rRNAs that contributed to the signal, we have used isolated transcriptionally active rRNA genes as a substrate for the hybridization reaction. To this end Miller spreads of nucleolar chromatin from oocytes of the salamander *Pleurodeles waltl* were prepared on carbon coated and freshly glow discharged cover slips and hybridized with the 28S riboprobe. When examined in the fluorescence microscope, rows of tandemly arranged, slightly elongated and strongly fluorescent entities were seen (Fig. 6 *a*). The distance for one repeat unit (i.e., from the start of one of these fluorescent structures to the start of the next one) ranged from 2.8 to 3.8 μm with a mean and standard deviation of 3.34 ± 0.46 μm (*n* = 69) and hence corresponded closely to the size of one rDNA transcription unit plus adjacent intergenic spacer of *Pleurodeles* as seen in conventional EM spreads (Fig. 6 *c*; for quantitative data see Franke et al., 1979). However, the sizes of the fluorescent entities reached not more than about one quarter of the total transcription unit length of 2.4 μm. This was expected from the position of the probe sequence within the transcription unit. RNA polymerases had to transit ∼75% of the gene before the 28S hybridization probe could bind to the transcript (schematically depicted in Fig. 6 *d*). To verify that the fluorescent signals corresponded to well unfolded transcribing rRNA genes we have probed chromatin spreads prepared in parallel with antibodies to pol I. Though the fluorescence intensity was considerably weaker and often difficult to record, tandemly repeated linear elements with a total repeat unit length ranging from 2.6 to 3.9 μm (mean 3.12 ± 0.46 μm; *n* = 36) were also clearly seen (Fig. 6 *b*). The mean length of the pol I-positive fluorescent elements (2.02 ± 0.38 μm; range 1.5
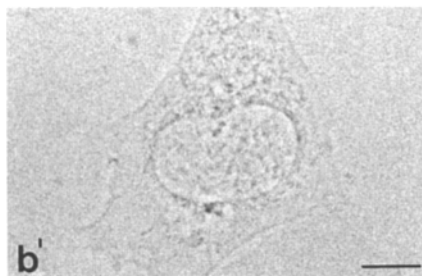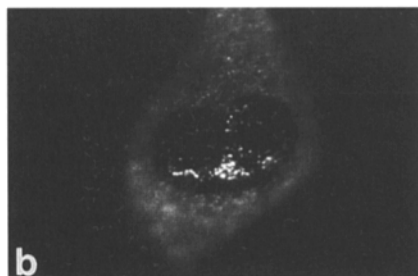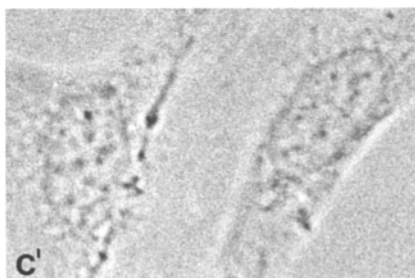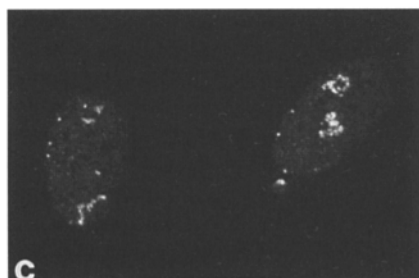
RV cells
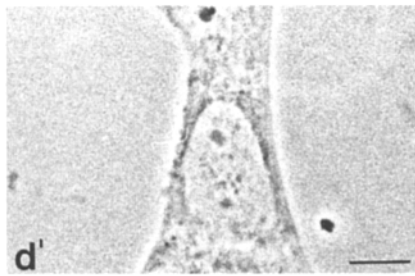
fluorescence   phase contrast

pol I
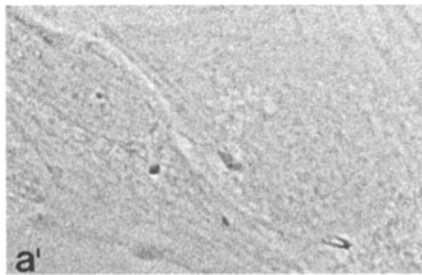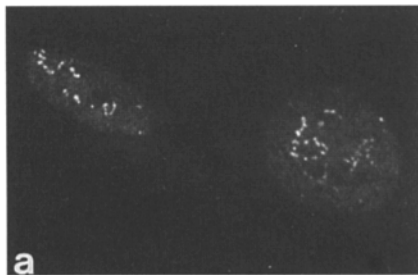
28 S

PtK$_2$ cells

pol I

28 S
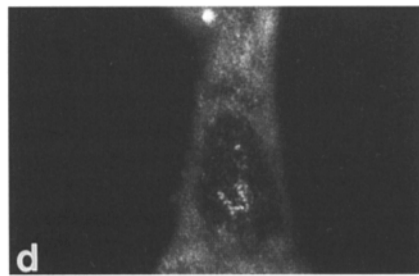
Figure 5. Visualization of rDNA transcription units of rat RV (*a* and *b*) and PtK2 (*c* and *d*) cells after DRB-induced unfolding of interphase nucleoli. Both in situ hybridization with the antisense 28S probe (*b* and *d*) and immunofluorescence microscopy using antibodies to pol I (*a* and *c*) reveal numerous fluorescent dot-like structures. The corresponding phase contrast images are also shown (*a'–d'*). Bars, 10 μm.

to 2.7 μm) approached that of a total rDNA transcription unit as measured in EM spreads (~2.4 μm), illustrating the well known fact that the axes of transcribed rRNA genes are entirely covered by densely spaced pol I molecules (Figs. 6, *c* and *d*). We thus conclude that our in situ hybridization approach is sensitive enough to detect individual transcription units of rRNA genes at the light microscopic level (for ultrastructural aspects of in situ hybridization to nascent pre-rRNA transcripts, see O'Reilly et al., 1994).

