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# Characterization and Immunolocalization of a Nucleolar Antigen with Anti-NOR Serum in HELA Cells

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**We have used a serum from a patient with rheumatoid arthritis and found it to immunoblot with a 92- to 88-kDa protein doublet with an isoelectric point of around 7.5 after mono- and two-dimensional electrophoresis in whole HeLa cells. By means of immunofluorescence and immunoelectron microscopy we have found it to specifically react with the nucleolar fibrillar component. After quantitative analysis under the electron microscope, we have demonstrated a similar labeling both in the fibrillar centers and the dense fibrillar component, using two different gold-coupled markers. When transcription was inhibited under physiological conditions (mitosis) or after AMD treatment the antigen remained, as shown by immunoblotting and immunolabeling with anti-NOR serum. These biochemical characteristics, which coincide with those of the ribosomal transcription human upstream binding factor, together with the immunolocalization with anti-NOR serum, allow us to discuss the possible role of these antigens in rDNA transcription.** 

## INTRODUCTION

The nucleolus is an essential structure within the cell nucleus where the synthesis of pre-rRNA and the assembly of ribosomal particles occur. The different steps of ribosome biogenesis take place within morphologically well-defined components, namely the fibrillar centers, the dense fibrillar component, and the granular component [26]. Nucleolar morphology and ribosomal gene activity mainly depend on the cell type and the metabolic state of the cell. The study of the different nucleolar components under various physiological or experimental conditions and their chemical composition and function are still clear targets for the research of several investigators [8, 11, 14, 20, 27, 54, 64].

Numerous approaches have been used to gain more insight into the different problems arising from the study of the nucleolus, an interesting one being the use of antibodies as markers of the different nucleolar components. Antibodies against cellular components are spontaneously produced by patients with systemic autoimmune diseases such as systemic lupus erythematosus, dermatomyositis, Sjögren's syndrome, and scleroderma [49]. Such cellular autoantigens include DNA, RNA, histones, and a number of nuclear, nucleolar, and cytoplasmic proteins and ribonucleoproteins [4, 60]. Several of these antibodies are reactive with the different nucleolar components [41, 47, 50] and previously were used in immunofluorescence microscopy to gain insight into the structural organization of the nucleolus. However, only very recently have antinucleolar autoantibodies been used to identify and localize different antigens within the nucleolus in order to provide some answers to basic questions concerning the mechanisms associated with ribosome biogenesis.

Recently, the mapping of nucleolar antigens revealed the presence of new markers of the different nucleolar components. A 34-kDa antigen has been localized to the nucleolar fibrillar regions and has been called fibrillarin [39]. An antigen with a molecular weight of 116 kDa has been detected in the fibrillar component in nucleoli of PTK1 and HeLa cells [31], and another antigen of 94 kDa is found in nucleolar regions where processing of preribosomal chains occur [21]. Some autoimmune sera, such as V11, have been used as markers of fibrillar centers, and others, such as G04 specifically react with the granular component [22]. Human autoantibodies against RNA polymerase I have also been found, being the antigen–antibody–gold exclusively detected in the fibrillar centers [49]. That is not an exhaustive list of the different autoantibodies discovered, but allows us an insight into the great importance of the information about the nucleolus and the role of each of its components that they can provide.

In the present work we have used an autoimmune serum, from a patient with rheumatoid arthritis, against a specific nucleolar protein localized in the fibrillar com-

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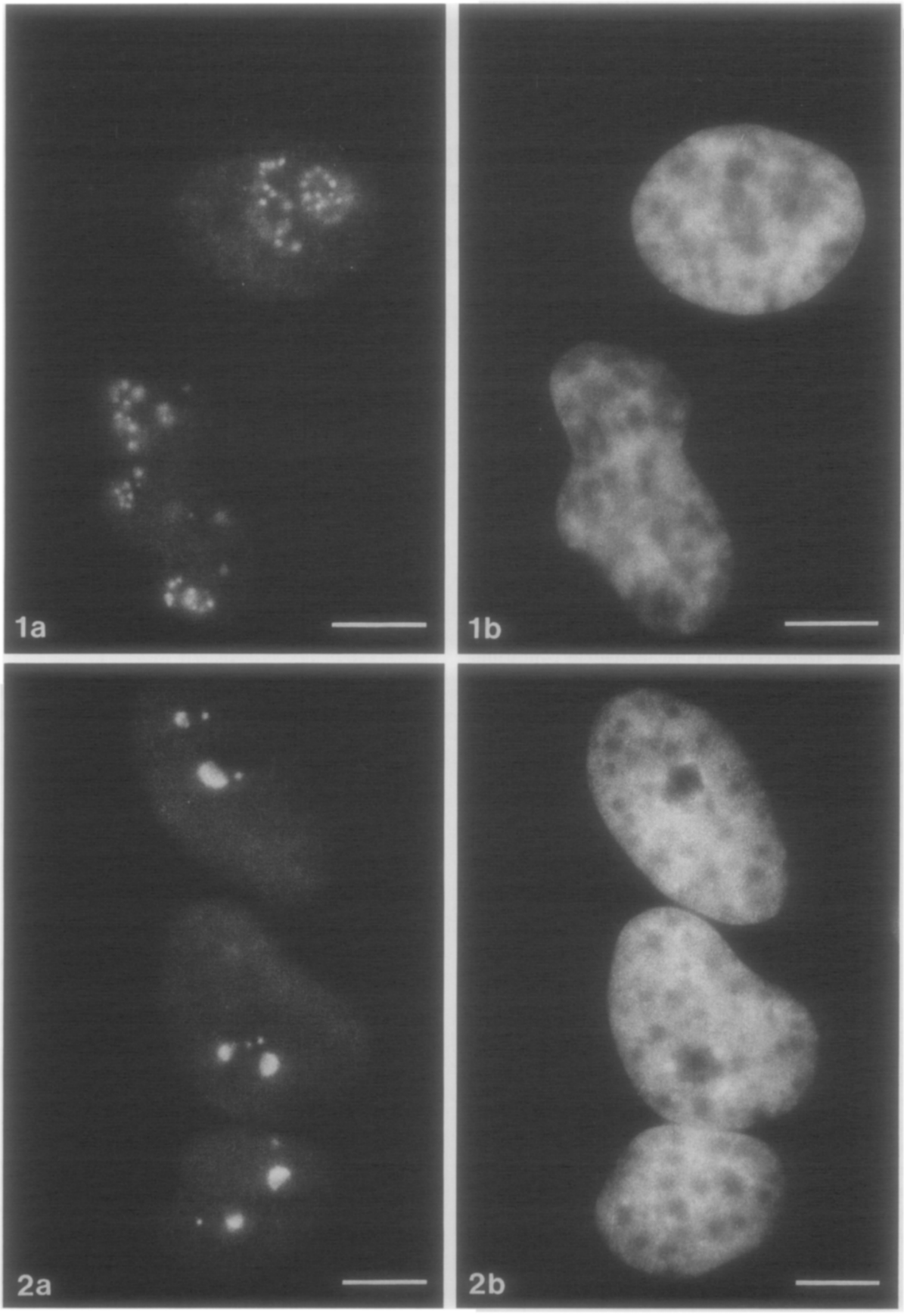
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ponent. By means of a quantitative study we have demonstrated that immunolabeling is similar both in the fibrillar centers and dense fibrillar component. This antigen localization coincide with that of Ag-NOR proteins, but its biochemical characterization has revealed a 92- to 88-kDa protein doublet with an isoelectric point of around 7.5 that might correspond to the ribosomal transcription factor hUBF [5], suggesting a possible role of these antigens in rDNA transcription.

