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Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunocytochemistry

(nucleolus/nucleolus organizer/fibrillar centers/rRNA genes/anti-RNA polymerase I antibodies)

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ABSTRACT **Rabbit antibodies to RNA polymerase I from a rat hepatoma have been used to localize the enzyme in a variety of cells at the light and electron microscopic level. In interphase cells the immunofluorescence pattern indicated that polymerase I is contained exclusively within the nucleolus. That this fluorescence, which appeared punctated rather than uniform, represented transcriptional complexes of RNA polymerase I and rRNA genes was suggested by the observation that it was enhanced in regenerating liver and in a hepatoma and was markedly diminished in cells treated with actinomycin D. Electron microscopic immunolocalization using gold-coupled second antibodies showed that transcribed rRNA genes are located in, and probably confined to, the fibrillar centers of the nucleolus. In contrast, the surrounding dense fibrillar component, previously thought to be the site of nascent pre-rRNA, did not contain detectable amounts of polymerase I. During mitosis, polymerase I molecules were detected by immunofluorescence microscopy at the chromosomal nucleolus organizer region, indicating that a considerable quantity of the enzyme remains bound to the rRNA genes. From this we conclude that rRNA genes loaded with polymerase I molecules are transmitted from one cell generation to the next one and that factors other than the polymerase itself are involved in the modulation of transcription of DNA containing rRNA genes during the cell cycle.**

Nucleoli are morphologically defined as distinct spheroidal bodies of interphase nuclei in which the synthesis of precursors of rRNA (pre-rRNAs), their processing, and their assembly with specific proteins take place (reviewed in refs. 1 and 2). Although the morphological features of nucleoli can vary widely in different cell types and in different metabolic states, three major components are usually distinguished (1): the granular component, the dense fibrillar component, and the "fibrillar centers." The latter usually appear as small islets of rounded structures of relatively low contrast embedded in the dense fibrillar component (1, 3, 4).

Autoradiographic and biochemical studies have indicated that material containing primary transcripts from the rRNA genes is mainly recovered in the dense fibrillar component, whereas the granular portions contain rRNA–protein complexes in later stages of maturation (for review, see ref. 5). Although some steps of ribosome biogenesis can be correlated with certain morphologically distinct regions of the nucleolus, the localization of the transcriptionally active rRNA genes has remained uncertain. Electron microscopic autoradiography has shown that pulse-labeled RNA molecules are located in the dense fibrillar component of the nucleolus (4, 6–8). On the other hand, *in situ* hybridization experiments have indicated the presence of DNA containing the genes for rRNA (rDNA) in the fibrillar centers (9). Because the latter technique detects rDNA sequences independent of transcriptional activity, it has been proposed that the fibrillar centers contain transcriptionally inactive rDNA, whereas transcribed regions are looped out and extend into the surrounding dense fibrillar component, thus becoming undetectable by light microscopic *in situ* hybridization (4, 7–9). According to these authors, the dense fibrillar component contains both the transcription units of the rRNA genes and the newly formed ribonucleoprotein fibrils.

Transcribed rRNA genes are usually characterized by the presence of a high number of densely spaced transcription complexes [i.e., the nascent ribonucleoprotein fibrils anchored to the chromatin axis by 12- to 15-nm-large particles containing the RNA polymerase I complex (10)], whereas nontranscribed rDNA is free of such material (for references, see ref. 11). In order to unequivocally localize the basal portion of the transcriptional complexes and, hence, the sites of transcription of the rDNA, we applied immunocytochemical techniques using antibodies directed against the RNA polymerase specific for pre-rRNA synthesis—i.e., RNA polymerase I (12). Here we report that these antibodies allow the detection of transcribed rRNA genes exclusively in one specific nucleolar component, the fibrillar center. Furthermore, we show that the RNA polymerases I remain attached to this nucleolar component throughout mitosis and form a part of the nucleolus organizer region (NOR).

MATERIALS AND METHODS

Antibodies. Rabbit antibodies against purified RNA polymerase I from rat Morris hepatoma have been characterized (12). Human antibodies to nucleolar antigens were obtained from the serum of a patient suffering from scleroderma autoimmune disease (13). IgG was prepared by chromatography on DEAE-cellulose.