Pre-rRNA Processing Components Are Not Detectable on NORs

The characteristic terminal "balls" observed at the leading end of the nascent transcripts of rRNA genes have recently been shown to represent 5' ETS processing complexes which catalyze the first step in pre-rRNA maturation (Mougey et al., 1993a). U3 snoRNPs participate in this early processing event and are associated with the processing complexes (Kass et al., 1990; Mougey et al., 1993b). Furthermore, U3 RNA has been localized by in vivo crosslinking to the 5' ETS of human and rat pre-rRNA (Maser and Calvet, 1989; Stroke and Weiner, 1989). Fibrillarin, a major protein component of U3 snoRNP, has also been detected by immunogold EM at the leading 5' ends of nascent pre-rRNA transcripts (Scheer and Benavente, 1990). We therefore examined the distribution of U3 and fibrillarin in mitosis as an
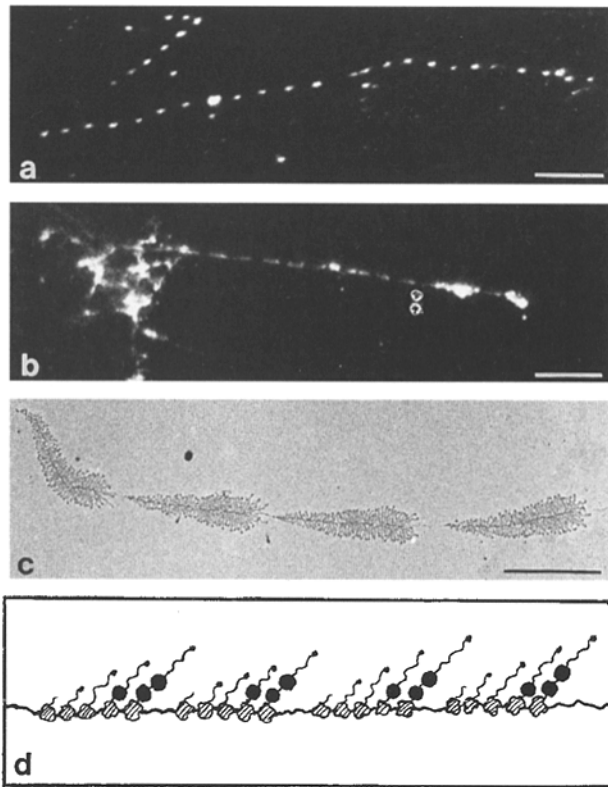
Figure 6. Visualization of individual rDNA transcription units by fluorescent in situ hybridization using antisense probe 28S (*a*), immunofluorescence microscopy with antibodies against pol I (autoimmune serum S18; *b*) or EM of spread nucleolar chromatin (*c*). Dispersed nucleolar chromatin of *Pleurodeles waltl* oocytes was deposited either on carbon coated, glow-discharged coverslips (*a* and *b*) or EM grids (*c*). Following in situ hybridization and incubation with antibodies to pol I, arrays of fluorescent entities are visible (*a* and *b*) whose tandem arrangement mirrors that of transcriptionally active rRNA genes as seen in EM spreads (*c*). Note the length difference of the fluorescent structures in *a* and *b* which is explained in the diagram (*c*). The expected labeling pattern after hybridization of the 28S antisense probe to nascent pre-rRNA transcripts is shown as solid circles assuming that the overall organization of the *Pleurodeles* rRNA genes is similar to that of *Xenopus laevis*. In *Xenopus*, the target sequences recognized by probe 28S are transcribed from residues +5,919 to +7,122 of the 7.8-kb transcription unit. In contrast, antibodies to pol I decorate the axes of the rDNA transcription units. Bars, 10 μm (*a* and *b*); 2 μm (*c*).

alternative approach to localize nascent pre-rRNA transcripts.

U3 was visualized by two different approaches, in situ hybridization and immunofluorescence microscopy. For in situ hybridization we used a digoxigenin-labeled riboprobe against *Xenopus* U3 which, due to evolutionarily conserved sequences (Gerbi et al., 1990) also recognized U3 of PtK2 cells. As shown in Fig. 7 *a*, U3 was concentrated in interphase nucleoli and dispersed in the cytoplasm of mitotic cells. The absence of U3 from mitotic chromosomes has also been noted in other mammalian cells using similar approaches (Azum-Gelade et al., 1994; Jiménez-Garcia et al., 1994). There was some enrichment of U3 in the perichromosomal region (for a detailed account of the structure and composi-

tion of the perichromosomal layer see Hernandez-Verdun and Gautier, 1994). Essentially the same results were obtained on *Xenopus* A6 cells (data not shown).

Antibodies directed against the trimethyl-guanosine cap at the 5' end of most small nuclear RNA (snRNAs) recognize U3 and other snoRNAs, in addition to nucleoplasmic snRNAs (Reuter et al., 1984). Following immunofluorescence microscopy, interphase nuclei revealed a distinctly speckled appearance reflecting the distribution of snRNAs (Fig. 7 *b*; see also Reuter et al., 1984). In addition, the nucleoli were clearly labeled as expected from the nucleolar location of U3 and other snoRNAs (Fournier and Maxwell, 1993). During mitosis, labeling became dispersed throughout the cell plasm indicating that little if any capped snoRNAs (U3, U8, U13) remain associated with the NORs (Fig. 7 *b*).

The distribution of fibrillarin during the cell cycle largely coincided with that of U3, including its enrichment in the perichromosomal region (Fig. 7 *c*; see also Reimer et al., 1987*b*). Most importantly, fibrillarin was virtually absent from the NORs (for contrasting data see, however, Ochs et al., 1985*a*).

Nucleolin Is Not Detectable at the NORs

As pre-rRNA chains emerge from the polymerases, they become packaged with specific proteins into RNP structures (Chooi and Leiby, 1981). Prominent among these early binding proteins is nucleolin (formerly designated C23 or 100-kD nucleolar protein; Olson, 1990). Available data on the mitotic distribution of nucleolin are contradictory. Several authors have detected nucleolin on the chromosomal NORs (Lischwe et al., 1981; Ochs et al., 1983, 1985*b*; Spector et al., 1984; Gas et al., 1985; Roussel et al., 1992), whereas others were unable to confirm this observation (Pfeifle et al., 1986). Obviously, the presence of nucleolin on NORs would be difficult to reconcile with the apparent absence of nascent transcripts unless one assumes that it also binds to nucleolar chromatin (for models of interactions of nucleolin with nucleolar structures see Jordan, 1987; Olson, 1990). Hence, we have reinvestigated this important issue by using three different antibodies to nucleolin and three different cell lines. The resulting fluorescence patterns closely resembled those of U3 and fibrillarin (Fig. 8, *a–c*). In contrast to the strongly labeled interphase nucleoli, mitotic chromosomes including NORs were always negative. Taken together, our results strongly suggest that nucleolin dissociates from the NORs during mitosis.