## MATERIALS AND METHODS

### *Human Serum*

Autoimmune serum was obtained from a patient with rheumatoid arthritis.

### *Cell Culture*

HeLa cells were grown and maintained in cell culture flasks containing DMEN supplemented with 10% FBS, 2 m*M* glutamine, 1 m*M* sodium pyruvate, 100 U/ml penicillin–streptomycin, 1% fungizone, and 1% antiPPLO.

For actinomycin D (AMD) experiments, HeLa cells were cultured onto either sterile glass coverslips or flasks. When the cells reached ~75% confluence, they were incubated for 5 h with 2 μg/ml AMD diluted from a 0.2 mg/ml stock solution made in DMSO. After this treatment, cells were processed for immunofluorescence and immunoelectron microscopy. Cell extracts were also obtained after AMD treatment for immunoblot analysis.

### *Immunofluorescence*

Cells grown in culture flasks were trypsinized (0.1% trypsin–EDTA, Sigma Chemical Co., St Louis, MO) and seeded onto sterile glass coverslips in bacteriological grade 90-mm plastic petri dishes. Cells on glass coverslips were rinsed in PBS and processed for indirect immunofluorescence in one of three ways: (1) absolute methanol fixation for 10 min at −20°C; (2) 3% formaldehyde fixation in PBS for 20 min at room temperature followed by permeabilization with 0.1% Triton X-100 in PBS for 2 min; or (3) in cold acetone for 7 min at −20°C.

All coverslips were then incubated with a 1:100 to 1:1000 dilution of anti-NOR serum in PBS for 45 min at 37°C in a humidified chamber, washed in PBS (four changes, 10 min each), incubated with 1:30 dilution of affinity-purified goat anti-human IgG FITC (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in PBS for 45 min at 37°C, washed in PBS, and stained with 5 μg/ml Hoechst 33342 (Calbiochem-Behring Corp., La Jolla, CA) in 1:9 PBS/glycerol. Two negative control experiments were performed, the first one in the absence of anti-NOR serum and the second one by using a normal human serum in the first incubation step.

To perform the isolation of metaphase chromosomes, cells grown in culture flasks were treated with 0.1 μg/ml Colcemid (GIBCO, UK) for 24 h. This was followed by a hypotonic treatment with 75 m*M* KCl for 10 min at 37°C. After a mild centrifugation the pellet was fixed three to four times with freshly mixed methanol:acetic acid (3:1) for 10 min at 4°C. The chromosome preparations were made by dropping the concentrated suspensions in fresh fixative onto sterile glass coverslips and processed for indirect immunofluorescence as described before. Chromosomes were stained with 1 μg/ml propidium iodide.

Slide specimens were analyzed on a Zeiss fluorescence microscope equipped with an Axiophot camera system. Micrographs were prepared with a Kodak TRI-X-PAN film push processed to 1600 ASA.

### *Electron Microscopy*

HeLa cells grown and maintained in culture flasks, as already described, were processed for morphological, cytochemical, and immunocytochemical electron microscopic studies.

For the morphological study, cells were fixed in 1.6% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, for 60 min at 4°C and were postfixed in 1% osmium tetroxide for 60 min at 4°C. Samples were dehydrated in acetone at progressively higher concentrations and embedded in resin following the method of Spurr [59].

For the cytochemical study, cells were fixed in 1.6% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.3, for 10 min at room temperature, followed by a postfixation in Carnoy's solution (1:3 (v/v) acetic acid:ethanol) for 10 min at 4°C.

For immunolocalization, cells were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.3, for 60 min at 4°C.

All fixations were performed on culture flasks. Subsequently, cells were scraped with a rubber policeman and pelleted by centrifugation. Samples were washed in 0.1 *M* cacodylate buffer, pH 7.3, and incubated in 0.5 *M* $NH_4Cl$ for 4 h at room temperature to block free aldehyde groups. They were then washed in the same buffer again.

*Lowicryl K4M-embedded sections.* Low-temperature embedding with the hydrophilic resin Lowicryl K4M (Chemische Werke Lowi., Waldkraiburg, Germany) was performed, with slight modifications, according to the method described by Roth [52]. Samples were dehydrated in a series of graded methanols at progressively lower temperatures. During infiltration with Lowicryl K4M at −20°C, 90% methanol was used as the dehydration agent. Lowicryl was polymerized by indirect long-wave (360 nm) uv irradiation from a 15 W Philips fluorescent lamp for 24 h.

Semithin and ultrathin sections were cut on a Reichert–Jung Ultracut E ultramicrotome. Some semithin sections were mounted on slides, and after NOR-silver staining were photographed in a Carl Zeiss photomicroscope. Ultrathin sections were mounted on 300-mesh copper or nickel grids and photographed in a Philips CM-10 transmission electron microscope (Servicio de Microscopía Electrónica, University of Seville).