Biological Material and Immunofluorescence Microscopy. Cryostat sections through frozen liver tissues from various species and through regenerating rat liver (18 hr after partial hepatectomy) and solid rat Novikoff hepatoma were air-dried, dipped briefly in cold (−20°C) acetone, and air-dried again. Established cell lines from man (HeLa), rat (RVF-SMC cells; ref. 14), rat kangaroo (PtK2 from male *Potorous tridactylis*), and various other species were grown on coverslips, washed briefly in phosphate-buffered saline (P_i/NaCl), fixed for 5 min in cold (−20°C) methanol, dipped in cold acetone, and finally air-dried. Metaphase chromosomes from PtK2 cells were prepared by a method avoiding fixation in acids (15). Briefly, cells treated for 2 hr with 10 μM Colce-

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Abbreviations: rDNA, DNA containing the genes for ribosomal RNA; pre-rRNA, ribosomal RNA precursor; NOR, nucleolus organizer region.

mid were collected by trypsination and washed with P_i/NaCl. The cell pellet was suspended in 75 mM KCl at 4°C, centrifuged (*ca.* 500 × *g* for 5 min), and resuspended in the buffer described by Adolph (ref. 16; his "method 1"). Finally, the material was deposited on acetone-cleaned microscope slides by using a cytocentrifuge (Cytospin; Shandon Labortechnik, Frankfurt), briefly fixed in cold acetone, and air-dried.

All preparations were incubated for 30 min with the specific antibodies or control IgG at 70 μg/ml and washed in P_i/NaCl, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG diluted 1:20 (Miles-Yeda, Rehovot, Israel).

Electron Microscopic Immunolocalization. Cryostat sections of 5-μm thickness through frozen rat liver and Novikoff hepatoma were prepared as described above. After acetone fixation and air-drying, the tissue sections were first treated for 30 min with goat IgG at 1 mg/ml to block nonspecific sites of protein adsorption and then rinsed with P_i/NaCl, followed by incubation with antibodies to RNA polymerase I (50 μg/ml) for 6 hr at room temperature. The preparations were thoroughly washed in P_i/NaCl and incubated overnight with goat anti-rabbit IgG coupled to colloidal gold (5-nm diameter; Janssen Life Sciences, Beerse, Belgium) diluted 1:50 with P_i/NaCl containing goat IgG at 1 mg/ml. After several washes in P_i/NaCl, the specimens on coverslips were fixed in cold 2.5% glutaraldehyde buffered with 50 mM sodium cacodylate (pH 7.2) for 15 min, washed in cacodylate buffer, and then postfixed in 2% osmium tetroxide (15 min). Specimens were then washed in distilled water, dehydrated through graded ethanol solutions, and immersed in an Epon/propylene oxide mixture, 1:1 (vol/vol), followed by pure Epon 812 at room temperature. Finally, an Epon-filled gelatine capsule was placed inverted over the preparation. After polymerization, the coverslips were removed by brief immersion of the capsules into liquid nitrogen, and ultrathin sections were prepared close to the flat surface of the Epon blocks. The sections were double-stained according to standard methods.

RESULTS

The antibodies used reacted with most of the subunits of rat RNA polymerase I but not with polypeptides of RNA polymerase II (12). With immunofluorescence microscopy, it was evident that these antibodies specifically and intensely interacted with the nucleoli of rat cells in tissues and in cell cultures (Figs. 1 and 2 *a* and *b*). By contrast, antibodies to RNA polymerase II were excluded from nucleoli (not shown; compare ref. 17). In frozen sections of rat liver, the nucleoli often revealed several small distinct fluorescent entities (Fig. 1*c*). The number of these brightly fluorescing intranucleolar spots was considerably higher in regenerating rat liver and Novikoff hepatoma cells than in normal hepatocytes (Fig. 1 *d* and *e*). A similar punctate fluorescence pattern was seen in nucleoli of cultured rat cells (Fig. 2*b*). Inhibition of rDNA transcription by treatment of such cells with actinomycin D resulted in a redistribution of the fluorescent intranucleolar structures into cap-like aggregates at the nucleolar periphery (Fig. 2 *c* and *d*). After prolonged exposure of the cells to the drug, the fluorescence disappeared completely or, in some cells, was reduced to a miniscule residual spot (Fig. 2 *e* and *f*).

