

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

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ABSTRACT Upon incubation of cultured rat cells with the adenosine analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), nucleoli reversibly dissociate into their substructures, disperse throughout the nuclear interior, and form nucleolar "necklaces". We have used this experimental system, which does not inhibit transcription of the rRNA genes, to study by immunocytochemistry the distribution of active rRNA genes and their transcriptional products during nucleolar dispersal and recovery to normal morphology. Antibodies to RNA polymerase I allow detection of template-engaged polymerase, and monoclonal antibodies to a ribosomal protein (S1) of the small ribosomal subunit permit localization of nucleolar preribosomal particles. The results show that, under the action of DRB transcribed rRNA genes spread throughout the nucleoplasm and finally appear in the form of several rows, each containing several (up to 30) granules positive for RNA polymerase I and argyrophilic proteins. Nucleolar material containing preribosomal particles also appears in granular structures spread over the nucleoplasm but its distribution is distinct from that of rRNA gene-containing granules. We conclude that, although transcriptional units and preribosomal particles are both redistributed in response to DRB, these entities retain their individuality as functionally defined subunits. We further propose that each RNA polymerase-positive granular unit represents a single transcription unit and that each continuous array of granules ("string of nucleolar beads") reflects the linear distribution of rRNA genes along a nucleolar organizer region. Based on the total number of polymerase I-positive granules we estimate that a minimum of 60 rRNA genes are active during interphase of DRB-treated rat cells.

DNA and chromatin of interphase nuclei occur in topologically well-defined arrangements which may play an essential role in transcriptional and replicative events (for reviews see 1 and 2). The most impressive example for the constraint of genes at a specific nuclear locus is provided by the genes coding for the precursor molecules of ribosomal RNA (rRNA genes) which are clustered in a spheroidal body known as nucleolus (for a review see 3). To gain more insight into the forces that maintain this highly ordered arrangement of the nucleolar genes and their products we have studied the drug-

induced nucleolar disintegration and dissociation of nucleolar subunits.

A large number of chemicals are known to interfere with ribosome formation and to induce characteristic alterations of the nucleolar architecture (4, 5). The halogenated adenosine analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB)¹ is especially remarkable. (a) When DRB is added to

¹ *Abbreviation used in this paper:* DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole.

chick fibroblasts grown in culture the normally compact nucleoli unravel into extended beaded strands which have been termed "nucleolar necklaces" by Granick (6, 7). (b) The DRB-induced dispersal of these nucleolar subunit structures is fully reversible, i.e., when DRB is removed from the medium the nucleoli regain their normal compact state (6, 7). (c) While DRB efficiently suppresses the synthesis of mRNA (e.g., 8–13) transcription of the rRNA genes is not, or only moderately, inhibited (6, 10, 14, 15). (d) DRB causes extensive degradation of the RNA of precursor particles to the large ribosomal subunit, probably due to the deficiency of essential preribosomal proteins, but does not interfere with the production of the small ribosomal subunit (6, 7).

By using antibodies to purified RNA polymerase I, we have recently localized transcriptionally active rRNA genes to morphologically distinct subcompartments of nucleoli, the so-called "fibrillar centers" (16). In the present study we have employed immunocytochemical techniques to identify the active rRNA genes during DRB-induced nucleolar disintegration. We have compared the RNA polymerase I immunofluorescence pattern with the distribution of nucleolar silver staining proteins which are considered to be associated with active rRNA genes (reviewed in 17). In normally functioning nucleoli the transcribed rRNA genes are intimately associated with, and surrounded by, other nucleolar structures containing the transcription products in the form of preribosomal particles which are processed through a series of maturation steps to become the cytoplasmic ribosomal subunits (for reviews see 3, 18, 19). Thus to examine the spatial relationship between the transcriptional machinery and its transcriptional products during the drug-induced fragmentation of the nucleolus we have used a monoclonal antibody directed against a defined ribosomal protein (S1; see reference 20), a component of both the small subunit of cytoplasmic ribosomes as well as of nucleolar preribosomal particles.

