Mapping Nucleolar Proteins with Monoclonal Antibodies

JOERG KISTLER, YVONNE DUNCOMBE, and ULRICH K. LAEMMLI
Departments of Molecular Biology and Biochemistry, University of Geneva, 1211 Geneve 4, Switzerland. Dr. Kistler's present address is Department of Cell Biology, University of Auckland, Auckland, New Zealand.

ABSTRACT Using monoclonal antibodies as probes, we have characterized three antigens with respect to localization in the nucleolus, molecular weight and solubility. Two proteins, of 110,000 and 94,000 apparent molecular weight, were found associated with the ribonucleoprotein fibers. A third protein, with a molecular weight of 40,000, was accumulated at the nucleolar periphery, was present in the nucleoplasm, and may be involved in pre-ribosome maturation and transport.

Nucleoli are domains in the nucleoplasm where precursor ribosomal RNA is synthesized, processed to the mature rRNA's and assembled with proteins to pre-ribosomal particles. The latter are then transported through the nucleoplasm and across the nuclear envelope into the cytoplasm, concomitant with their activation to functional ribosomes. Detailed knowledge exists on the structure of the highly repeated rRNA genes (17). However, the protein composition of nucleoli from mammalian cells appears complex and little is known about the arrangement and function of individual nucleolar proteins. Isolated nucleoli have been fractionated and the proteins displayed and categorized by two-dimensional polyacrylamide electrophoresis (21). Purification of a few proteins have been achieved and their nucleolar location confirmed by immunoelectron microscopy (4, 14, 16, 20).

We used monoclonal antibodies as probes to study the distribution of nucleolar proteins in human cells. This approach is particularly efficient because unfractionated nuclear material can be used for immunization, and hybridomas specific for nucleoli can easily be identified by immunofluorescence microscopy. The results from immunofluorescence microscopy, immunoelectron microscopy, and solubilization studies are discussed in view of functional aspects of three selected nucleolar antigens.

MATERIALS AND METHODS

Isolation of Polyamine Nuclei: HeLa cells were grown in suspension culture with Roswell Park Memorial Institute-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% newborn calf serum. Where applicable, [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) was added to the culture at 1 μ Ci/ml for 18 h before harvesting the cells.

For isolation of polyamine nuclei (12) ~5 × 10^8 cells from 1l culture medium were pelleted at 2,000 rpm for 10 min in a Sorvall GSA rotor (Beckman Instruments, Spinco Div., Palo Alto, CA). Each of two pellets was washed 3× in solution 1 which contained 3.7 mM Tris-HCl (pH 7.4), 0.05 mM spermine, 0.13 mM spermidine, 0.5 mM EDTA and 20 mM KCl, and pelleted in a Clay Adams Dynac centrifuge (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ). The pellets of swollen cells were then chilled on ice, 40 ml solution 2 (4× concentrated solution 1 plus 0.1% digitonin) was added, and the cells were disrupted immediately by 10 strokes of a 50 ml capacity Dounce homogenizer using a type A pestle. Cytoplasmic material was removed from nuclei by one wash in solution 2 and two washes in solution 3 (5 mM Tris-HCl (pH 7.4), 0.25 mM spermidine, 2 mM EDTA, 2 mM KCl, 0.1% digitonin) at 500 *g* for 5 min in a cooled Clay Adams Dynac centrifuge (Clay Adams). All final pellets were resuspended in 2 ml solution 3 and assayed for purity by phase-contrast light microscopy.

All solutions contained 0.1 mM phenylmethylsulfonyl fluoride and 10 KIE/ml Trasylol (Bayer [Bayer Co., New York, NY]). Solutions 2 and 3 were prepared according to established procedures (13).