Discussion

In previous immunocytochemical studies it has been shown that pol I molecules remain associated with the chromosomol NORs as cells progress through mitosis despite little or no RNA being synthesized during this phase of the cell cycle (Scheer and Rose, 1984; Reimer et al., 1987*a*; Haaf et al., 1988). We entertained the idea that the polymerases transiently stall during mitosis, perhaps in the form of "frozen" transcriptional units, and are reactivated toward the end of mitosis (Scheer and Rose, 1984). Such behavior would be compatible with the rapid resumption of rRNA synthesis at telophase which precedes the reactivation of non-nucleolar genes (Morcillo et al., 1976). Further support came from
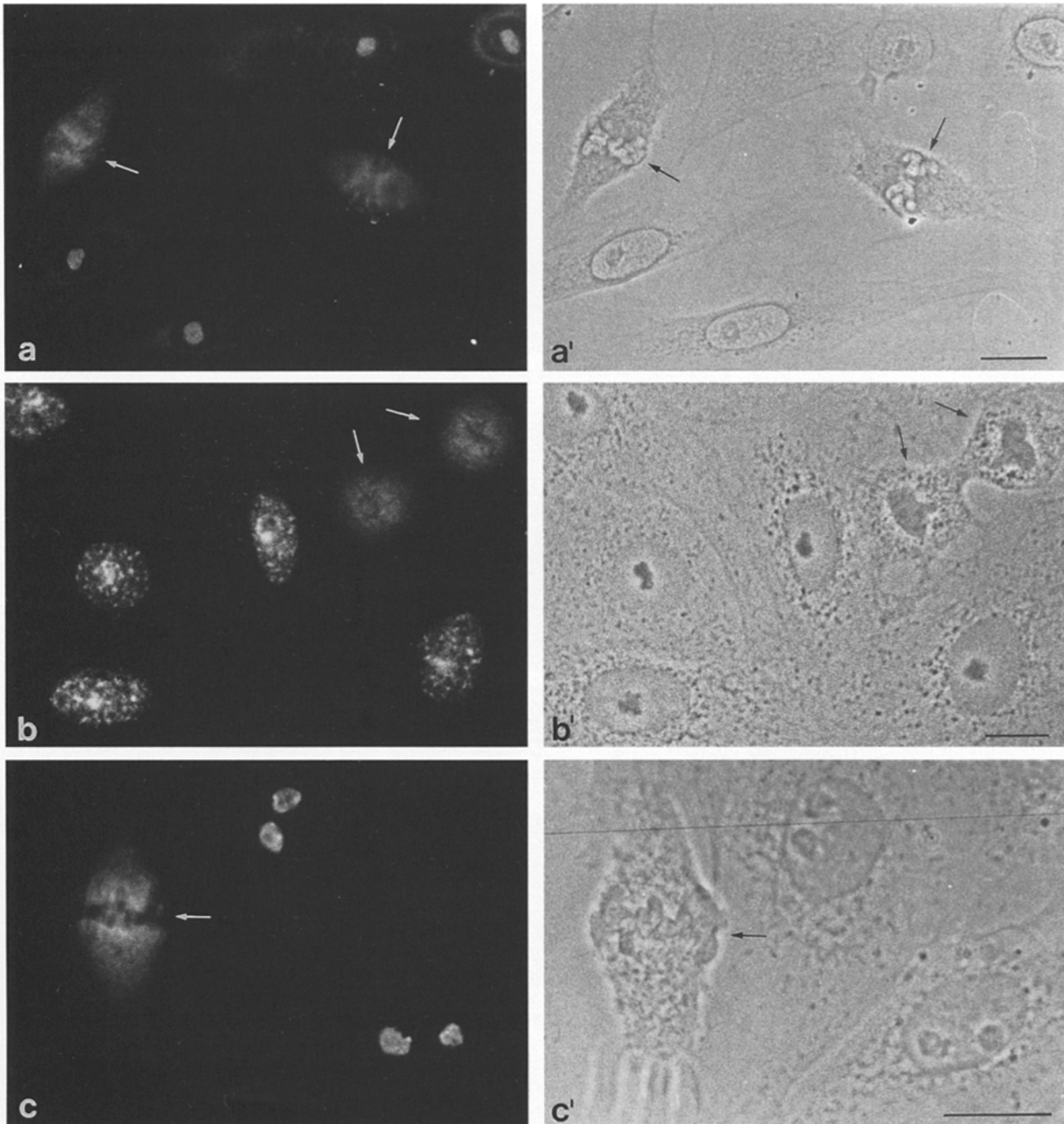
Figure 7. Components of the early pre-rRNA processing machinery are absent from the chromosomal NORs of mitotic PtK2 cells. The distribution of U3 snoRNA is revealed by fluorescent in situ hybridization (*a*); U3 and other capped snoRNAs are detected using antibodies against the trimethyl-guanosine cap of snRNAs (*b*). Besides capped snoRNAs these antibodies also recognize nucleoplasmic snRNAs as indicated by the speckled fluorescence pattern of the nucleoplasm (*b*; for further details see Reuter et al., 1984). The nucleolar protein fibrillarin is visualized by immunofluorescence microscopy using mAb 72B9 (*c*). Both U3 snoRNA and fibrillarin are enriched in interphase nucleoli but are virtually absent from the NORs of mitotic chromosomes (dividing cells are indicated by *arrows* in *a–c*). The corresponding phase contrast micrographs are shown (*a'–c'*). Bars, 20 μm.

biochemical experiments indicating that in mitotic HeLa cells pol I molecules exist as initiated complexes with rDNA (Matsui and Sandberg, 1985). The rapid turning on and off of rRNA synthesis during the cell cycle represents an extremely interesting circumstance through which mechanisms mediating gene regulation can be studied. With the

development of fluorescent in situ hybridization techniques it became feasible to critically examine the "frozen tree" model.

Our results do not provide evidence for the association of pre-rRNA transcripts with the chromosomal NORs. Two sets of controls showed that our in situ hybridization ap-
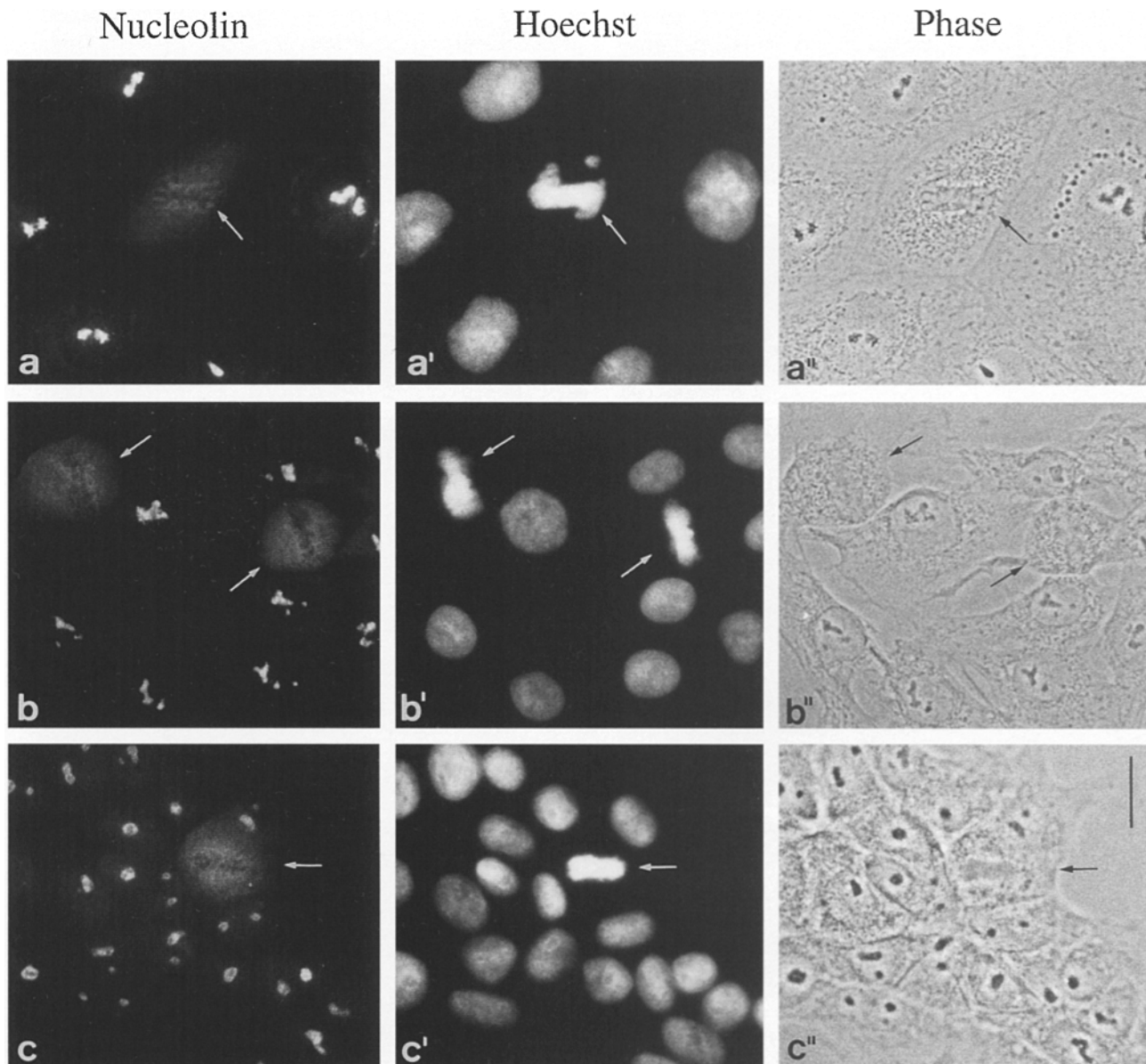
Figure 8. Distribution of nucleolin during interphase and mitosis of PtK2 (*a*), rat RVF-SM (*b*), and *Xenopus* A6 (*c*) cells as revealed by immunofluorescence microscopy with three different anti–nucleolin antibodies. (*a*) Affinity purified antibodies against mouse nucleolin (anti–ppl05, Pfeifle et al., 1986); (*b*) affinity purified antibodies against calf thymus nucleolin (Sapp et al., 1986); (*c*) mAb b6-6E7 against *Xenopus* nucleolin (Messmer and Dreyer, 1993). In all examples shown, nucleolin is strongly enriched in the interphase nucleoli but virtually absent from the NORs of mitotic chromosomes (mitotic cells are indicated by *arrows* in *a–c*). The corresponding DNA (Hoechst; *a'–c'*) and phase contrast images (*a"–c"*) are shown. Bar, 20 μm.