MATERIALS AND METHODS

Drug and Antibodies: DRB was purchased from Calbiochem-Behring Corp. (Giessen, Federal Republic of Germany) and was used at a final concentration of 50 $\mu\text{g}/\text{ml}$. Antibodies to purified RNA polymerase I from a rat hepatoma were raised in rabbits and have been extensively characterized

(16, 21; for limited reactivity between different species see also 22). Serum was precipitated with ammonium sulfate and the immunoglobulin fraction was used for immunofluorescence at a concentration of 100 $\mu\text{g}/\text{ml}$. The hybridoma clone RS1-105 producing monoclonal antibodies (IgM) to protein S1 of the small ribosomal subunit (c.f. 20) was the result of a fusion of mouse myeloma cell line Ag8.653 with the spleen cells of a BALB/c mouse that had been immunized with a cytoskeletal residue preparation from mouse liver tissue (c.f. 23). A detailed description, including immunoblotting experiments on ribosomal and nucleolar proteins will be published elsewhere (Hügler, B., R. Hazan, U. Scheer, and W. W. Franke, manuscript in preparation). For immunofluorescence microscopy, hybridoma supernatants were used undiluted.

Cells and Immunofluorescence Microscopy: RVF-SMC cells, an established cell line derived from vascular smooth muscle cells of rat vein, were grown as monolayer cultures as described (24). For light microscopy, coverslips with attached cells were placed inverted onto a microscope slide and photographed immediately with phase contrast optics. For indirect immunofluorescence microscopy, cells grown on coverslips were treated as described (16). Double immunofluorescence microscopy was performed by incubation of cells with antibodies in the following sequence (10 min each): Anti-RNA polymerase I, fluorescein isothiocyanate-labeled anti-rabbit IgG (1:20; Miles-Yeda, Rehovot, Israel), anti-S1, rhodamine-labeled anti-mouse IgG/IgM (1:20, Medac, Hamburg, Federal Republic of Germany). Photographs were taken with a Zeiss Photomicroscope III equipped with epifluorescence optics and appropriate filters selective for fluorescein and rhodamine fluorescence.

Silver Staining: RVF-SMC cells grown on coverslips were fixed in Carnoy's mixture and stained with a silver nitrate solution essentially as described (25, 26).

RESULTS

When DRB was added to the culture medium of rat cells at a concentration of 50 $\mu\text{g}/\text{ml}$, the first signs of an altered nucleolar morphology were detectable within 15–20 min. In phase-contrast microscopy, nucleoli of living cells no longer appeared as distinct and highly refractile bodies as in untreated cells (Fig. 1 a) but instead showed less well-defined boundaries apparently reflecting initial stages of a progressive structural loosening and expansion. 6 h after drug treatment most of the nuclear interior was filled with a network of irregularly contoured strands of widths from 0.7 to 1 μm (Fig. 1 b). Removal of the drug by addition of fresh medium to the cells caused condensation of the strands and, within 3 h, the original nucleolar morphology was fully restored (Fig. 1 c).

In untreated cells antibodies to RNA polymerase I reacted exclusively with the nucleoli and exhibited a punctate pattern as shown by indirect immunofluorescence microscopy (Fig.

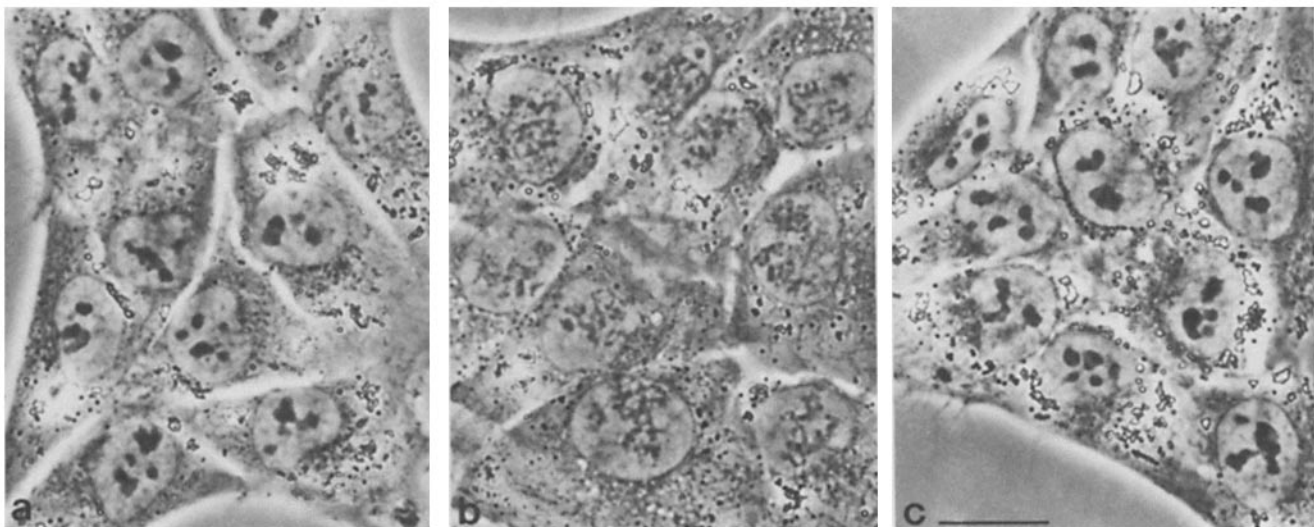


FIGURE 1 Phase-contrast micrographs of living rat RVF-SMC cells grown as monolayers on coverslips. (a) untreated cells; (b) 6 h after addition of 50 $\mu\text{g}/\text{ml}$ DRB; (c) after 6 h of DRB treatment the drug was removed by addition of fresh medium and the cells were incubated for an additional 3 h. Bar, 20 μm . $\times 750$.