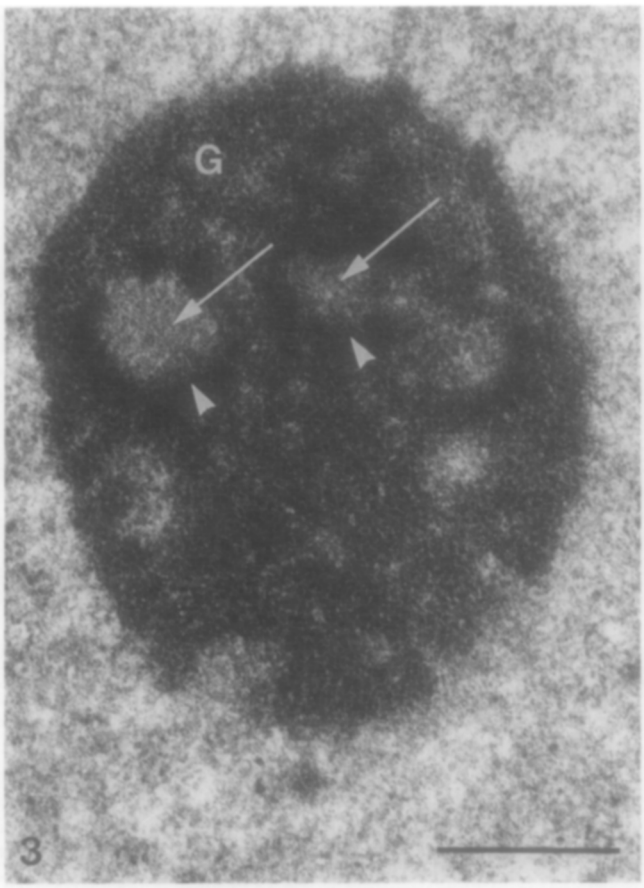
*One-step silver staining.* This technique was applied, following Howell and Black [23] and Ploton *et al.* [42], with the modifications of Moreno *et al.* [32] or Moreno *et al.* [36] for the observation at the ultrastructural or at the light microscope level, respectively. Sections were washed in distilled water for 5 min and treated for 5 min at room temperature with a mixture containing 1 vol of 2% gelatin in 1% formic acid and 2 vol of 50% silver nitrate solution (Fluka, Buchs, Switzerland). They were rinsed in distilled water, floated in 5% sodium thiosulfate solution for 10 min, and thoroughly rinsed in distilled water again. To demonstrate the specificity of the staining, sections were incubated for 60 min at 37°C in one of the following enzymatic solutions: 0.1% DNase in PBS containing 1 m*M* $MgCl_2$, 0.1% RNase in PBS, or 0.1% pronase in PBS.

*Immunocytochemical labeling.* Grids were incubated by floating them, cell sections down, on a drop of 0.1 *M* PBS, pH 7.4, containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20 (PBTT), for 5 min

**FIGS. 1 and 2.** Immunofluorescence microscopy on HeLa cells fixed in 3% formaldehyde after incubation with anti-NOR serum. Bars, 5 μm.

**FIG. 1.** (a) Fluorescence is localized to small nucleolar spots. (b) Corresponding Hoechst dye.

**FIG. 2.** (a) HeLa cells treated with 2 μg/ml AMD for 5 h showing an intense labeling restricted to cap-like structures located at the nucleolar periphery. (b) Corresponding Hoechst dye.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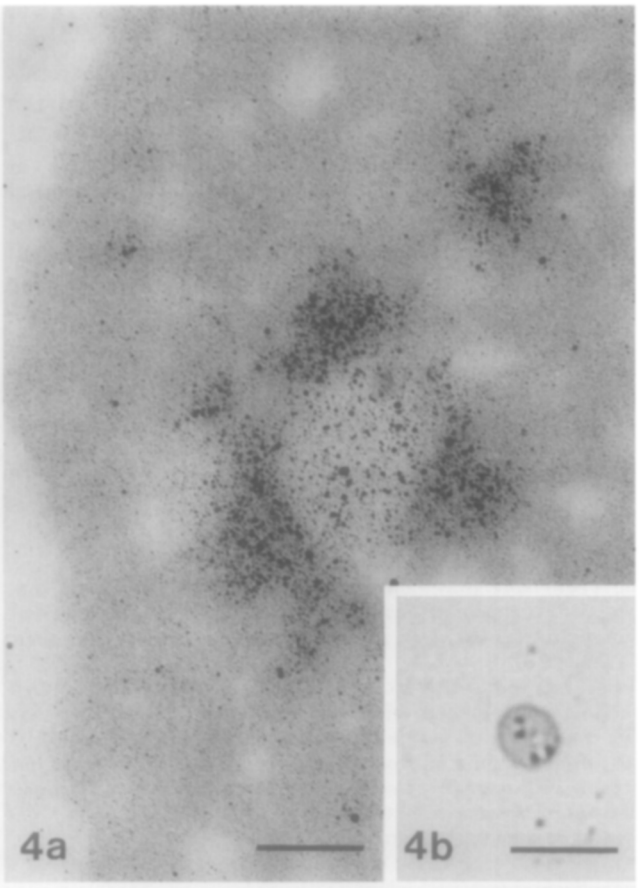
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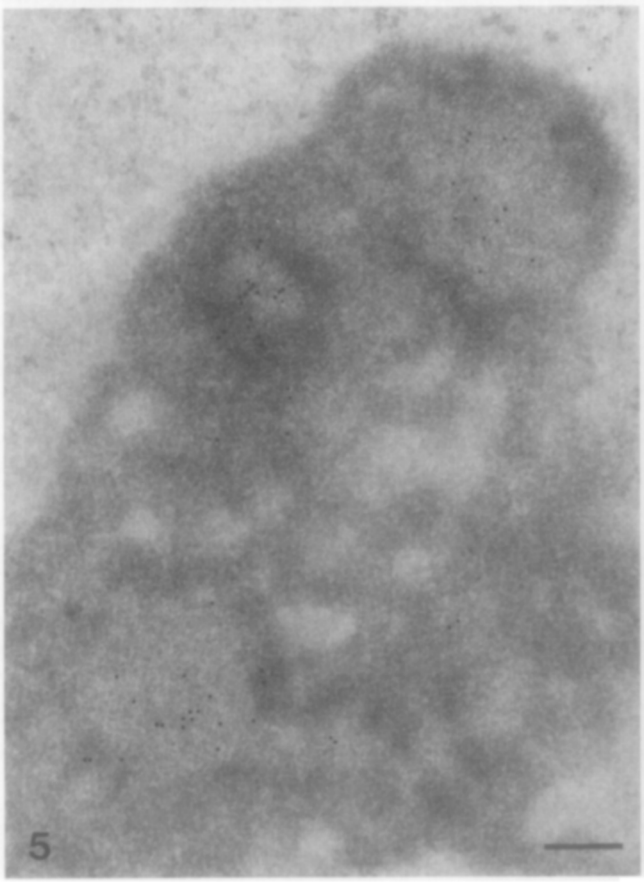
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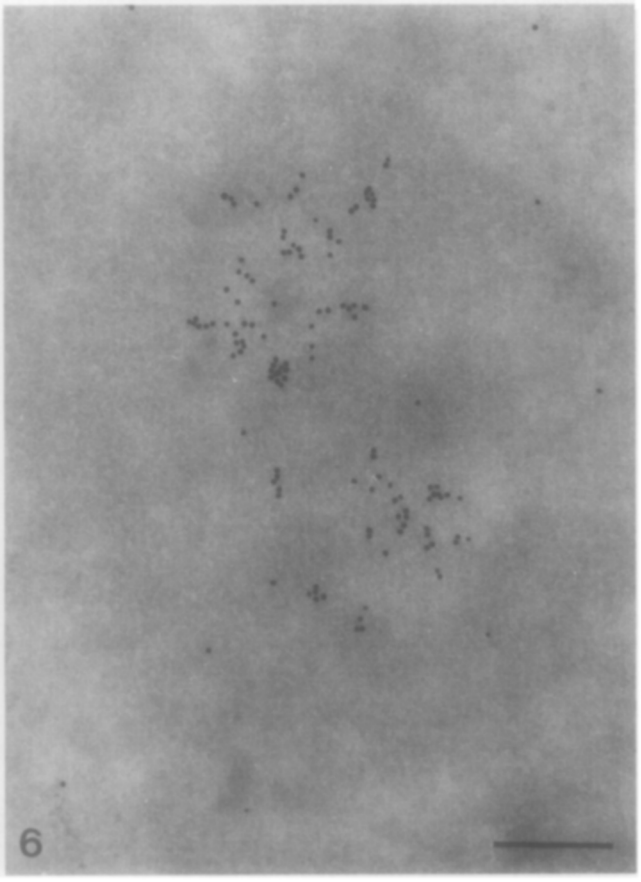
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at room temperature, and washed in PBS. The next step of the treatment was an incubation in anti-NOR serum diluted 1:100 to 1:1000 in PBS for 60 min at room temperature. After washing in PBS, the second incubation was performed in protein A–gold complex prepared following Bendayan *et al.* [3] diluted in PBTT, until an optical density of 0.06 (525 nm) was reached, for 60 min at room temperature. In other cases goat anti-human IgG ($\gamma$-chain specific) coupled to 10 nm diameter colloidal gold (Sigma Chemical Co.) diluted 1:25 in 0.1 *M* PBS containing 0.2 mg/ml polyethylene glycol 20000 was used in the second incubation step, for 60 min at room temperature. In this latter case, after rinsing in PBS, sections were postfixed in 2% glutaraldehyde in PBS for 5 min. All grids were washed with distilled water and contrasted with 7.5% uranyl acetate in distilled water. To demonstrate the specificity of the labeling controls were performed by omitting the incubation with the anti-NOR serum or by using a normal human serum during the first incubation step.