Isolation of Polyamine Nucleoli: For isolation of nucleoli, nuclei were disrupted by sonication and fractionated on a density gradient. 20 μl of 20 mM spermine/50 mM spermidine was added to 2 ml resuspended nuclei to condense nucleolar chromatin. Nucleoli were released from nuclei by microsonication (three to four 10-s sonication pulses). Samples were cooled with ice water. Subsequently, the 2-ml aliquots were complemented with 7 ml solution 3, 350 μl 20 mM spermine/50 mM spermidine, 25 ml of 89% (vol/vol Percoll (Pharmacia Fine Chemicals, Div of Pharmacia Inc., Piscataway, NJ) in 5 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.8 mM spermine, 2 mM spermidine, 0.1% digitonin, and homogenized with 10 strokes of a Dounce homogenizer using a type A pestle. This mixture was centrifuged at 20,000 rpm for 30 min in a Sorvall SS-34 rotor; a density gradient was established that separated two bands with the nucleoli near the bottom of the gradient, and the nucleoplasmic fibrous material in the upper third of the gradient. Percoll was removed from the nucleoli by two washes in solution 3 (centrifuged at 1,500 *g* for 30 min in a Clay Adams Dynac centrifuge) (Clay Adams), and the nucleoli were resuspended in solution 4 which contained 5 mM Tris-HCl (pH 7.4), 0.25 mM spermidine, 2 mM KCl, 0.1% digitonin (13). 0.1 mM phenylmethylsulfonyl fluoride and 10 KIE/ml Trasylol were added to all solutions.

Digestion and Salt Extraction of Nucleoli: To probe for nucleic acid-protein interactions, nucleoli were treated with DNase I and/or RNase A followed by salt extraction and analysis by SDS PAGE. 0.5-ml aliquots of nucleoli in solution 4 (A^{260} = 1–2) were adjusted to 5 mM $MgCl_2$ and treated with either 40 μg/ml DNase I (Worthington, Biochemical Corp., Freehold, NJ), or 10 μg/ml boiled RNase A, or both, respectively, and incubated for 1 h at 0°C. Nucleoli were extracted with 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.1% digitonin, and either 100 mM, 400 mM, or 2 M NaCl, and then centrifuged for 10 min in a Beckman microfuge. Proteins in the supernatants

were first precipitated with trichloroacetic acid and then, like the pellets, resuspended in SDS PAGE sample buffer at a concentration 0.5 A^{260} U/50 μl.

SDS PAGE and Immunoblotting: SDS PAGE was carried out as described by Laemmli (8) with the following modification: 4 M urea, 1 mM EDTA, 0.1% thiodiglycol were added to both the stacking and the running gels, and the electrophoresis buffer contained 0.1% mercaptoethanol. 50 μl samples were loaded per well. Proteins were visualized by staining gels with Coomassie Blue.

Gels for immunoblotting were incubated in 4 M urea, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 50 mM NaCl, 0.1 mM dithiothreitol for 2 h, and transferred to nitrocellulose sheets (Schleicher and Schuell Inc., Keene, NH) according to established procedures (3). The procedure for immunolabeling was as described by Towbin et al. (28) and Renart et al. (24) with the following modifications: nitrocellulose filters with the SDS PAGE protein replica were treated for 1 h in 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 150 mM NaCl, 0.5% bovine serum albumin, 0.05% Triton X-100, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone-40 to saturate binding sites. Undiluted hybridoma supernatants were applied for 1–2 h and the filters subsequently washed several times in 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100. The filters were then incubated for 1–2 h in the same buffer which contained 0.1 μCi/ml sheep anti-mouse Ig $^{125}I(Fab')_2$ (Amersham Corp.). This was followed by two 20-min washes with 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 M NaCl, 0.1% bovine serum albumin, and 0.1% Triton X-100 and one rinse with 10 mM Tris-HCl (pH 7.4) to remove the salt. Filters labeled with different antibodies were kept separate at all times as particular high affinity antibodies were found to cross-contaminate other filters when the latter were washed together. Dried nitrocellulose filters were exposed at −80°C with pre-flashed Kodak X-Omat autoradiography film in cassettes equipped with intensifying screens (9).