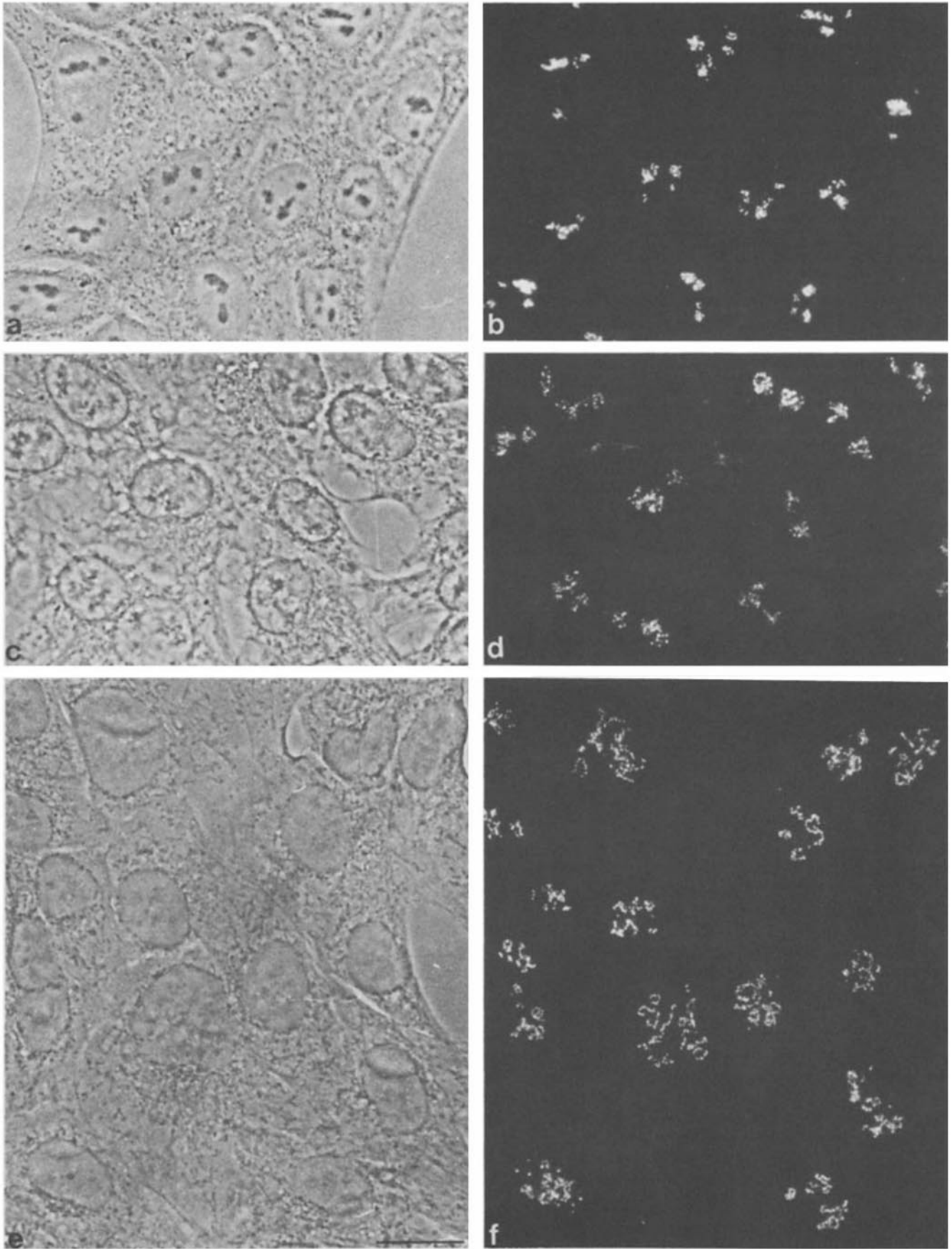


FIGURE 2 Effect of DRB (50 $\mu\text{g}/\text{ml}$) on nucleolar morphology of cultured rat cells 1 h (c and d) and 6 h (e and f) after addition of the drug as seen in phase-contrast optics (a, c, e) and indirect immunofluorescence microscopy using antibodies to RNA polymerase I (b, d, f). Control cells are shown in a and b. Bar, 20 μm . $\times 800$.