*Morphometric and stereological analysis.* This study was carried out on 50 images per group of nucleoli chosen at random, taken from ultrathin immunolabeled sections. These images were digitalized, processed, and analyzed in the IMAGO automatic image analysis system (Servicio de Microscopía Electrónica, University of Seville). The parameters measured were the area of each unit of fibrillar component (fibrillar center plus the dense fibrillar component surrounding it) and the area of each of its subcomponents, both the fibrillar centers and the dense fibrillar component. The number of gold particles per each fibrillar component, fibrillar center, or dense fibrillar component area units (0.1 $\mu m^2$) was also measured.

Data were analyzed statistically using the following programs written for the IBM computer consoles: nested analysis of variance, Student's *t* test or, for nonhomogeneous data, the Kolmogorov–Smirnov and the U-test. A statistical difference was considered when $P < 0.001$.

*Mono-dimensional gel electrophoresis [sodium dodecyl sulfate (SDS)–PAGE].* HeLa cells were trypsinized from culture flasks after attaining 75% confluence, washed in PBS, and pelleted after a mild centrifugation. Extraction of proteins was performed after treating the pellet with SDS–PAGE sample buffer for 10 min at 97°C. Electrophoresis was performed, as described by Laemmli [28], on 7.5–12% SDS–polyacrylamide gels using the Mini-Protean II electrophoresis cell (Bio-Rad, Richmond, CA). A set of molecular weight standards were obtained from Bio-Rad Laboratories: myosin (200 kDa), $\beta$-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.69 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). When electrophoresis was carried out to subsequently perform transfers of proteins to nitrocellulose paper, a set of prestained molecular weight standards were used (Sigma Chemical Co.): $\alpha$2-macroglobulin (180 kDa), $\beta$-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), and triosephosphate isomerase (26.6 kDa).

Total proteins in gels were revealed with Coomassie blue staining.

*Two-dimensional gel electrophoresis.* HeLa cells were pelleted as for mono-dimensional SDS–PAGE. We used the O'Farrell lysis buffer [37] as the isoelectric focusing (IEF) sample buffer. The concentrations of proteins in the IEF sample buffer were determined with a modified Bradford assay, which allows quantitation of proteins in the presence of urea, carrier ampholytes, nonionic detergents, and thiol compounds [43]. The volume of sample loaded per capillary gel tube contained a concentration of 30 $\mu g$ total proteins.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) was performed according to O'Farrell [37] using the Mini-Protean 2D electrophoresis cell (Bio-Rad) as described previously [34]. The two-dimensional gel electrophoresis in the presence of SDS was carried out as for mono-dimensional SDS–PAGE electrophoresis.

Total proteins in gels were revealed by using the Bio-Rad silver stain Kit.

*Electrophoretic transfer and immunoblotting.* Proteins were transferred electrophoretically from polyacrylamide gels to nitrocellulose paper [66] and incubated in 3% gelatin in TBS (15 m*M* Tris, 200 m*M* NaCl) for 24 h at room temperature. After two washes of 10 min each in TBS containing 0.05% Tween-20 (TTBS), an incubation in anti-NOR serum diluted 1:100 in TTBS containing 1% gelatin was performed for 24 h at room temperature. Blots were rinsed in TTBS and incubated in peroxidase-labeled goat antihuman IgG (Boehringer-Mannheim Biochemicals) diluted 1:2000 in TTBS, for 2 h at room temperature. After washing in TTBS and TBS, the reaction was visualized with chloronaphthol substrate (50 ml TBS containing 0.03 g 4-chloro-1-naphthol in 10 ml methanol and 20 $\mu l$ of 30% hydrogen peroxide). Color development was stopped in distilled water and blots were allowed to air dry. Control trials were performed by omitting the incubation with the anti-NOR serum.

NOR-silver staining on transfers was performed after blocking nitrocellulose paper in 3% gelatin in TBS for 24 h at room temperature, following the cytochemical technique described before.