proach was sensitive enough to visualize individual rDNA transcription units. First, by exposure of cultured cells to the drug DRB, nucleoli were induced to unravel into beaded strands or "necklaces" while continuing transcription. Each of the beads is considered to represent a single rDNA transcription unit (Scheer et al., 1984; Haaf et al., 1991). After hybridization with the 28S probe, the beads appeared as distinctly fluorescent dots. Since this probe visualized only the later transcripts at the distal ends of the rRNA genes (located 80% or more of the gene length downstream from the transcription start; see Fig. 1), one fifth of the transcription unit is sufficient to produce a positive signal. Mammalian rRNA

genes are transcribed simultaneously by 100–130 pol I molecules (Puvion-Dutilleul, 1983); hence ∼25 nascent transcripts should be recognizable as a fluorescent dot provided that our initial assumption (one fluorescent dot of the DRB nuclei represents one rDNA transcription unit) is correct. Second, hybridization of the 28S probe to Miller spreads of nucleolar chromatin from salamander oocytes revealed, in the light microscope, linear arrays of strongly fluorescent entities separated by non-fluorescent gaps. Their tandem arrangement, dimensions, and reactivity with antibodies to pol I has firmly established the identity of these fluorescent structures as transcriptionally active rRNA genes or "Christ-

mas trees" known from EM spread preparations (Miller, 1981). Again, as indicated in Fig. 6 *d*, the hybridization probe binds only to relatively late nascent transcripts, i.e., those that have reached 75% of the length of a completed transcript. Since there are ∼100 transcriptional complexes per rRNA gene (Fig. 6 *c*), 25 transcripts are sufficient to produce a strong signal. We expect that the light microscopic analysis of Miller spreads by fluorescent in situ hybridization and immunofluorescence microscopy will provide a powerful tool for the analysis of the biochemical composition of transcriptionally active rRNA genes and the topology of transcription relevant proteins. Most importantly, this approach should help to identify those "early" ribosomal and nonribosomal proteins including processing factors that bind already to the nascent pre-rRNA chains. Furthermore, the interactions of rDNA transcription units with other nucleolar components involved in the formation and maintenance of specific subnucleolar domains may also be studied using less disruptive spreading conditions which preserve the basic architecture of nucleoli (Garcia-Blanco et al., 1995).

The tandemly repeated rRNA genes are so densely clustered at the NORs of mitotic chromosomes that 25 transcripts should not have gone undetected. Assuming a minimum of 100 copies of rRNA genes per NOR of PtK2 cells (for quantitative data see Long and Dawid, 1980), these data indicate that the vast majority of the transcripts is released from the rDNA as cells enter mitosis. Although we cannot formally exclude the possibility that nascent transcripts persist at the NORs but escaped detection because the condensed chromosomes limit access of the hybridization probe, we consider this unlikely for the following reasons. First, nascent transcripts of the Drosophila *Ubx* gene have been visualized on condensed metaphase chromosomes using a similar approach with digoxigenin-tagged hybridization probes (Shermoen and O'Farrell, 1991). Second, antibodies against rRNAs also failed to decorate the NORs in immunofluorescence. This negative result cannot be explained by restricted accessibility to the NOR because other antibodies recognized NOR-associated proteins such as pol I, DNA topoisomerase I, UBF, and ppl35 (Fig. 2).

However, our in situ data do not exclude the possibility that a few transcription complexes may remain poised on the transcription initiation site of the rRNA genes. A growing RNA chain has to reach a length of ∼100 nt until its 5′ end emerges from the DNA–enzyme complex (Hanna and Meares, 1983). Therefore it is conceivable that very early transcripts are not accessible to the hybridization probe and remain undetected.

Nascent transcripts of the rRNA genes carry a terminal "knob" or "ball" at their leading end. These structures have been shown to be the morphological equivalent of 5′ ETS processing complexes (Mougey et al., 1993*a*) and to contain, in addition to several proteins, U3 snoRNA and fibrillarin (Kass et al., 1990; Scheer and Benavente, 1990; Mougey et al., 1993*b*). Following in situ hybridization with a U3-specific probe and immunofluorescence with antibodies against the trimethyl-guanosine cap of U3 and against fibrillarin, we were unable to detect U3 or fibrillarin on the NORs. This finding also speaks against the persistence of nascent pre-rRNA transcripts on the chromosomal NORs and supports our results obtained with the rRNA hybridization probes. However, since terminal ball formation requires a minimum transcript length of 113 nt in *Xenopus* and ∼650 nt in mammalian species (Mougey et al., 1993*a*), U3 snoRNPs are probably absent from the earliest pre-rRNA transcripts. Hence our data do not exclude the existence of some short nascent transcripts close to the transcription initiation site of the rRNA genes.

Another marker protein for nascent pre-rRNA transcripts seems to be nucleolin, which has been implicated in the early stages of ribosome assembly (Olson, 1990). Several authors have localized nucleolin at the mitotic NORs (Lischwe et al., 1981; Ochs et al., 1983, 1985*b*; Spector et al., 1984; Gas et al., 1985; Roussel et al., 1982) whereas others have failed to do so (Pfeifle et al., 1986). We have used three different anti–nucleolin antibodies and several cell lines to clarify, by immunofluorescence microscopy, the distribution of nucleolin during mitosis. The results were clearcut. In no instance could we detect NOR-associated nucleolin. We ascribe the previous positive reports to the fact that nucleolin accumulates in early telophase in numerous dot-like prenucleolar bodies (Ochs et al., 1985*b*) which have been erroneously identified as NORs. If nucleolin does, in fact, bind to nascent pre-rRNA transcripts, then we must conclude from our data that at least the majority of the transcripts are released from the rDNA template at the onset of mitosis. Furthermore, our immunofluorescence results do not support the view that nucleolin remains bound to rDNA during mitosis and serves to maintain the nucleolar chromatin in a decondensed state (Gas et al., 1985; see also Belenguer et al., 1990). A recent quantitative analysis has also shown that in dividing HeLa cells the mitotic chromosomes are almost devoid of nucleolin (Roussel and Hernandez-Verdun, 1994).