Nucleoli of all other mammalian species tested were also positive (rat kangaroo, mouse, bovine, and human). Certain cell types such as HeLa showed with special clarity that the fluorescence was not evenly distributed over the whole nucleoli but was restricted to a single or a few roundish subnucleolar regions (Fig. 2 *g* and *h*). The antibodies did not cross-react with the polymerases of nonmammalian species (chicken, amphibia, and insects).

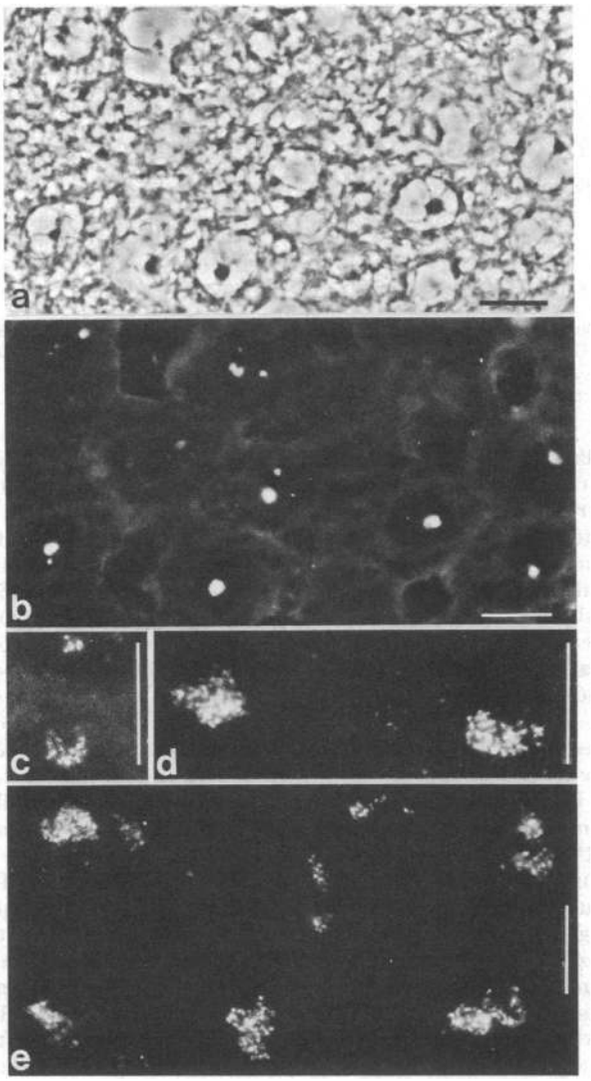

FIG. 1. Immunofluorescence microscopy of cryostat sections through normal rat liver (*b* and *c*), regenerating rat liver (*d*), and rat Novikoff hepatoma (*e*), all after staining with antibodies to RNA polymerase I. For comparison, the section through normal rat liver is also shown in phase-contrast optics (*a*). Positive reaction is restricted to nucleoli. At higher magnification the nucleoli exhibit a distinct punctate fluorescent pattern (*c–e*). (Bars = 10 μm.)

In order to identify the nucleolar component reacting with the antibodies, we used various electron microscopic post-embedding immunolabeling techniques (18, 19). None of them gave satisfactory results because of the loss of immunoreactivity during treatment with aldehyde fixatives. Therefore, we utilized an electron microscopic procedure that followed the light microscopic immunofluorescence protocol as closely as possible. The antibodies bound were detected by secondary antibodies coupled to 5-nm colloidal gold particles. After the immunoreaction the tissue sections were fixed and processed for the preparation of ultrathin sections. With this procedure the individual morphological components of the nucleolar body were easily recognized (Fig. 3*a*). Gold–antibody complexes were selectively enriched over the fibrillar centers and were absent from the other nucleolar components (Fig. 3 *a* and *b*). In ultrathin sections through conventionally fixed Novikoff hepatoma cells, fibrillar centers could be especially well identified by their associated patches of the dense fibrillar material (Fig. 3*c*). Again, after immunolabeling of such sections, the gold-complexed antibodies were seen exclusively over the fibrillar