Photographs of gels and transfers were taken with an AGFA Copex Rapid A.H.U. film processed to 9 ASA.

## RESULTS

Under immunofluorescence microscopy and after immunolabeling with a 1:300 dilution of anti-NOR serum on fixed HeLa cells only small nucleolar spots showed fluorescence (Fig. 1a). On the other hand, cells treated with AMD showed an intense labeling restricted to nucleolar cap-like structures (Fig. 2a). In both cases we did not observe labeling over the nucleoplasm or in the cytoplasm. Finally, controls performed did not emit any fluorescence.

At the ultrastructural level HeLa cells disclose nucleoli with a compact morphology, mainly composed of granules and fibrils, which, respectively, constitute the granular and fibrillar components. The fibrillar component is formed by two subcomponents, one being some

**FIG. 3.** HeLa cell nucleolus processed for conventional electron microscopy disclosing a compact type morphology where the different nucleolar components can be distinguished. Granular component (G), fibrillar centers (arrows), and dense fibrillar component (arrowheads). Bar, 0.5 $\mu m$.

**FIG. 4.** One-step NOR-silver staining on material fixed in 1.6% glutaraldehyde–Carnoy and embedded in Lowicryl. (a) At the ultrastructural level, the specific silver precipitate is localized over the fibrillar centers and the dense fibrillar component. Bar, 0.2 $\mu m$. (b) Semithin section showing NOR-silver staining on distinct nucleolar spots. Bar, 5 $\mu m$.

**FIGS. 5 and 6.** Immunoelectron microscopic localization with anti-NOR serum on ultrathin sections of HeLa cells fixed in 0.1% glutaraldehyde–4% paraformaldehyde and embedded in Lowicryl. Gold particles are detected over the fibrillar centers and dense fibrillar component.

**FIG. 5.** Protein A–gold labeling. Bar, 0.2 $\mu m$.

**FIG. 6.** Gold-coupled goat anti-human labeling. Bar, 0.2 $\mu m$.

**TABLE 1**

Quantitative Analysis of Immunolocalization with Anti-NOR Serum and Labeling with Protein A–Gold (PA–gold) or Goat Anti-Human–Gold (GAH–gold) on the Fibrillar Component of HeLa Cell Nucleoli

| | Area ($\mu m^2$) | PA–gold particles/ 0.1 $\mu m^2$ | GAH–gold particles/ 0.1 $\mu m^2$ |
|---|---|---|---|
| Fibrillar component unit | 0.1582 ± 0.0132 | 11.00 ± 0.9280 | 36.94 ± 3.5943 |
| DFC | 0.0612 ± 0.0050* | 11.79 ± 1.6242 | 37.64 ± 5.5027 |
| FC | 0.0538 ± 0.0061* | 13.38 ± 1.4865 | 38.95 ± 4.7169 |

*Note.* $N = 50$; mean ± SEM.
* $P < 0.001$ compared with the fibrillar component unit.

lighter structures called fibrillar centers (FCs) which are found at least partly surrounded by a more dense zone that correspond to the other subcomponent, namely the dense fibrillar component (DFC) (Fig. 3).

At the ultrastructural level, NOR-silver staining was localized over the fibrillar centers and dense fibrillar component, the latter showing a more intense silver precipitate (Fig. 4a). On semithin sections we detected, under the light microscope, a dot-like silver-specific nucleolar stain (Fig. 4b). Controls performed to demonstrate the specificity of NOR-silver staining revealed an absence of reaction after incubation with pronase, while the staining was maintained after treatment with both RNase and DNase.

When we proceeded to immunolocalization with a 1:500 anti-NOR serum dilution and labeling with protein A–gold or gold-coupled goat anti-human IgG (GAH), we observed gold particles over the fibrillar centers and dense fibrillar component (Figs. 5 and 6). The granular component, chromatin, nucleoplasm, and cytoplasm were devoid of any labeling. We neither observed any gold particles in the controls performed.

In order to carry out a quantitative analysis of the immunolabeling with the anti-NOR serum we previously initiated a morphometric study to determine the size of the fibrillar component units and of their subcomponents (Table 1). Next, we proceeded to the quantitation of gold particles localized over the fibrillar component units, fibrillar centers, and dense fibrillar component, after applying protein A–gold or GAH–gold, with the aim of establishing the relative proportion of immunolabeling in each of the fibrillar subcomponents. We observed, independently of the marker used, a similar distribution of the labeling in both the fibrillar centers and dense fibrillar component. However, we found a 2.5 times more intense labeling, approximately, when GAH–gold was applied (Table 1).

In metaphasic cells, where there is no transcriptional activity of rDNA, immunolabeling with our serum appears localized to the chromosomal NOR (Figs. 7a and 7b). Following a 5-h exposure of HeLa cells to 2 $\mu g/ml$ AMD, electron microscopy revealed a characteristic pro-