Immunization and Monoclonal Antibody Production: Histone-depleted nuclei in native form or modified with formaldehyde were used for immunization. Polyamine nuclei were resuspended in solution 4 and treated with 5 mM $MgCl_2$ and 80 μg/ml DNase I for 2 h at 0°C. The digested nuclei were then stabilized with 0.1 mM $CuSO_4$ under a N_2 atmosphere (13) for 10 min and extracted with 10 mM Tris-HCl (pH 9), 10 mM EDTA, 2 M NaCl, 0.1% digitonin. The lysis mixture was divided into aliquots of 20 A^{260} units and pellets of native histone-depleted nuclei (20,000 rpm for 30 min in a Sorvall SS-34 rotor) were stored at −80°C. Protein modification with formaldehyde was as follows: native histone-depleted nuclei were treated with 2% formaldehyde, 10 mM PIPES (pH 7.4), 0.1% digitonin overnight at 4°C, and dialysed with several buffer changes against 0.1 M Tris-HCl (pH 7.4), 2 M NaCl, 0.1% digitonin. Pellets were recovered by centrifugation and were stored as aliquots of 20 A^{260} units.

2-mo-old BALB/c mice were immunized with doses of 20 A^{260} units of extracted nuclei in either native or modified form in Freund's complete adjuvant, and boosted 4 wk later with the same amount in incomplete adjuvant. 8 wk after initial immunization, the same amounts of antigen in phosphate-buffered saline (PBS)[1] were injected on three consecutive days (26). All injections were intraperitoneal. 2 d later, spleen cells from immunized mice were fused with PAI myeloma cells (gift from Dr. T. Staehelin and Dr. J. W. Stocker, Hoffman La Roche [Basel, Switzerland]) and directly cloned according to established methods (6, 27).

Screening for Nucleolar Specificity: Hybridomas were screened for antibody specificity by immunofluorescence microscopy (see below) on HeLa cell monolayers grown on multiwell glass slides (Flow Laboratories, Inc., McLean, VA). Fluorescence-positive supernatants were further tested by immunoblotting, and hybridomas that recognized a single protein band were then subcloned by limited dilution to ensure their monoclonal character. We derived hybridomas H10-C10-D10 from a fusion for which spleen cells from a mouse immunized with native histone-depleted nuclei were used; of 329 immunofluorescence-screened hybridomas, two were positive for nucleoli and 23 for nucleoplasm and/or cytoplasm. We derived hybridomas H21-F11-C11 and H15-D12-B10 from a mouse immunized with modified histone-depleted nuclei; of 155 immunofluorescence-screened hybridomas, 16 revealed antinucleolar specificity, and 14 were directed against antigens in nucleoplasm and/or cytoplasm. Of the 16 hybridomas that labeled nucleoli by immunofluorescence, antibodies from five independent clones displayed staining patterns on immunoblots identical to H21-F11-C11, and antibodies from another five hybridomas displayed staining patterns identical to H15-D12-B10.

Immunofluorescence Microscopy: HeLa cell monolayers were grown on glass slides and prepared for immunofluorescence microscopy according to the procedure of Epstein and Clevenger (5). The slides were washed free of medium with PBS, and the cells were fixed for 20 min in PBS that contained 2% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA). A brief rinse in PBS preceded a 3-min exposure to −20°C acetone to permeabilize the cells. After several washes in PBS, undiluted hybridoma supernatants were applied and the slides were kept in a moist chamber at 37°C for 1 h. Unbound antibodies were removed by three 10-min washes in PBS and the cells were then reacted with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig antibodies, (Nordic Immunological Laboratories, El Toro, CA) 1:60 in PBS for 1 h. Following three 10-min washes in PBS, the slides were dipped into Evans Blue (Bio Mérieux, Charbonnières les Bains, France) at 1:10,000 in PBS for 5 min and then washed again in PBS. The final mounting was in 50% glycerol and 10 mM p-phenylenediamine as anti-fading agent (7). Specimens were observed in a Leitz Orthoplan fluorescence microscope with a 50× objective (Planneofluotar) and photographs were recorded on Ektachrome 400 film.