2, *a* and *b*; see also references 16 and 22). This punctate pattern of intranucleolar fluorescence indicated that each nucleolus represented an aggregate of a number of transcriptionally active subunits (Fig. 2 *b*). After DRB treatment the area containing fluorescent aggregates was markedly enlarged (Fig. 2 *d*; 1 h after treatment) and, after prolonged periods of

incubation, appeared as linear arrays of fluorescent entities spread over the nucleoplasm (Figs. 2 *f* and 3 *b*; 6 h after treatment). Although the number of these fluorescent granules varied considerably in the whole cell population most nuclei contained, 6 h after DRB treatment, 50–75 (mean value 63) granules, each with a discrete size of 0.4–0.6 μm (Figs. 2 *f* and

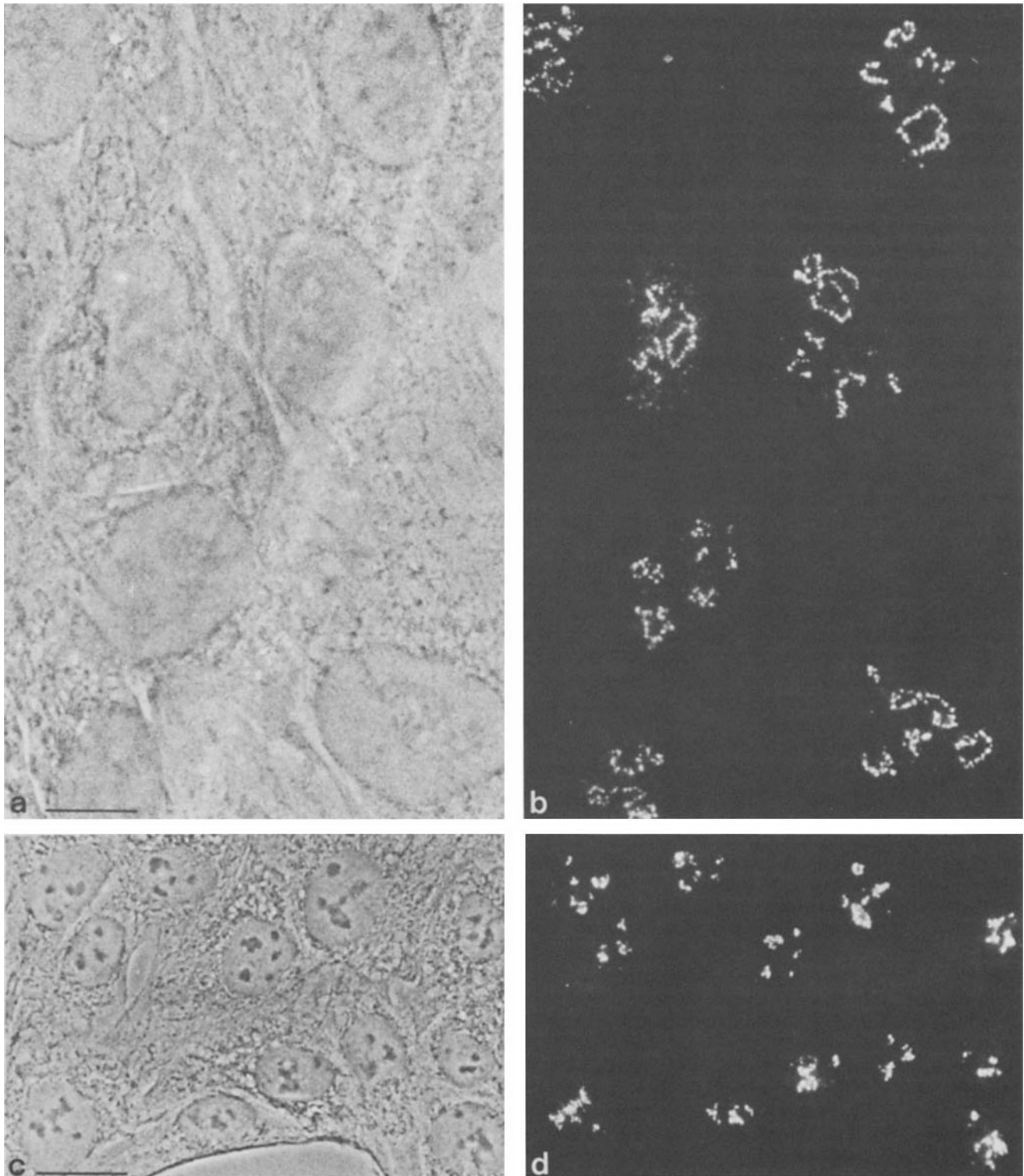


FIGURE 3 (a and b) High magnification of the nucleolar necklaces induced by 6-h treatment of rat cells with DRB as seen in phase contrast (a) and immunofluorescence microscopy (b) using antibodies to RNA polymerase I. Each nucleus usually contains six separate rows each containing a variable number of fluorescent granules (b). (c and d) After removal of DRB the original nucleolar morphology is fully restored within 3 h as seen in phase-contrast optics (c) and immunofluorescence microscopy (d). Bars, 10 μm (a); 20 μm (c). a, b, $\times 1,600$; c, d, $\times 780$.

3 b). The total complement of fluorescent entities per cell nucleus did not form a single continuous row but instead occurred as several (usually six) distinct linear arrays of variable lengths (Fig. 3 b). The longest uninterrupted strings of granules measured 15–22 μm and contained 15–30 fluorescent units. Other chains contained only a few granules and, occasionally, solitary granules were also observed. After removal of DRB, these extended “necklaces” retracted and, after 3 h, dense aggregates of fluorescent granular entities associated with the reformed compact nucleoli were observed (Fig. 3 d).