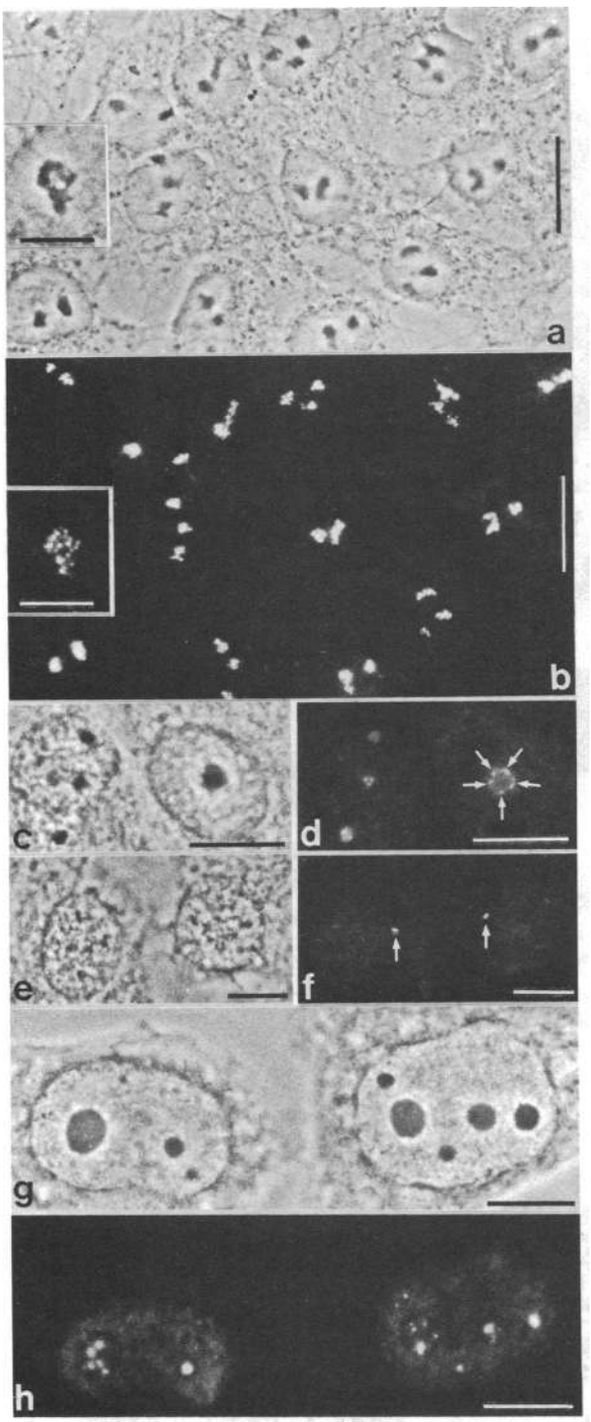

FIG. 2. Cultured cells (RVF-SMC) of rat (*a–f*) and HeLa (*g* and *h*) processed for immunofluorescence with antibodies to RNA polymerase I are shown in phase contrast (*a*, *c*, *e*, and *g*) and epifluorescence (*b*, *d*, *f*, and *h*) optics. Nucleoli are selectively fluorescing (*b*) and, at higher magnification, reveal numerous dot-like substructures (*b Inset*). Treatment of RVF cells with actinomycin D (5 μg/ml) leads to a redistribution of the antibody-decorated intranucleolar entities to the nucleolar periphery (arrows in *d*) 30 min after addition of the drug and, after prolonged treatment (4 hr), to an almost complete loss of fluorescence (arrows in *f* show two residual fluorescent spots). In HeLa cells, nucleoli show either a single fluorescent sphere or a group of distinct subnucleolar components (*h*). (Bar in *a* = 20 μm; bars in *a Inset* and *c–h* = 10 μm.)

centers (Fig. 3 *d* and *e*). Control preparations treated identically, but with preimmune IgG instead of polymerase antibodies, were free of gold particles.