Immunoperoxidase Staining for Electron Microscopy: Antigens were localized in nucleoli using an indirect peroxidase labeling technique and electron microscopy. To ensure penetration of antibodies into the cells and minimum unspecific antibody binding, reaction steps and washing steps were done on cell monolayers and the cells were only pelleted before osmification.

HeLa cells attached to cell culture dishes were washed free of medium with PBS and fixed with 2% formaldehyde, 0.1% Triton X-100 in PBS for 20 min. Excess fixative was inactivated by washing the cells with PBS that contained 0.5 mg/ml $NaBH_4$. After treatment with 0.1% Nonidet P-40 and 0.05% sodium deoxycholate for 10 min, cells were washed in PBS and then incubated with undiluted hybridoma supernatant for 1 h. Unbound antibodies were removed with five 5-min PBS washes, and the cells were treated with 1/100 normal rabbit serum. Peroxidase-conjugated rabbit anti-mouse Ig antibodies (Nordic) were applied at 1/200 in PBS for 1 h, followed by five 5-min PBS washes. The material was then fixed with 0.2% glutaraldehyde in PBS for 20 min, washed several times with PBS and impregnated with 0.25 mg/ml diaminobenzidine in PBS. After 10 min, the solution was replaced with 0.25 mg/ml diaminobenzidine, 0.02% H_2O_2 in PBS, and the reaction allowed to continue for 30 min. The cells were thoroughly washed, scraped off the culture dish, pelleted and resuspended into 1% OsO_4 in H_2O at 4°C for 20 min. After leaving the cells in H_2O overnight (4°C) the peroxidase-labeled cells were dehydrated with ethanol and embedded into Epon according to standard procedures. Thin sections were viewed without further staining with a Philips 300 electron microscope operating at 60kV. A 50 μm objective aperture was used and photographs were recorded on Kodak 4489 electron microscope film.

RESULTS

Polyamine Nucleoli

Nucleoli were isolated in polyamine buffers previously described for the isolation of metaphase chromosomes (2, 13), and for the isolation of nuclei (12). One step in the isolation protocol deserves special attention: nucleoli in solution 3 should be viewed in phase-contrast light microscopy before sonication and sufficient spermine/spermidine should be added so that nucleoli appear condensed and strongly refractile. Expanded nucleolar chromatin is more susceptible to rupture during sonication, and nucleoli may break apart. Liberated intact nucleoli appear with "hairy" contours in the light microscope, which are due to adherent nucleoplasmic remnants. Douncing in a mixture of colloidal silica (Percoll) and polyamine buffer removes these adherent remnants. Analysis of the density gradient profile by phase-contrast light microscopy shows a nucleoli rich fraction in the lower gradient band, and fibrous material and unbroken nuclei in the upper band (Fig. 1 *a* and *b*). The [^{3}H]thymidine gradient profile reveals the separation of the two bands (Fig. 1 *c*). Polyamine nucleoli retained an average 8% of total nuclear DNA. The DNA sequences coding for the 18S and 28S rRNA's (which account for <1% of total nucleolar DNA, [1]) were found 4× enriched in polyamine nucleoli with respect to intact nuclei (France Ballivet, unpublished observations).

The partitioning of proteins between lower and upper gradient bands has been visualized by SDS PAGE (Fig. 2). A number of Coomassie Blue–stained bands present in the nuclei lane are weak or absent in nucleoli, but strong in the

[1] *Abbreviation used in this paper*: PBS, phosphate buffered saline.