Staining of untreated rat cells with silver nitrate revealed a large number of argyrophilic granules present in each nucleolus (Fig. 4 a; see also reference 25). They were indistinguishable by their sizes, numbers, and packing patterns from the intranucleolar granular entities reacting with antibodies to RNA polymerase I (compare Fig. 4 a with Fig. 2 b). Correspondingly, DRB-induced nucleolar necklaces were also selectively stained by the silver impregnation procedure and revealed linear arrays of argyrophilic granules (Fig. 4 b), similar to those of the polymerase-containing elements (compare Fig. 4 b with Fig. 3 b).

In contrast to the distribution of RNA polymerase I and the silver-staining granules, ribosomal protein S1 occurred

both in the nucleolus and, at lower local concentrations, in the cytoplasm of untreated cells (Fig. 5 b). Usually the nucleoli were surrounded by several fluorescent dots, probably representing nuclear particles containing precursors to the small ribosomal subunit which are en route to the nuclear envelope and the cytoplasm. The nucleolar fluorescence obtained by applying antibodies to S1 was rather dense and uniform and did not reveal a punctate subunit pattern as described above for RNA polymerase I. Further, double immunofluorescence microscopy after consecutive incubation with rabbit antibodies to RNA polymerase I (Fig. 5 d) and murine antibody to S1 (Fig. 5 e) showed a strikingly different distribution of both antigens in response to DRB. Specifically, S1-containing material was recognized in the form of numerous fluorescent granular units, which were almost uniformly distributed throughout the nucleoplasm and did not reveal any indication of linear arrangements (Fig. 5 e). They did not co-localize with the RNA polymerase I-containing granules which in the same cells were organized in rows (Fig. 5 d).

DISCUSSION

Our results provide evidence that transcriptionally active rRNA genes, which are normally tightly clustered in a specific nucleolar subcompartment (the fibrillar center; see reference