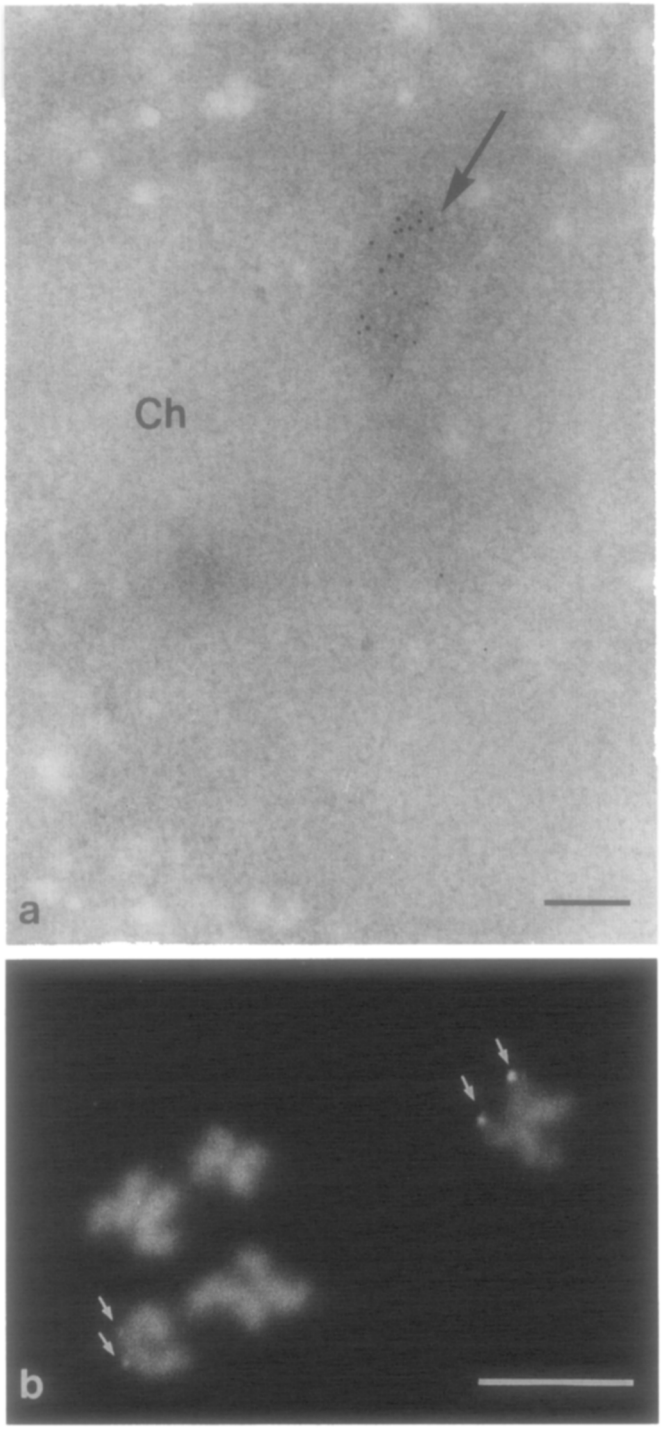


**FIG. 7.** Immunolabeling with anti-NOR serum localized to the chromosomal NOR. (a) Immunoelectron labeling in metaphasic HeLa cells (arrow). Chromosome (Ch). Bar, 0.2 $\mu m$. (b) Immunofluorescence microscopy on HeLa metaphasic chromosomes (arrows). Bar, 5 $\mu m$.

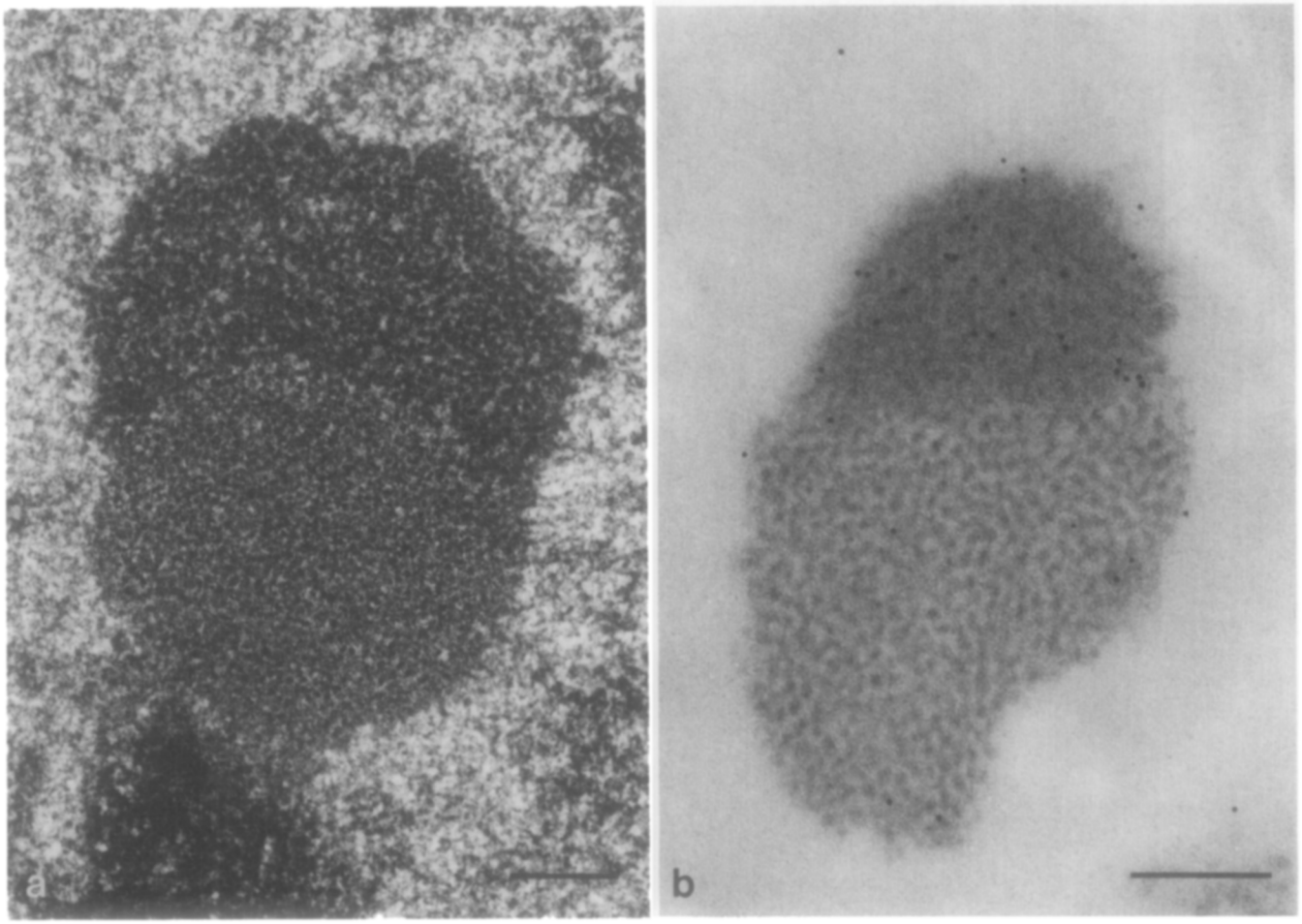


**FIG. 8.** HeLa cells treated with 2 μg/ml AMD for 5 h. (a) Segregated nucleolus under conventional electron microscopy. Bar, 0.2 μm. (b) Immunoelectron microscopic labeling with anti-NOR serum and protein A–gold, mainly localized on the fibrillar component. Bar, 0.2 μm.

gressive segregation of nucleolar main components. In Fig. 8a, a segregated nucleolus with the fibrillar component forming a cap-like structure in the nucleolar periphery, separated from the granular component, is illustrated. Under these experimental conditions, immunolocalization with anti-NOR serum demonstrated that gold particles remained attached mainly to the fibrillar component, being localized to the cap-like structures (Fig. 8b).