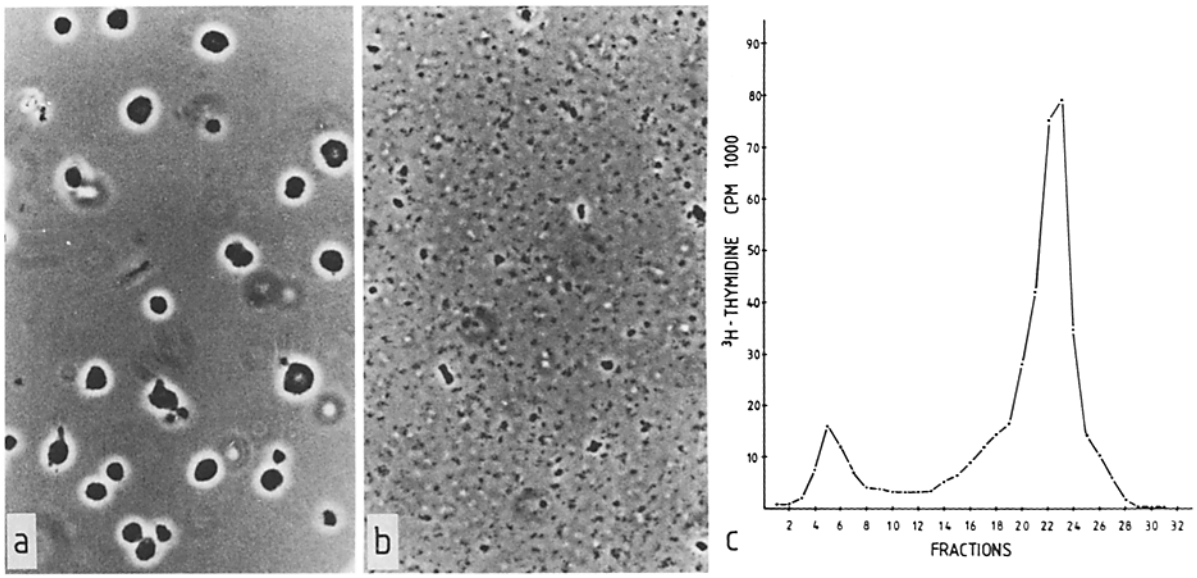

FIGURE 1 Fractionation of sonicated nuclei on Percoll density gradient. (*a*) Phase-contrast light micrograph of nucleoli enriched in the lower gradient band, washed free of Percoll and resuspended in solution 3. (*b*) Micrograph of the material accumulated in the upper gradient band, treated as above. This fraction contains fibrous structures and intact nuclei (if any remain after sonication). (*c*) Gradient profile of sonicated nuclei of which the DNA was labeled with [^{3}H]thymidine. 1.1-ml fractions were collected and 10 μl of each were counted. Bottom of gradient is left in the graph.