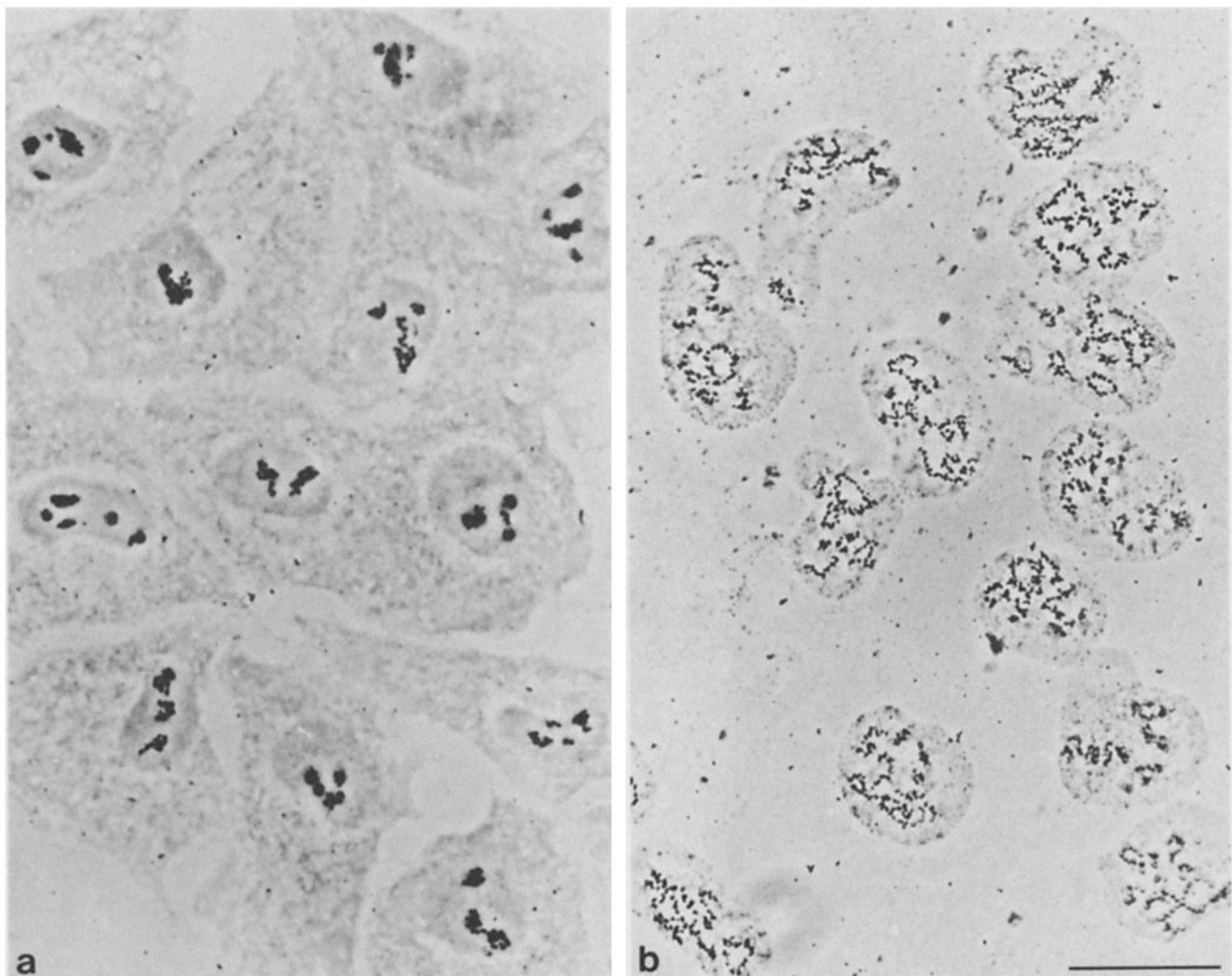


FIGURE 4 Rat RVF-SMC cells before (a) and 6 h after treatment with DRB (b) stained with the silver impregnation technique. Bar, 20 μm . $\times 1,100$.