The apparent absence of nucleolin from the NORs casts some doubts on the widely held view that this protein is responsible for the selective staining of NORs with silver nitrate (e.g., Howell, 1982; Roussel et al., 1992). More likely candidates for the silver staining of NORs in cytological preparations are pol I (Williams et al., 1982), the transcription initiation factor UBF, and/or ppl35, all of which remain associated with the NORs during mitosis (see Fig. 2). Of course, other as yet unknown proteins might determine the argyrophilic character of the NORs.

How can we incorporate all these observations into a coherent model of rRNA gene regulation during mitosis? From our failure to detect transcripts on the NORs of mitotic chromosomes, we rule out the "frozen tree" model in which pol I molecules are stalled (Fig. 9 *a*). Based on the following arguments, we further conclude that the mitotic repression of the rRNA genes is mechanistically different from the growth-dependent regulation of rRNA gene expression during interphase. In mammalian cells, the control of interphase rRNA synthesis occurs primarily at the level of transcription initiation mediated by positively and negatively acting transcription initiation factors that work in concert with pol I (for reviews see Paule, 1994; Moss and Stefanovsky, 1994). During amphibian oogenesis, rRNA gene expression is also modulated through the frequency of initiation events by pol I (Scheer et al., 1976). If similar mechanisms were involved in the mitotic repression of the rRNA genes, they would affect assembly of preinitiation complexes or recruitment of pol I to the promoter, but allow the already engaged polymerases to terminate properly. Consequently, after a time
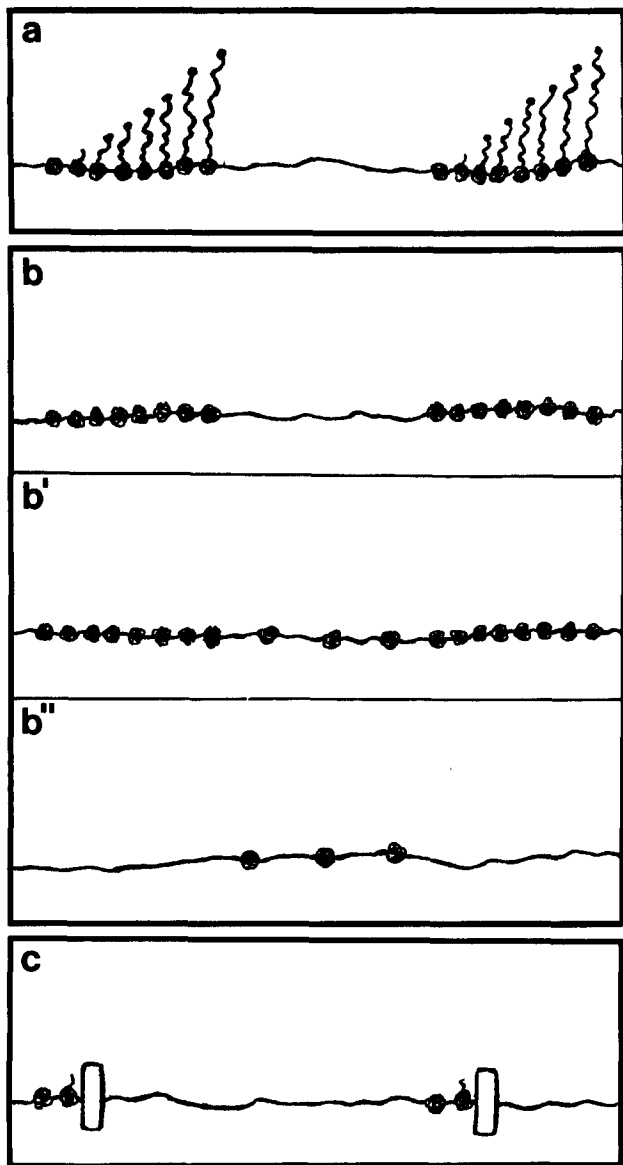
Figure 9. Alternative models for the mitotic repression of rRNA gene expression. The "roadblock" shown in version *c* is meant to illustrate regulation at the elongation level. It does not imply a specific mechanism of transcriptional elongation blockage. For further details see text.

lag of ∼5 min (i.e., the time required for a polymerase to traverse a mammalian rRNA gene with an average elongation rate of 2.5 × 10³ nt/min), all rRNA genes should be cleared of pol I. This is not the case. Rather, pol I molecules remain bound to the chromosomal NORs (Fig. 2 *a*), apparently in the form of initiated transcription complexes as judged from their ability to elongate in the presence of the polyanion heparin (Matsui et al., 1979; Matsui and Sandberg, 1985) or relatively high concentrations (0.4%) of the anionic detergent Sarkosyl (Gariglio et al., 1974). It is known that only initiated transcription complexes are capable of elongating in the presence of such high detergent concentrations while the assembly of the transcription initiation

complex at the rDNA promoter is inhibited (Schnapp and Grummt, 1991).

We envisage two ways to reconcile the presence of initiated pol I enzyme complexes with the apparent absence of nascent transcripts on the NORs of mitotic chromosomes. One model, depicted in Fig. 9 *b*, incorporates the premature release of nascent transcripts from the rDNA while the stalled polymerase complexes remain stably bound. Two variants of this model are conceivable. Pol I molecules are also stalled along the intergenic regions (NTS) reflecting transcription from spacer promoters during the preceding interphase (Fig. 9 *b'*; for identification of spacer transcription in mammalian species see Cassidy et al., 1987; Kuhn and Grummt, 1987; Tower et al., 1989). In this case the entire rDNA repeat unit will have inactive pol I associated with it. Alternatively, pol I remains bound to the intergenic region only (Fig. 9 *b"*). In any event, our in situ hybridization results indicate that during mitosis potential spacer-bound pol I complexes lack nascent transcripts. In the mouse, spacer transcription originates from a promoter located ∼2-kb upstream of the gene promoter (Kuhn and Grummt, 1987; Tower et al., 1989) and, hence, should be detected with probe 974b which includes this NTS region (see Fig. 1). However, the NORs of mouse mitotic chromosomes were negative after in situ hybridization with probe 974b. We note that models *b–b"* which assume that pol I enzymes remain bound to the rDNA template, are not compatible with the well known fact that transcriptional inhibition of rRNA genes during interphase leads to an increase of template-free pol I molecules (Chesterton et al., 1975; Grummt et al., 1976). However, the mechanisms involved in the repression of rDNA transcription during mitosis might be different from those modulating rRNA synthesis during interphase.