During mitosis the chromosomal bodies of all mammalian species studied were completely negative in immunofluorescence preparations, with the only exception being certain strongly fluorescing spots present on some of the chromosomes. For a given cell type, the number of these fluorescent spots was constant per chromosome complement, but it varied from species to species. In PtK2 cells, for example, there was only one fluorescent spot per chromosome set (Fig. 4 *b–e*). In order to identify the antibody binding site in greater detail, metaphase chromosome plates were prepared without the use of acetic acid. Only the secondary constriction of the X chromosome, which is known to contain the NOR [i.e., the rRNA genes of this species (20, 21)], revealed bright and selective fluorescence (Fig. 4 *f–i*). In favorable spreadings of metaphase plates, even the separate spots per chromatid could be resolved (Fig. 4*i*).

DISCUSSION

In electron microscopic spread preparations of nucleolar chromatin, most of the RNA polymerases associated with the chromatin strands bear a nascent ribonucleoprotein fibril and, hence, are actively engaged in transcription (ref. 10; for review, see ref. 11). Thus, it can be assumed that the distribution of antibodies to RNA polymerase I as observed in our immunocytochemical preparations reflects the distribution of transcriptionally active rRNA genes in interphase cells. Our present observation of an actinomycin D-induced loss of fluorescence is in line with this conclusion and also indicates that the immunocytochemical methods used detect only transcriptionally active polymerase I molecules and not their free forms because this drug is known to cause a rapid, premature release of the transcriptional complexes from the template (22, 23). Moreover, the sensitivity toward actinomycin D makes it highly unlikely that a specific storage pool of DNA-associated but nontranscribing polymerases is present in the nucleoli.

Our observations show that transcriptionally active rRNA genes are concentrated in clusters of several distinct nucleolar entities. These active nucleolar subunits seem to be, at least in rat liver, more frequent in states of increased metabolic activity, such as during regeneration and malignant proliferation, apparently reflecting the recruitment of more rRNA genes for transcription.

The nucleolar regions containing polymerase I have been identified, at the electron microscopic level, as a morphologically distinct component of the nucleolus, the fibrillar center. In order to decide whether this highly selective labeling reflects the true distribution of the polymerases or is a consequence of hindered accessibility of immunoglobulins to other nucleolar regions, we used antibodies to nucleolar constituents other than polymerase I, such as scleroderma autoantibodies. Gold–antibody complexes were detected in considerable quantities in the more condensed regions surrounding the fibrillar centers, indicating that these nucleolar regions are well accessible to immunoglobulins. Thus, we conclude that the absence of labeling in the dense fibrillar component after reaction with antibodies to RNA polymerase I reflects the absence of polymerases in this nucleolar component. This conclusion is also in accord with *in situ* hybridization studies that have localized rDNA in fibrillar centers (9). At first glance, our conclusion appears to be in contradiction to autoradiographic studies reporting that transcription of rRNA genes takes place in the dense fibrillar component (4, 7, 8). However, in experiments in which short labeling times of RNA (2–3 min) have been applied, silver grains indeed have been found also over the fibrillar centers, especially their peripheral portions, while the surrounding dense fibrillar structures were labeled only after approximately 5 min (5, 6, 24). These data are in agreement with our immunolocalization findings in that they also indicate that

