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

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**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) 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 TFIIB. 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

1. **Abbreviations used in this paper:** DRB, 5,6-dichloro-l-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.
Pleurodeles waltl dichloro-l-/~-D-ribofuranosylbenzimidazole (DRB; Sigma, Deisenhofen, Germany) was used in this study as a mitotic arrest agent. Rat kidney (PtK2) tissues were cultured in DME (GIBCO BRL, Eggenstein, Germany) supplemented with 10% FCS at 37°C in a 5% CO2 incubator.

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) 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 ppi3 (affinity-purified rabbit antibodies against mouse ppi3; 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 pMr794 (Grunmt et al., 1979) and pMr100 (Tiemeier et al., 1977) were kindly provided by Ingrid Grunmt (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 −5715 to +5635 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-Sall fragment, comprised a short NTS sequence and a large part of the 5′ ETS (residues −175 to +3,084). Probe "TSI," a 228-nt BamHI-EcoRI fragment, was derived from internal transcribed spacer sequences (ITSi, 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 (Jepesen 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, ITSi, 28S). Some diagnostic restriction sites are indicated (E, EcoRI; S, Sall).
(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 containing 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.
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 1-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 nucleolus 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 cytoplasmic fluorescence was observed in metaphase (Fig. 4 b). The 28S and 974b probes labeled the cytoplasmic ribosomes (a–c). The corresponding DNA staining with Hoechst (a'–e') and phase contrast images (a''–e'') are shown. Bars, 20 μm.
Figure 4. Distribution of rRNAs and their precursors in PK2 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 recently 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 walti 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
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.

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 snoRNP's 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...
Understanding the nucleolar organization in mitotic cells

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 nuclear 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).

Discussion

In previous immunocytochemical studies it has been shown that pol I molecules remain associated with the chromosomal 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., 1987b; 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
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.

The approach 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 (Shermon 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., 1993a) and to contain, in addition to several proteins, U3 snoRNA and fibrillarin (Kass et al., 1990; Scheer and Benavente, 1990; Mougey et al., 1993b). 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., 1993b), 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, 1985b; 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., 1985b) 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 blockade. For further details see text.

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 blockade. For further details see text.
fore envisage that at mitosis rDNA transcription complexes pause or terminate ~210-bp downstream of the promoter un-
plexes (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 accommodate 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-pro-
cessive 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 phosphoryla-
tion of the universal transcription factor, the TATA-binding protein (Gottesfeld et al., 1994). While mitotic phosphoryla-
tion 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.

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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 nuclear ribonucleoproteins (snoRNPs) by fluorescence in situ hybridization and immunocytochemistry. Mol. Biol. Cell. 5:1289–1299).

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