The other model is based on the assumption that regulation acts at the level of early transcription elongation rather than at the initiation step (Fig. 9 *c*). There is a growing list of eukaryotic protein coding genes whose expression is controlled during the elongation phase (for reviews see Krumm et al., 1993; Lis and Wu, 1993; Wright, 1993). We suggest that related mechanisms also modulate the expression of the rRNA genes during the mitotic cycle. A central feature of the elongation control mechanisms is that after initiation the transcriptional complexes enter an abortive elongation pathway and pause and/or terminate prematurely within several hundred nucleotides of the promoter, thus generating short, truncated transcripts (Krumm et al., 1993; Wright, 1993). An indication for a polarized distribution of pol I complexes along the rRNA genes at mitosis stems from the finding that promoter-containing rDNA fragments were specifically enriched in scaffolds prepared from metaphase chromosomes of HeLa cells whereas the entire rDNA repeat was found associated with the matrix prepared from interphase nuclei (Keppel, 1986). The author concluded that pol I complexes which seem to mediate the binding of rDNA to matrix/scaffold structures are clustered on promoter-proximal regions during mitosis. Moreover, short RNAs derived from the 5' end of rDNA transcription units, designated small nuclear polymerase I RNA (snPI RNA), have been found in mouse and human cells by nuclear run-on analyses (Reichel et al., 1982; Reichel and Benecke, 1984). The most abundant class of these truncated pre-RNAs comprise the first 210 nt of the 5' ETS (Reichel and Benecke, 1984). We there-

fore envisage that at mitosis rDNA transcription complexes pause or terminate ~210-bp downstream of the promoter until transactivating factors convert them into a processive form capable of generating full-length pre-rRNA transcripts. A pol I transcription factor, termed TIF-IC, has recently been described which is capable of converting stalled rDNA transcription complexes into elongation–competent complexes (Schnapp et al., 1994). Transcription factors acting in a similar way are also known to regulate the elongation of several Drosophila pol II–dependent genes (e.g., positive transcription elongation factor; Marshall and Price, 1992).

A pausing region of 210 bp could accomodate three or four densely spaced pol I complexes with diameters of ~15-nm each. As discussed above, the short transcripts are either wholly or partially buried within the pol I complexes and, hence, most likely escape detection by in situ hybridization. Moreover, their 5' ends lack the pre-rRNA processing machinery (Mougey et al., 1993a) and thus cannot be labeled with antibodies to fibrillarin and U3 snoRNA.

Our data do not allow us to determine whether non-processive rDNA transcription is a result of elongation-deficient transcription complexes or, alternatively, steric hindrance of elongation, possibly imposed by chromatin structure or specific DNA-binding proteins. However, they do not support the view that cells use a common mechanism to inactivate all nuclear genes during mitosis, possibly by phosphorylation of the universal transcription factor, the TATA-binding protein (Gottesfeld et al., 1994). While mitotic phosphorylation of the TATA-binding protein or associated factors have been shown to affect the initiation frequency of pol III–dependent genes (Gottesfeld et al., 1994), alternative strategies operating at the postinitiation level are apparently used for the transient inactivation of the rRNA genes during the mitotic cycle. It is tempting to speculate that the continuous presence of the pol I–dependent transcriptional machinery prevents the condensation of nucleolar chromatin at mitosis (for ultrastructural aspects of the mitotic NORs see Hsu et al., 1967; Goessens, 1984) and allows the immediate and effective resumption of pre-rRNA synthesis in the newly formed daughter nuclei.

Note Added in Proof. A recent study also provided evidence that both U3 and U8 snoRNAs are released from the NORs during mitosis (Matera, A. G, K. T. Tycowski, J. A. Steitz, and D. C. Ward. 1994. Organization of small nucleolar ribonucleoproteins (snoRNPs) by fluorescence in situ hybridization and immunocytochemistry. *Mol. Biol. Cell.* 5:1289–1299).

References

Azum-Gelade, M.-C., J. Noaillac-Depeyre, M. Caizergues-Ferrer, and N. Gas. 1994. Cell cycle redistribution of U3 snRNA and fibrillarin. *J. Cell Sci.* 107:463–475.

Belenguer, P., M. Caizergues-Ferrer, J.-C. Labbe, M. Doree, and F. Amalric. 1990. Mitosis-specific phosphorylation of nucleolin by p34^{cdc2} protein kinase. *Mol. Cell. Biol.* 10:3607–3618.

Cassidy, B. G., H.-F. Yang-Yen, and L. I. Rothblum. 1987. Additional RNA polymerase I initiation site within the nontranscribed spacer region of the rat rRNA gene. *Mol. Cell. Biol.* 7:2388–2396.

Chan, E. K. L., H. I. 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 transcription upstream binding factor. *J. Exp. Med.* 174:1239–1244.

Chesterton, C. J., B. E. H. Coupar, P. H. W. Butterworth, J. Buss, and M. H. Green. 1975. Studies on the control of ribosomal RNA synthesis in HeLa cells. *Eur. J. Biochem.* 57:79–83.

Chooi, W. Y., and K. R. Leiby. 1981. An electron microscopic method for localization of ribosomal proteins during transcription of ribosomal DNA: a method for studying protein assembly. *Proc. Natl. Acad. Sci. USA.* 78:4823–4827.

Cox, K. H., D. V. DeLeon, L. M. Angerer, and R. C. Angerer. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev. Biol.* 101:485–502.

Fan, H., and S. Penman. 1971. Regulation of synthesis and processing of nucleolar components in metaphase-arrested cells. *J. Mol. Biol.* 59:27–42.

Fey, E. G., G. Krochmalnic, and S. Penman. 1986. The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* 102:1654–1665.

Fischer, D., D. Weisenberger, and U. Scheer. 1991. Assigning functions to nucleolar structures. *Chromosoma (Berl.).* 101:133–140.

Fournier, M. J., and E. S. Maxwell. 1993. The nucleolar snRNAs: catching up with the spliceosomal snRNAs. *Trends Biochem. Sci.* 18:131–135.

Franke, W. W., U. Scheer, H. Spring, M. F. Trendelenburg, and H. Zentgraf. 1979. Organization of nucleolar chromatin. *In* The Cell Nucleus. Vol. 7. H. Busch, editor. Academic Press, New York. 49–95.

Franke, W. W., E. Schmid, J. Vandekerckhove, and K. Weber. 1980. A permanently proliferating rat vascular smooth muscle cell with maintained expression of smooth muscle characteristics, including actin of the vascular smooth muscle type. *J. Cell Biol.* 87:594–600.

Garcia-Blanco, M. A., D. D. Miller, and M. P. Sheetz. 1995. Nuclear Spreads: I. Visualization of bipartite ribosomal RNA domains. *J. Cell Biol.* 128:15–27.

Gariglio, P., J. Buss, and M. H. Green. 1974. Sarkosyl activation of RNA polymerase activity in mitotic mouse cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 44:330–333.

Gas, N., M.-L. Escande, and B. J. Stevens. 1985. Immunolocalization of the 100 kDa nucleolar protein during the mitotic cell cycle in CHO cells. *Biol. Cell.* 53:209–218.

Gerbi, S. A., R. Savino, B. Stebbins-Boaz, C. Jeppesen, and R. Rivera-Leon. 1990. A role for U3 small nuclear ribonucleoprotein in the nucleolus? *In* The Ribosome - Structure, Function and Evolution. W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner, editors. American Society for Microbiology, Washington, D.C. 452–469.

Goessens, G. 1984. Nucleolar structure. *Int. Rev. Cytol.* 87:107–158.

Gottesfeld, J. M., V. J. Wolf, T. Dang, D. J. Forbes, and P. Hartl. 1994. Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. *Science (Wash. DC).* 263:81–84.

Grummt, I., V. A. Smith, and F. Grummt. 1976. Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase. *Cell.* 7:439–445.

Grummt, I., C. Soellner, and I. Scholz. 1979. Characterization of a cloned ribosomal fragment from mouse which contains the 18 S coding region and adjacent spacer sequences. *Nucleic Acids Res.* 6:1351–1369.