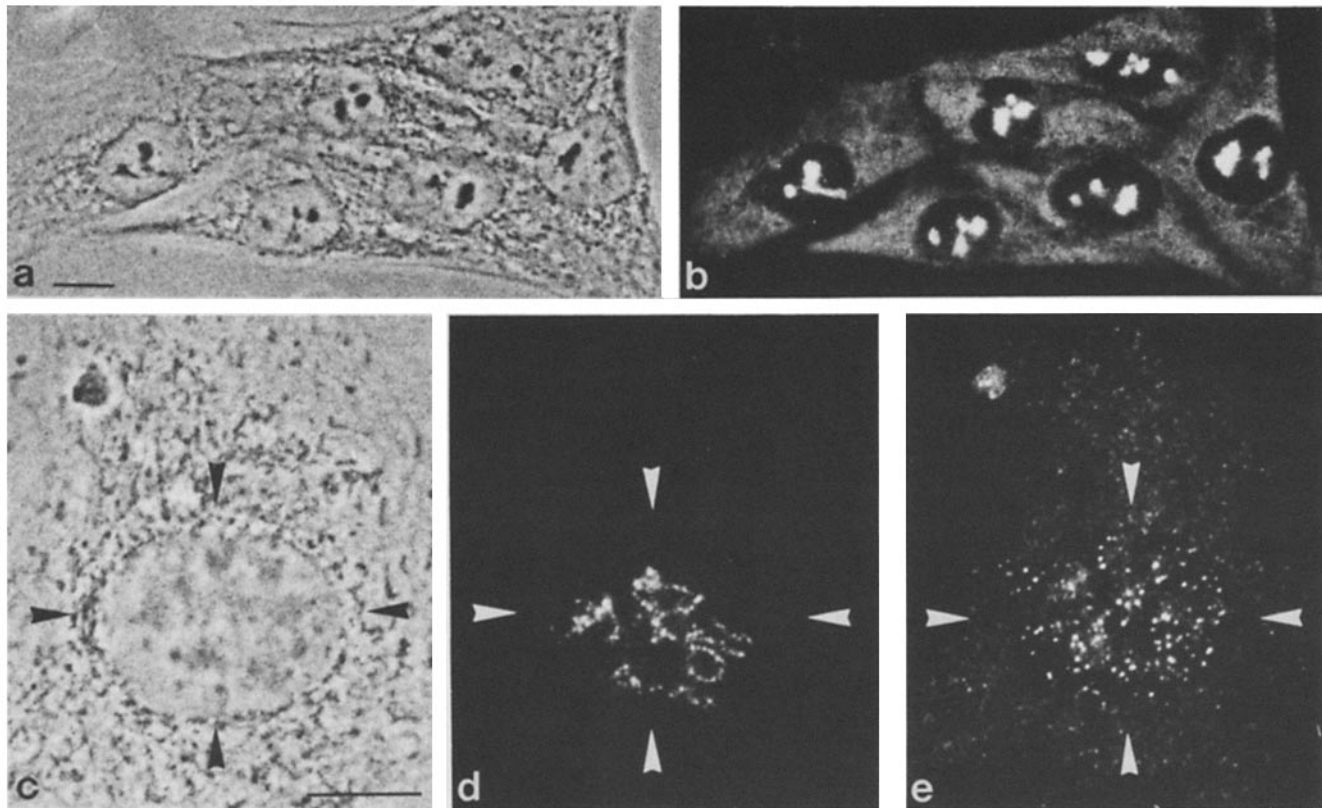


FIGURE 5 (a and b) Rat cells incubated with monoclonal antibodies to the ribosomal protein S1, seen in phase-contrast optics (a) and by epifluorescence (b). Note the strong nucleolar fluorescence as well as the cytoplasmic fluorescence (b). (c–e) Double immunofluorescence of DRB-treated cells (6 h) using antibodies to rat polymerase I (d) and S1 (e). Both fluorescence patterns are clearly different. The corresponding phase-contrast image is shown in c. Arrowheads in c–e indicate the position of the nuclear envelope. Bars, 10 μm . (a and b) $\times 825$; (c–e) $\times 1,500$.

16) are released from their specific topological constraints during DRB-induced nucleolar fragmentation and rapidly spread throughout the nuclear interior. We propose that the nucleolar necklaces observed a few hours after exposing cultured rat cells to DRB represent extended linear arrays of active rRNA genes based on the following findings: (a) The necklaces contain RNA polymerase I complexes in the form of linear arrays of granular bodies. Since the specific procedure used allows only the detection of template-bound and not of free forms of RNA polymerase I molecules (16), we conclude that the fluorescent entities contain transcribing RNA polymerase I and therefore, by implication, transcriptionally active rRNA genes. (b) Measurements of the topology of the RNA polymerase I-containing granules agree with those for rat rDNA transcription units. Specifically, transcription units of rRNA genes from rat cells have axial lengths of 4–4.5 μm as measured in electron microscopic spread preparations (27). A single transcription unit contains ~ 120 RNA polymerase I molecules attached to the rDNA, and each of these polymerases is associated with a lateral fibril of nascent preribosomal RNP which can attain a maximum length of 0.25 μm (27). In view of the considerable size of such “Christmas trees” and the known tendency of the lateral fibrils to stick together (see Fig. 2 in reference 28), it is reasonable to assume that individual transcription units can be resolved at the light microscopic level as bodies of a minimum diameter of 0.5 μm . This is even more likely as it has been reported that DRB causes a significant increase in size of the transcription units of mammalian rRNA genes (14, 15). In this context it should also be

noted that a group of 120 closely spaced polymerase molecules provides sufficient antibody binding sites for the generation of a strong fluorescence signal in indirect immunofluorescence microscopy.

Although adjacent granules are usually well resolved and seem to be separated from each other, their conspicuous regular arrangement, together with the continuous filament structure seen in phase contrast, indicates the presence of interconnecting elements. In line with our interpretation, each intergranular intercept free of RNA polymerase then would contain the nontranscribed spacer (with a total length of $\sim 8 \mu\text{m}$ of DNA; 29), whereas each fluorescent bead would represent an individual transcription unit of rDNA. (c) The silver staining technique also reveals a granular subunit organization of the nucleolar necklaces. Argyrophilic granules are arranged in linear arrays extending throughout the nuclei, similar to the rows of polymerase I-positive granules. The observation by Angelier et al. (30) that argyrophilic proteins occur in association with transcription units of rRNA genes but are absent from the nontranscribed spacers lends further support to our conclusion. (d) The calculated values for the rate of ribosome formation agree with those estimated from the present data. In particular, the number of granules reacting with antibodies to RNA polymerase I ranges from 50 to 75 per nucleus (mean value: 63). In nucleoli of untreated cells that happened to exhibit a somewhat loosened arrangement of the intranucleolar RNA polymerase I-positive granules, similar numbers have been counted. Thus we conclude that, on the average, 63 rRNA genes from a total of ~ 200 (31, 32)