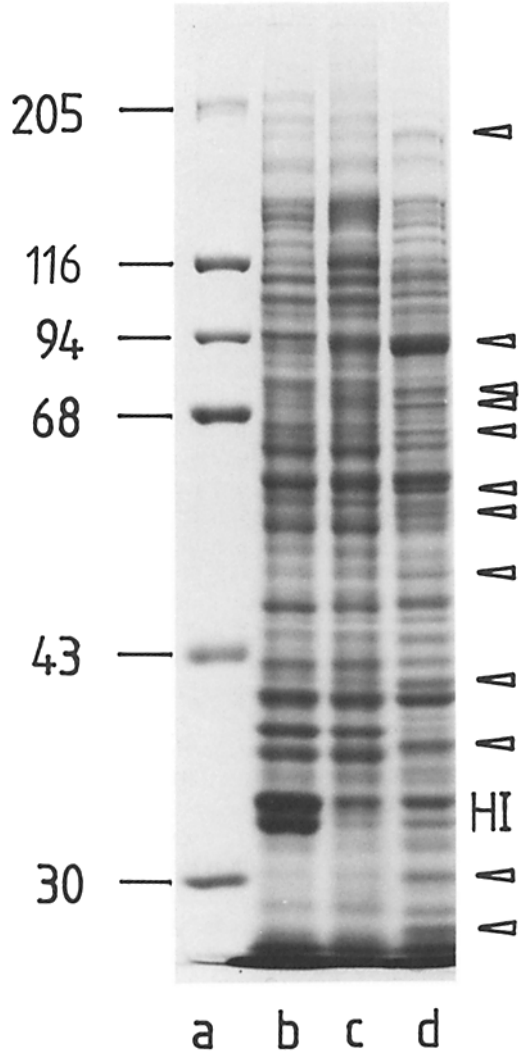

FIGURE 2 Partitioning of nuclear proteins into the upper and lower gradient bands and comparison with total nuclear proteins. 7.5% PAGE. (Lane *a*) Markers (mol wt × 10^{-3}): (205) Myosin; (116) β-galactosidase; (94) phosphorylase B; (68) BSA; (43) ovalbumine, (30) carbonic anhydrase. (Lane *b*) Upper gradient band 0.5 A^{260} U. (Lane *c*) Polyamine nuclei 0.5 A^{260} U. (Lane *d*) Lower gradient band 0.5 A^{260} U. Arrowhead: some of the enriched proteins typically found in the nucleolar fraction.

upper gradient band fraction. The respective proteins are therefore components of the fibrous material derived from sonicated nucleoplasm. In contrast, nucleolar proteins are represented by bands that are strong in the nucleoli lane, and weak or absent in the upper gradient fraction. It is difficult to assess the purity of the isolated nucleoli in quantitative terms because many proteins appear to be contained in both nucleolar and nucleoplasmic compartments and thus partition into both gradient bands. Electron microscopy is not a reliable means to look at polyamine nucleoli because their structure is variable as a function of spermine/spermidine-induced condensation of nucleolar matter. Phase-contrast light microscopy does, however, demonstrate the effectiveness of Percoll density gradient centrifugation for the quantitative removal of fibrous material from nucleoli.

Localization of Nucleolar Proteins with Monoclonal Antibodies

Three hybridomas producing antibodies specific to nucleolar proteins were selected (see Materials and Methods) due to their specificity in the immunofluorescence assay and Western blot analysis. Hybridoma H10-C10-D10 makes antibodies of IgG_1 subclass against a protein of 110,000 apparent molecular weight (Fig. 3*d*) as identified by superposition of the autoradiograph of the immunoblot with the corresponding complementary nitrocellulose sheet that had its protein pattern visualized by amidoblack staining (two replicas of the identical gel are obtained by diffusion transfer [3]). Immunofluorescence microscopy on HeLa cell monolayers that contained cells at various stages of the cell cycle reveals brightly fluores-