Grunstein, M. 1990. Histone function in transcription. *Annu. Rev. Cell. Biol.* 6:643–678.

Guldner, H. H., C. Szostecki, H. P. Vosberg, H. J. Lakomek, E. Penner, and R. A. Bautz. 1986. Scl 70 autoantibodies from scleroderma patients recognize a 95 kDa protein identified as DNA topoisomerase I. *Chromosoma (Berl.).* 94:132–138.

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.

Haaf, T., D. L. Hayman, and M. Schmid. 1991. Quantitative determination of rDNA transcription units in vertebrate cells. *Exp. Cell Res.* 193:78–86.

Hanna, M. M., and C. F. Meares. 1983. Topography of transcription: path of the leading end of nascent RNA through the Escherichia coli transcription complex. *Proc. Natl. Acad. Sci. USA.* 80:4238–4242.

Hartl, P., J. M. Gottesfeld, and D. J. Forbes. 1993. Mitotic repression of transcription in vitro. *J. Cell Biol.* 120:613–624.

Hernandez-Verdun, D., and T. Gautier. 1994. The chromosome periphery during mitosis. *BioEssays.* 16:179–185.

Howell, W. M. 1982. Selective staining of nucleolus organizer region (NOR). *In* The Cell Nucleus. Vol. 11. H. Busch and L. Rothblum, editors. Academic Press, New York. 89–142.

Hsu, T. C., B. R. Brinkley, and F. E. Arrighi. 1967. The structure and behavior

of the nucleolus organizers in mammalian cells. *Chromosoma (Berl.).* 23:137–153.

Hsu, T. C., S. E. Spirito, and M. L. Pardue. 1975. Distribution of 18+28S ribosomal genes in mammalian genomes. *Chromosoma (Berl.).* 53:25–36.

Jeppesen, C., B. Stebbins-Boaz, and S. A. Gerbi. 1988. Nucleotide sequence determination and secondary structure of Xenopus U3 snRNA. *Nucleic Acids Res.* 16:2127–2148.

Jimenez-Garcia, L. F., M. L. Segura-Valdez, R. L. Ochs, L. I. Rothblum, R. Hannan, and D. L. Spector. 1994. Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. *Mol. Biol. Cell.* 5:955–966.

Johnson, T. C., and J. J. Holland. 1965. Ribonucleic acid and protein synthesis in mitotic HeLa cells. *J. Cell Biol.* 27:565–574.

Jordan, E. G. 1987. At the heart of the nucleolus. *Nature (Lond.).* 329:489–490.

Kass, S., K. Tyc, J. A. Steitz, and B. Sollner-Webb. 1990. The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell.* 60:897–908.

Keppel, F. 1986. Transcribed human ribosomal RNA genes are attached to the nuclear matrix. *J. Mol. Biol.* 187:15–21.

Kornberg, R. D., and Y. Lorch. 1992. Chromatin structure and transcription. *Annu. Rev. Cell. Biol.* 8:563–587.

Krumm, A., T. Meulia, and M. Groudine. 1993. Common mechanisms for the control of eukaryotic transcriptional elongation. *BioEssays.* 15:659–665.

Kuhn, A., and I. Grummt. 1987. A novel promoter in the mouse rDNA spacer is active in vivo and in vitro. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3487–3492.

Lis, J., and C. Wu. 1993. Protein traffic on the heat shock promoter: parking, stalling, and trucking along. *Cell.* 74:1–4.

Lischwe, M. A., R. L. Richards, R. K. Busch, and H. Busch. 1981. Localization of phosphoprotein C23 to nucleolar structures and to the nucleolus organizer regions. *Exp. Cell Res.* 136:101–109.

Long, E. O., and I. B. Dawid. 1980. Repeated genes in eukaryotes. *Annu. Rev. Biochem.* 49:727–764.

Marshall, N. F., and D. H. Price. 1992. Control of formation of two distinct classes of RNA polymerase II elongation complexes. *Mol. Cell. Biol.* 12:2078–2090.

Maser, R. L., and J. P. Calvet. 1989. U3 small nuclear RNA can be psoralen crosslinked in vivo to the 5' external transcribed spacer of pre-ribosomal RNA. *Proc. Natl. Acad. Sci. USA.* 86:6523–6527.

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.

Matsui, S., 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.

McIntosh, J. R. 1991. Structural and mechanical control of mitotic progression. *Cold Spring Harbor Symp. Quant. Biol.* 56:613–619.

Messmer, B., and C. Dreyer. 1993. Requirements for nuclear translocation and nucleolar accumulation of nucleolin of Xenopus laevis. *Eur. J. Cell Biol.* 61:369–382.

Miller, O. L. 1981. The nucleolus, chromosomes, and visualization of genetic activity. *J. Cell Biol.* 91:15s–27s.

Miller, O. L., and A. H. Bakken. 1972. Morphological studies of transcription. *Acta. Endocrinol. [Suppl].* 168:155–177.

Morcillo, G., C. de la Torre, and G. Gimenez-Martin. 1976. Nucleolar transcription during plant mitosis. *Exp. Cell Res.* 102:311–316.

Moreno, S., and P. Nurse. 1990. Substrates for p34^{cdc2}: in vivo veritas? *Cell.* 61:549–551.

Moss, T., and V. Y. Stefanovsky. 1994. Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I. *In* Progress in Nucleic Acid Research. W. E. Cohn, and K. Moldave, editors. Academic Press, San Diego. In press.

Mougey, E. B., M. O'Reilly, Y. Osheim, O. L. Miller, A. Beyer, and B. Sollner-Webb. 1993a. The terminal balls characteristic of eukaryotic rRNA transcription units in chromatin spreads are rRNA processing complexes. *Genes & Dev.* 7:1609–1619.

Mougey, E. B., L. K. Pape, and B. Sollner-Webb. 1993b. A U3 nuclear ribonucleoprotein-requiring processing event in the 5' external transcribed spacer of Xenopus precursor rRNA. *Mol. Cell. Biol.* 13:5990–5998.

O'Reilly, M. M., S. L. French, M. L. Sikes, and O. L. Miller. 1994. Ultrastructural in situ hybridization to nascent transcripts of highly transcribed rRNA genes in chromatin spreads. *Chromosoma (Berl.).* 103:122–128.

Ochs, R. L., M. A. Lischwe, P. O'Leary, and H. Busch. 1983. Localization of nucleolar phosphoproteins B23 and C23 during mitosis. *Exp. Cell Res.* 146:139–149.

Ochs, R. L., M. A. Lischwe, W. H. Spohn, and H. Busch. 1985a. Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. *Biol. Cell.* 54:123–134.

Ochs, R. L., M. A. Lischwe, E. Shen, R. E. Carroll, and H. Busch. 1985b. Nucleologenesis: composition and fate of prenucleolar bodies. *Chromosoma (Berl.).* 92:330–336.

Olson, M. O. J. 1990. The role of proteins in nucleolar structure and function. *In* The Eukaryotic Nucleus. Molecular biochemistry and macromolecular assemblies. Vol. 2. P. R. Strauss and S. H. Wilson, editors. The Telford Press,

Caldwell, NJ. 519–559.

Paule, M. R. 1994. Transcription of ribosomal RNA by eukaryotic RNA polymerase I. *In* Transcription: Mechanisms and Regulation. R. C. Conaway and J. W. Conaway, editors. Raven Press, New York. 83–106.