are transcriptionally active in DRB-treated and in untreated rat cells. The reported decrease of the rate of accumulation of 45S pre-rRNA in the presence of DRB (6, 10) is not necessarily a reflection of reduced transcriptional activity of the rRNA genes but could be also a consequence of increased pre-rRNA degradation. The latter alternative is supported by morphological studies showing that the frequency of rRNA transcriptional units and the packing density of the nascent transcripts is similar in DRB-treated and control cells (15). Assuming that all rRNA genes are fully loaded with polymerase (~120 per gene; 27) and that the elongation rate of the nascent pre-rRNA polynucleotide chain is in the range of 40–80 nucleotides per second (19), the synthesis rate would be 1,400–2,800 ribosomes per minute. This value is close to the biochemical estimates of rates of ribosome formation in various animal cells (19). (e) The number of rows of polymerase I-containing granules is congruous with the number of nucleolar organizers. Most frequently, we have observed six rows of tandemly arrayed granules in a given nucleus. These data indicate that the total complement of active rRNA genes is distributed in six different domains. In fact, diploid rat cells contain six separate nucleolar organizer regions (33, 34). (f) The nucleolar necklaces are not associated with precursor particles to the small ribosomal subunit, as shown by double immunofluorescence microscopy applying antibodies to RNA polymerase I and to the ribosomal protein S1. Instead, protein S1-containing material occurred in numerous other bodies dispersed throughout the nuclear interior which were clearly distinct from the nucleolar necklaces. This disconnection between antigens suggests that the nucleolar necklaces are mainly composed of rRNA genes and are structurally dissociated from the transcription products which normally are stored in the vicinity of the transcription units.

In aggregate, our observations show that rRNA genes are not invariably fixed at certain topological sites of interphase nuclei but, in the course of nucleolar fragmentation, can rapidly disperse throughout the nuclear interior. They also demonstrate that preribosomal particles are not necessarily accumulating in the immediate vicinity of the transcribed genes but can, at least in a drug-perturbed situation, be assembled into bodies distant from the genes.

Work in several laboratories has indicated a correlation between silver staining of chromosomal nucleolar organizer regions and the transcriptional activity of the rRNA genes during the preceding interphase (reviewed in reference 17). Moreover, a direct relationship between argyrophilia of interphase nucleoli and transcriptional activity of rRNA genes has been noted in a variety of cell types from different species (35–40). In cell types where the argyrophilic reaction is not uniform in the nucleoli but appears in punctate patterns a direct relationship between the number of these nucleolar argyrophilic granules and the synthetic rate of pre-rRNA has been established (25, 26). We have recently noted that the number of intranucleolar fluorescent granules in immunocytochemical preparations using antibodies to RNA polymerase I is significantly higher in regenerating rat liver hepatocytes and in Novikoff hepatoma cells as compared with normal rat hepatocytes (16). Based on our present finding of a striking similarity of the number and localization of argyrophilic and polymerase I-containing granules (for quantitation of argyrophilic granules in normal and tumor cells, see references 25 and 26), we suggest that with both cytochemical techniques individual transcription units of rRNA genes can be visual-

ized, and that the number of argyrophilic or polymerase I-containing granules is a direct measure of the number of transcriptionally active rRNA genes.

At the moment it is not clear which protein is actually responsible for the highly selective silver staining of the chromosomal nucleolar organizer regions and nucleolar components. Most studies have indicated that during interphase argyrophilic proteins are mainly, if not exclusively, localized in the fibrillar centers of nucleoli (41–43) which also contain extended, non-nucleosomal rDNA chromatin filaments (43, 44) and the transcribing RNA polymerase I molecules (16). By combining electron microscopic spread preparations with the silver staining technique, it has recently been directly shown that argyrophilic proteins are associated with transcribing rRNA genes (30; for polypeptide candidates see also 45). Although all these data are compatible with the notion that RNA polymerase I molecules themselves are responsible for the cytological silver staining, it cannot be excluded that other proteins associated with nucleolar transcription units such as protein C23 are also involved (e.g. 46, 47).

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