To characterize the nucleolar antigens recognized by the anti-NOR serum we carried out a mono-dimensional gel electrophoresis of whole HeLa cell extracts followed by electrophoretic transfer and found two polypeptides which respectively migrate at 92 and 88 kDa immunoblotted with anti-NOR serum. Corresponding Coomassie-stained gels of HeLa cell extracts showing the full complement of extracted proteins and negative control assay in which anti-NOR was excluded together confirm the blotting immunospecificity (Fig. 9).

As previously described, the antigens detected by the anti-NOR serum were found in the fibrillar centers and dense fibrillar component. As the Ag-NOR proteins are the major proteins so far detected in these localizations, we tested whether the proteins recognized with the anti-NOR serum coincided with some of the Ag-NOR proteins. On adjacent nitrocellulose strips, we found different labeling patterns with anti-NOR serum and NOR-silver staining on whole cell extracts. The silver staining revealed some major bands at 105 and 38 kDa (Fig. 9).

As the fibrillar component is believed to be the site of ribosomal transcription, we checked the effects on the nucleolar antigens recognized by the anti-NOR serum of impaired rRNA transcription under experimental conditions. Treatment of HeLa cells with AMD inhibited rRNA transcription, as demonstrated earlier by the segregated morphology of nucleoli, and after electrophoretic transfer and blotting with anti-NOR serum we clearly observed that the two polypeptides still remained at 92 and 88 kDa (Fig. 9).

In order to further characterize the two polypeptides revealed by the anti-NOR serum we carried out a two-dimensional electrophoresis followed by an electrophoretic transfer of the whole HeLa cell extract. We found, after immunoblotting with anti-NOR serum, the same

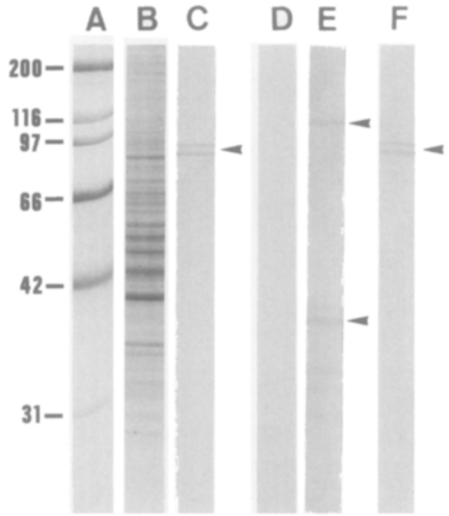


**FIG. 9.** Characterization by immunoblotting of the antigen recognized by the anti-NOR serum using 12% SDS–PAGE. (A) PAGE migration pattern of molecular weight standards. (B) Coomassie blue staining of PAGE-separated proteins present in whole HeLa cell extracts. (C) Two polypeptides, 92 and 88 kDa, are revealed upon probing with 1:100 diluted anti-NOR serum (arrowhead). (D) Corresponding negative control assay in which anti-NOR serum was omitted. (E) NOR-silver staining of Western-blotted proteins from whole HeLa cell extracts. Main positive bands are visible at 105 and 38 kDa (arrowheads). (F) Immunoblotting with 1:100 diluted anti-NOR serum on AMD-treated whole HeLa cell extracts. Arrowhead indicates the 92- and 88-kDa protein doublet.

polypeptide doublet at 92 and 88 kDa, disclosing an isoelectric point of around 7.5 (Fig. 10).

## DISCUSSION

The nucleolus, the site of ribosomal RNA synthesis, is a very complex structure with multiple components. However, despite the fact that the morphology of the nucleolus has been thoroughly studied, precise information about the functional role of its different components and the site of ribosomal transcription are still lacking. General agreement exists on the view that the three nucleolar main components, the fibrillar centers, the dense fibrillar component, and the granular component, correspond in some way to the different steps in the transcription and processing of rRNA into RNP. The problem appears when the function of each particular component has to be established. Disagreement already begins with the fundamental process of rRNA gene transcription. Some argue that this is restricted to the fibrillar centers, while others believe that it takes place in the dense fibrillar component or even in both of them [8, 11, 14, 20, 26, 54].

It would be very interesting to be able to differentiate between the nucleolar components by the use of specific markers that would allow insight into their respective functions to be gained and their distribution under various physiological conditions to be followed. As already reported, antibodies against nucleolar antigens, which are able to identify antigens localized in the different nucleolar subcomponents with a high specificity [1, 21, 22, 31, 39, 49], are present in autoimmune sera.

In the present study we have used a novel autoantibody able to react with a high specificity against an antigen localized to the fibrillar component. By immunofluorescence we first observed it to be located inside the nucleolus with a dot-like pattern. When we applied immunolocalization at the ultrastructural level we found that the antigen was exclusively detected both on the fibrillar centers and dense fibrillar component. These data indicate that the antigen detected with the anti-NOR serum must be localized in certain structures

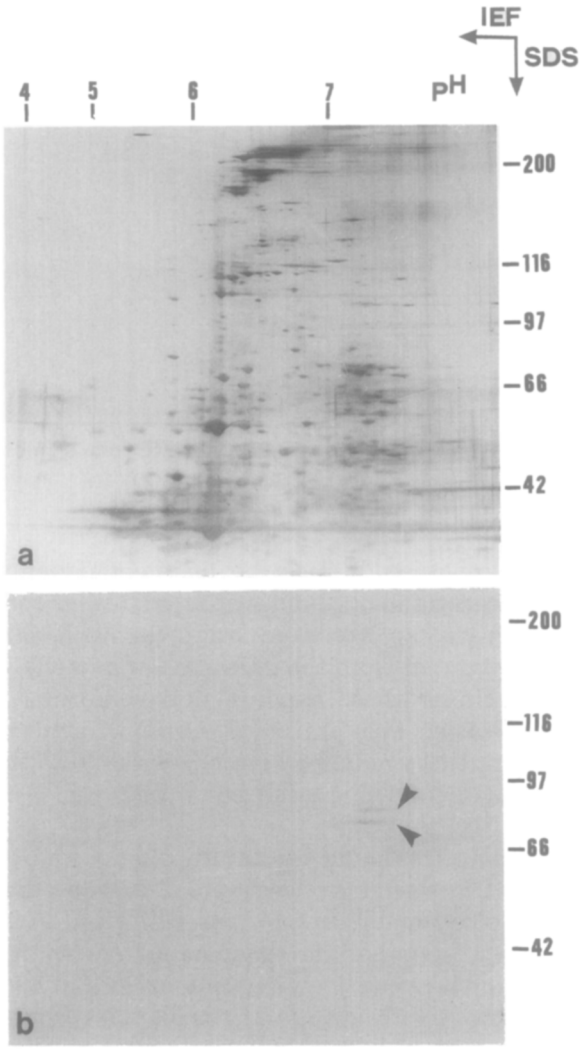


**FIG. 10.** Further characterization of the antigens by means of two-dimensional electrophoresis using 7.5% SDS–PAGE, followed by electrophoretic transfer of whole HeLa cell extracts. (a) Pattern of total proteins stained with the Bio-Rad silver stain kit. (b) Immunoblotting with a 1:100 dilution of anti-NOR serum. The 92- and 88-kDa protein doublet can be observed disclosing an isoelectric point of around 7.5 (arrowheads).

found within both subcomponents of the fibrillar component, and coincide with the localization of rDNA described by other authors.