Peng, H. B. 1991. Solutions and protocols. *In* Methods in Cell Biology. Vol. 36. B. K. Kay and H. B. Peng, editors. Academic Press, San Diego. 657–662.

Pfeifle, J., K. Boller, and F. A. Anderer. 1986. Phosphoprotein pp135 is an essential component of the nucleolus organizer region (NOR). *Exp. Cell Res.* 162:11–22.

Prescott, D. M. 1964. Cellular sites of RNA synthesis. *Prog. Nucleic Acids Res.* 3:33–57.

Puvion-Dutilleul, F. 1983. Morphology of transcription at cellular and molecular levels. *Int. Rev. Cytol.* 84:57–101.

Reichel, R., and B.-J. Benecke. 1984. Localization of small nuclear polymerase I RNA sequences at the 5' end of the human rDNA transcription unit. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:473–479.

Reichel, R., H.-J. Monstein, H.-W. Jansen, L. Philipson, and B.-J. Benecke. 1982. Small nuclear RNAs are encoded on the nontranscribed region of ribosomal spacer DNA. *Proc. Natl. Acad. Sci. USA.* 79:3106–3110.

Reimer, G., K. M. Rose, U. Scheer, and E. M. Tan. 1987a. Autoantibody to RNA polymerase I in scleroderma sera. *J. Clin. Invest.* 79:65–72.

Reimer, G., K. M. Pollard, C. A. Penning, R. L. Ochs, M. A. Lischwe, H. Busch, and E. M. Tan. 1987b. Monoclonal autoantibodies from a (New Zealand black × New Zealand white) F1 mouse and some human scleroderma sera target an Mr 34,000 nucleolar protein of the U3 RNP particle. *Arthr. Rheum.* 30:793–800.

Rendon, M. C., R. M. Rodrigo, L. G. Goenechea, G. Garcia-Herdugo, M. M. Valdivia, and F. J. Moreno. 1992. Characterization and immunolocalization of a nucleolar antigen with anti-NOR serum in HeLa cells. *Exp. Cell Res.* 200:393–403.

Reuter, R., B. Appel, P. Bringmann, J. Rinke, and R. Lührmann. 1984. 5'-terminal caps of snRNAs are reactive with antibodies specific for 2,2,7-trimethylguanosine in whole cells and nuclear matrices. *Exp. Cell Res.* 154:548–560.

Robert-Fortel, I., H.-R. Junera, G. Geraud, and D. Hernandez-Verdun. 1993. Three-dimensional organization of the ribosomal genes and Ag-NOR proteins during interphase and mitosis in PtK1 cells studied by confocal microscopy. *Chromosoma (Berl.).* 102:146–157.

Rose, K. M., J. Szopa, F.-S. Han, Y.-C. Cheng, A. Richter, and U. Scheer. 1988. Association of DNA topoisomerase I and RNA polymerase I: a possible role for topoisomerase I in ribosomal gene transcription. *Chromosoma (Berl.).* 96:411–416.

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.

Roussel, P., P. Belenguer, 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.

Roussel, P., C. Andre, C. Masson, G. Geraud, and D. Hernandez-Verdun. 1993. Localization of RNA polymerase I transcription factor hUBF during the cell cycle. *J. Cell Sci.* 104:327–337.

Sapp, M., R. Knippers, and A. Richter. 1986. DNA binding properties of a 110kDa nucleolar protein. *Nucleic Acids Res.* 14:6803–6820.

Scheer, U., and R. Benavente. 1990. Functional and dynamic aspects of the mammalian nucleolus. *BioEssays.* 12:14–21.

Scheer, U., and K. M. Rose. 1984. Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunochemistry. *Proc. Natl. Acad. Sci. USA.* 81:1431–1435.

Scheer, U., M. F. Trendelenburg, and W. W. Franke. 1976. Regulation of transcription of genes of ribosomal RNA during amphibian oogenesis. A biochemical and morphological study. *J. Cell Biol.* 69:465–489.

Scheer, U., B. Hügle, R. Hazan, and K. M. Rose. 1984. Drug-induced dispersal of transcribed rRNA genes and transcriptional products: Immunolocalization and silver staining of different nucleolar components in rat cells treated with 5,6-dichloro-β-D-ribofuranosylbenzimidazole. *J. Cell Biol.* 99:672–679.

Schnapp, A., and I. Grummt. 1991. Transcription complex formation at the mouse rDNA promoter involves the stepwise association of four transcription factors and RNA polymerase I. *J. Biol. Chem.* 266:24588–24595.

Schnapp, G., A. Schnapp, H. Rosenbauer, and I. Grummt. 1994. TIF-IC, a factor involved in both transcription initiation and elongation of RNA polymerase I. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4028–4035.

Shermoen, A. W., and P. H. O'Farrell. 1991. Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. *Cell.* 67:303–310.

Smith, J. C., and J. R. Tata. 1991. Xenopus cell lines. *In* Methods in Cell Biology. Vol. 36. B. K. Kay and H. B. Peng, editors. Academic Press, San Diego. 635–654.

Spector, D. L., R. L. Ochs, and H. Busch. 1984. Silver staining, immunofluorescence, and immunoelectron microscopic localization of nucleolar phosphoproteins B23 and C23. *Chromosoma (Berl.).* 90:139–148.

Stroke, I. L., and A. M. Weiner. 1989. The 5' end of U3 snRNA can be crosslinked in vivo to the external transcribed spacer of rat ribosomal RNA precursors. *J. Mol. Biol.* 210:497–512.

Tiemeier, D. C., S. M. Tilghman, and P. Leder. 1977. Purification and cloning of a mouse ribosomal gene fragment in coliphage lambda. *Gene.* 2:173–191.

Tower, J., S. L. Henderson, K. M. Dougherty, P. J. Wejksnora, and B. Sollner-Webb. 1989. An RNA polymerase I promoter located in the CHO and mouse ribosomal DNA spacers: functional analysis and factor and sequence requirements. *Mol. Cell. Biol.* 9:1513–1525.

Voit, R., A. Schnapp, A. Kuhn, H. Rosenbauer, P. Hirschmann, H. G. Stunnenberg, and I. Grummt. 1992. The nucleolar transcription factor mUBF is phosphorylated by casein kinase II in the C-terminal hyperacidic tail which is essential for transactivation. *EMBO (Eur. Mol. Biol. Organ.) J.* 11: 2211–2218.

Wassef, M., J. Burglen, and W. Bernhard. 1979. A new method for visualization of pre-ribosomal granules in the nucleolus after acetylation. *Biol. Cell.* 34:153–158.

Williams, M. A., J. A. Kleinschmidt, G. Krohne, and W. W. Franke. 1982.

Argyrophilic nuclear and nucleolar proteins of Xenopus laevis oocytes identified by gel electrophoresis. *Exp. Cell Res.* 137:341–351.

Wolffe, A. P. 1991. Developmental regulation of chromatin structure and function. *Trends Cell Biol.* 1:61–66.

Wright, S. 1993. Regulation of eukaryotic gene expression by transcriptional attenuation. *Mol. Biol. Cell.* 4:661–668.

Zatsepina, 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.

Zhang, H., J. C. Wang, and L. F. Liu. 1988. Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes. *Proc. Natl. Acad. Sci. USA.* 85:1060–1064.

Zlatanova, J., and K. van Holde. 1992. Histone H1 and transcription: still an enigma? *J. Cell Sci.* 103:889–895.