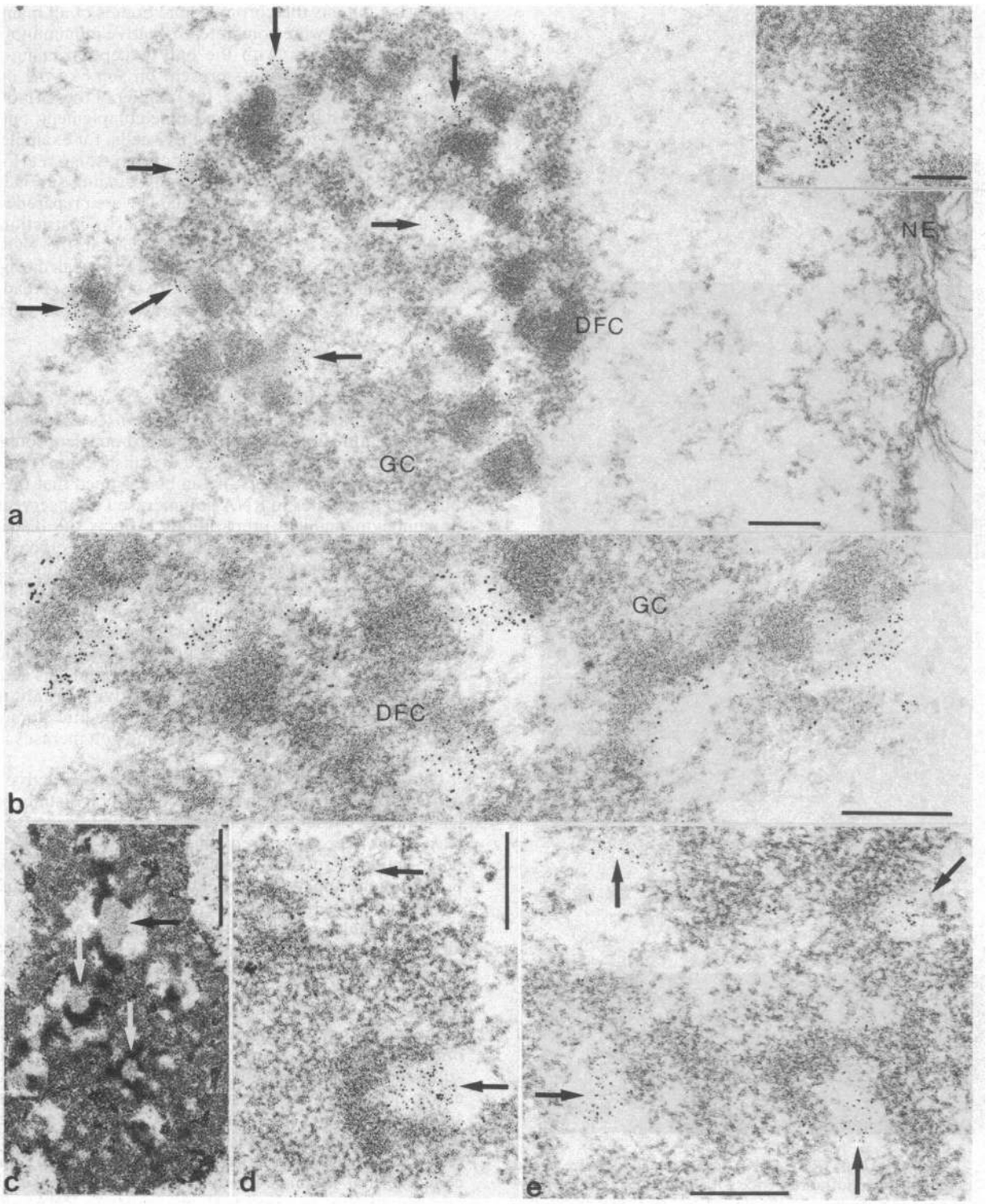

FIG. 3. Electron microscopic immunolocalization of RNA polymerase I in nucleoli of regenerating rat liver (*a* and *b*) and rat Novikoff hepatoma (*d* and *e*) after incubation of cryostat sections with antibodies to RNA polymerase I, followed by secondary antibodies coupled to 5-nm colloidal gold particles. After this immunoreaction, the tissue sections were processed for electron microscopy. The gold particles (small black dots) are selectively enriched over roundish zones of low contrast, the fibrillar centers (some are denoted by arrows in *a*, *d*, and *e*). (*a Inset*) A fibrillar center at higher magnification. Note that these fibrillar centers are always surrounded by the dense fibrillar component (DFC), which is not stained by the antibodies. A similar situation is found in conventionally fixed Novikoff hepatoma cells (*c*), where fibrillar centers are denoted by arrows. GC, granular component of the nucleolus; NE, nuclear envelope. (Bar in *a Inset* = 0.1 μm; bars in *a*, *b*, *d*, and *e* = 0.2 μm; bar in *c* = 1 μm.)

active rRNA genes are located in the fibrillar centers, perhaps more toward their periphery. The pre-rRNA then may be rapidly translocated to and accumulated in the surrounding dense fibrillar component.