A number of different experimental strategies have been adopted to trace the rRNA genes within the nucleolus, but the results obtained are still controversial. In animal cells, by means of cytochemical studies the presence of DNA inside the fibrillar centers has been detected [14, 30], and *in situ* hybridization at the light and ultrastructural level has established its ribosomal nature [25, 65]. Other authors, however, by means of the same techniques have only detected rDNA in the surrounding dense fibrillar component [10, 15, 67]. Some contradictory results have also been obtained after detecting DNA by means of anti-DNA monoclonal antibodies, as certain authors have localized DNA inside the fibrillar centers [55, 61, 63], while others just in small nucleolar areas which correspond to the invaginations of peri or intranucleolar chromatin usually present in the proximity of FCs, DFC, and nucleolar interstices [44]. Recently, after applying nick-translation using mild digestion with DNase I at the ultrastructural level, actively transcribing or potentially active genes have been found in the fibrillar centers and in the interstices surrounding them [62].

Another approach has been based on the immunodetection of enzymes involved in rRNA transcription as their distribution should accurately reflect the sites where the rRNA transcription units are located. By immunogold electron microscopy, RNA polymerase I has been detected mainly in the fibrillar centers [45, 57], the labeling obtained in the DFC being low and dependent on the cell type. A similar pattern of nucleolar distribution has also been found for topoisomerase I, an enzyme required for proper transcription of the rRNA genes [51], though others indicate it to be enriched in the DFC, their occurrence in FCs being much less [45]. By means of a treatment with 5,6-dichloro-$\beta$-D-ribofuranosyl benzimidazole and immunolabeling with antibodies against RNA polymerase I, Haaf *et al.* [18] have been able to detect the rDNA transcription units in vertebrate cells and affirm that this combination, or any other able to label rDNA, can provide a powerful method to determine their number in individual cell nuclei.

With the use of our anti-NOR serum we have demonstrated an immunodetection of the antigens localized in the FCs and the DFC, which, as already seen, are both candidates for the sites where ribosomal transcription occurs. In order to check more precisely the nucleolar localization of the antigens we carried out a quantitative study of the labeling and observed that the distribution of gold particles was similar in both the fibrillar centers and dense fibrillar component, independently of the marker used. However, when gold-coupled goat anti-human was applied, labeling was around 2.5 times higher than when protein A–gold was used, due, as it has already been described by other authors, to an amplification in the signal obtained [44].

This distribution of gold particles is similar to that obtained after NOR-silver staining, which is believed to correlate well with the transcriptional activity of rRNA genes [7, 9, 19, 33–35, 46, 53]. So far, ribosomal gene activity has generally been evaluated by silver staining of nucleoli, but some restrictions exist as the silver-stainable materials are acidic proteins associated with transcribed rRNA genes, and it is not yet clear which are the specific proteins actually involved in rDNA transcription. At least a part of the argyrophilic proteins seems to be independent of nucleolar transcriptional activity, perhaps being related to a structural function. However, Haaf *et al.* [18] still consider the use of NOR-silver staining as the more accessible and universal method to determine the number of rDNA transcription units. When rRNA synthesis was inhibited under physiological conditions (mitosis) or after using AMD, we observed that the antigens detected with the anti-NOR serum behaved in a way similar to the Ag-NOR proteins or RNA polymerase I, remaining attached to the metaphasic NOR and to the fibrillar component localized in crescents areas at the outermost margins of the interphasic nucleolus, respectively [20, 49].

We checked whether the antigens detected corresponded to the Ag-NOR proteins, but in fact this was not the case, since NOR-silver staining revealed a different pattern from that shown by the anti-NOR serum in adjacent strips of Western-blotted whole HeLa cell protein extracts. We found some major silver-stained bands at 105 and 38 kDa, while the molecular weight of the antigens recognized by the anti-NOR serum corresponded to a doublet at 92 and 88 kDa that also appeared when AMD-treated HeLa cell extracts were used.

We also performed two-dimensional gel electrophoresis of whole HeLa cell extracts to further characterize the antigens detected and found that, after Western-blotting with anti-NOR serum, they showed an isoelectric point of around 7.5. This latter data again confirm that the proteins detected are not Ag-NOR proteins since these show an acidic isoelectric point of approximately 5 [4, 6, 34, 58].

We have observed that the antigen recognized by the anti-NOR serum shows a biochemical behavior very similar to the one detected with the NOR-90 serum, obtained from a scleroderma patient and that has been identified as the human upstream binding factor (hUBF) [5]. Both sera are able to react specifically with antigens formed by a protein doublet which migrates in the same molecular weight rank. It has been demonstrated that hUBF binds specifically to the upstream control element and core of the rRNA gene promoter to activate transcription by RNA polymerase I in a binding

site-dependent manner [2, 16, 24, 38, 40], and its molecular weight has been calculated to be 89.4 kDa in human cells [24]. However, by SDS–PAGE analysis it has been shown to consist of two polypeptides with apparent molecular sizes of 97 and 94 kDa that comigrate with recombinant hUBF [24].

We have observed that the antigen detected with the anti-NOR serum remains joined to the fibrillar component when transcription is impaired. Such a behavior is similar to those shown by RNA polymerase I [49, 56], DNA topoisomerase I [17, 51], and nucleolin [4, 12, 13, 29]. The reason is, as it is generally accepted, that these proteins have to be maintained and conserved through some relatively short steps of the cell cycle where inhibition of transcription exists (mitosis), since in a specific moment, immediately after mitosis, they will be necessary to carry out intense rDNA transcriptional activity to enter G1.

All these data, together with the finding that the antigens detected by anti-NOR serum are localized in the fibrillar component of the nucleolus, the site of rRNA transcription, which coincides with the main place of hUBF action, strongly support that they are a genuine RNA polymerase I factor. However, we cannot exclude the possibility that these antigens play a role in the nucleolus in addition to being a transcription factor, for example in processing and transport of rRNA and preribosomal particles.

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