Of particular interest is the observation that, during mitosis, polymerase I molecules remain associated with the NOR. This finding is in agreement with biochemical data indicating that RNA polymerase I activity is largely recovered

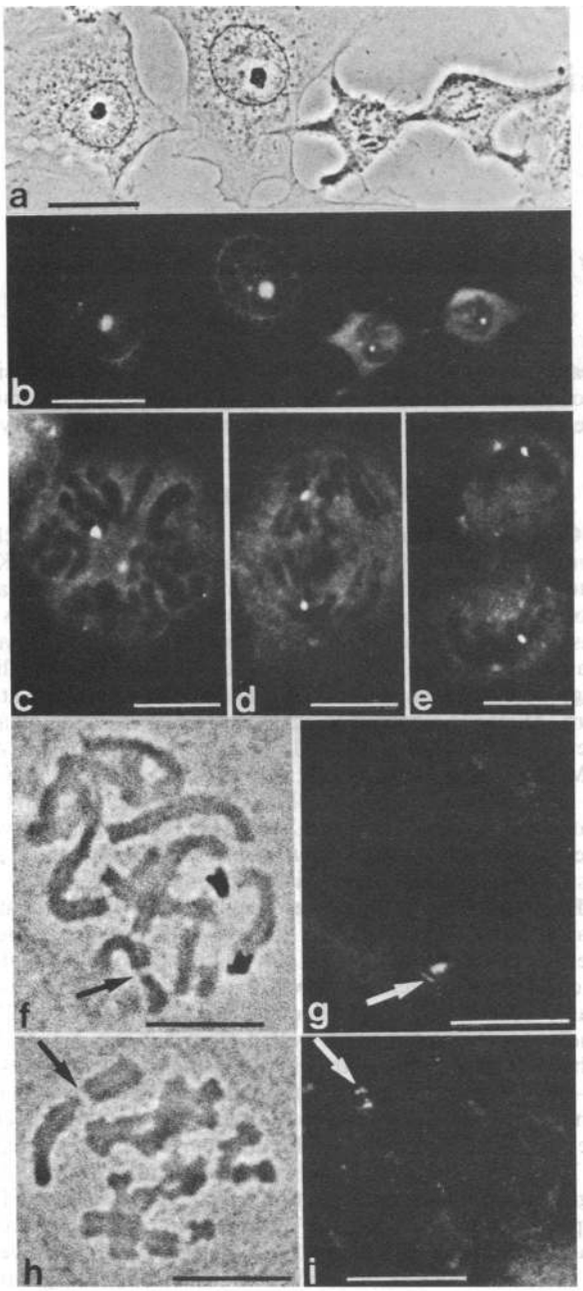

FIG. 4. Immunofluorescence microscopy of cells (*a–e*) and isolated metaphase plates (*f–i*) of PtK2 after staining with antibodies to rat RNA polymerase I (*b–e*, *g*, and *i*). Corresponding phase-contrast micrographs are shown for comparison (*a*, *f*, and *h*). Positive fluorescence is seen in nucleoli of interphase cells but also in one distinct site per chromosome set of mitotic cells (*b*). This is shown in metaphase (*c*), anaphase (*d*), and telophase (*e*). In isolated metaphase chromosomes, the secondary constriction (NOR) of the X chromosome is the only site of fluorescence (arrows in *f–i*). (Bar in *a* = 20 μm; bars in *c–i* = 10 μm.)

with isolated metaphase chromosomes (25). Our localization data now further show that these polymerase molecules are not randomly distributed on the mitotic chromosomes but remain in close association with the rRNA genes. The presence of polymerase I complexes on the NOR during metaphase suggests the notion that this structure is the equivalent to the fibrillar centers of interphase cells, thus confirming earlier conclusions based on morphological similarities and the presence of argyrophilic proteins in both structures (4, 26, 27).

Because little or no rRNA is synthesized at metaphase (28), the polymerase I bound to the NOR must be in a state of arrested transcription, perhaps in the form of "frozen" transcriptional complexes. The mechanism that prevents the polymerases from transcribing the rRNA genes during mitosis provides an interesting case to study the control of gene activity.

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