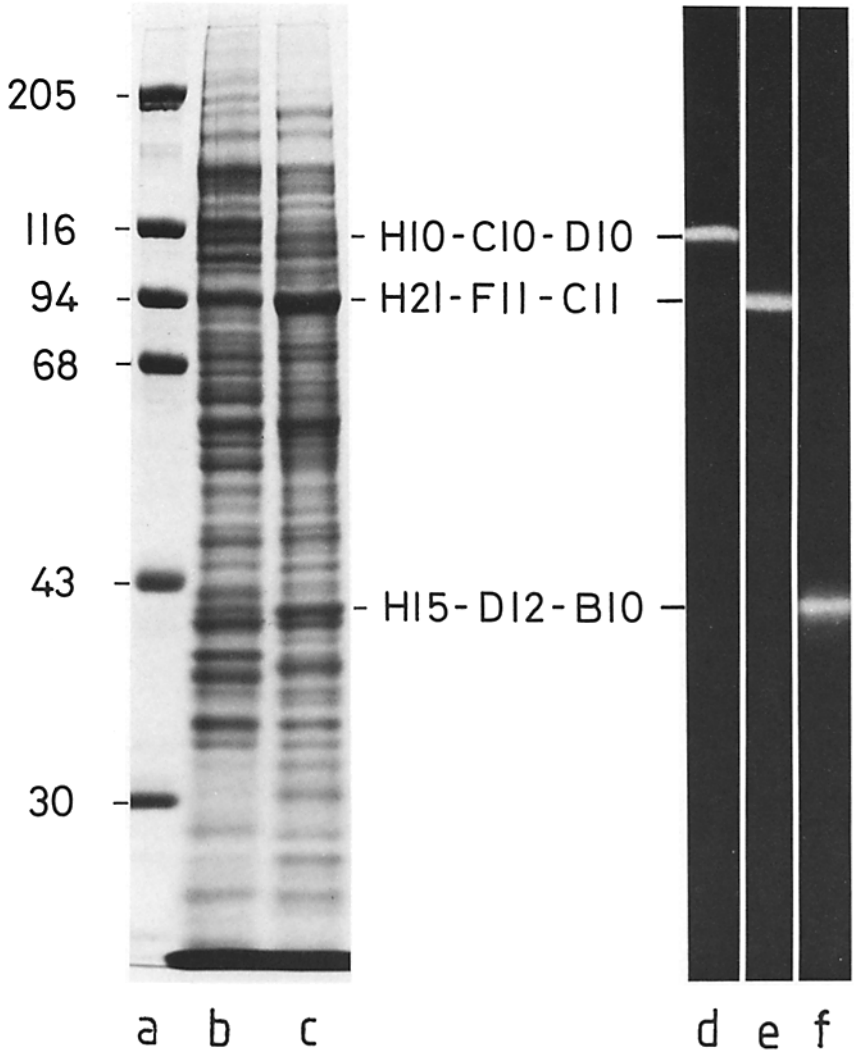

FIGURE 3 7.5% PAGE of nuclear proteins and identification of nucleolar proteins by immunoblotting. (Lane *a*) Markers (mol wt, $\times 10^{-3}$): (205): Myosin; (116) β-galactosidase; (94) phosphorylase B; (68) BSA; (43) ovalbumine; (30) carbonic anhydrase. (Lane *b*) Polyamine nuclei 0.5 A^{260} U. (Lane *c*) Polyamine nucleoli 0.5 A^{260} U. (Lanes *d–f*) Gel lanes like (lane *c*) were transferred to nitrocellulose and reacted with hybridoma supernatants. (Lane *d*) H10-C10-D10; (lane *e*) H21-F11-C11. (Lane *f*) H15-D12-B10. Antigens were identified by superposition of immunoblots with their corresponding (identical) amidoblack stained nitrocellulose strips.

cent nucleoli and a homogenous intensity throughout mitotic cells (Fig. 4*a*). Cross-reaction with nucleolar antigens in transformed *Xenopus* kidney cells, *Drosophila* fibroblasts, and mouse liver has been observed with the same method. Hybridomas H21-F11-C11 and H15-D12-B10 were derived from mice immunized with histone-depleted nuclei modified with formaldehyde. H21-F11-C11 produces IgG_1 subclass antibodies directed against a major nucleolar protein of 94,000 apparent molecular weight (Fig. 3*e*). Nucleoli were found labeled in the immunofluorescence assay (Fig. 4*b*). Hybridoma H15-D12-B10 secretes antibodies (also of IgG_1 subclass) against a 40,000-mol-wt protein (Fig. 3*f*). In immunofluorescence, these antibodies light up nucleoli and, less strongly, the surrounding nucleoplasm (Fig. 4*c*). They also stain the cytoplasm of mitotic cells. Frequently, a polar distribution of antigen was observed in mitotic cells, mostly as diffuse arcs and more rarely as arcs with a punctate pattern (Fig. 4*d*). When one correlates immunofluorescence micrographs with the corresponding phase-contrast micrographs (not shown), these fluorescent particles appear to be located between chromosomes. To decide whether the 40,000-mol-wt antigen is bound to the chromosome structure, we have analysed chromosome spreads labeled with the same antibodies. We have never observed a similar punctate pattern in those preparations.

Actinomycin D stops elongation of ribosomal transcripts. We exposed HeLa cell monolayers to 0.1 μg/ml actinomycin D for 1 h before processing the slides for immunofluorescence microscopy. Nucleoli appeared intact in phase-contrast after this treatment. The fluorescence pattern had changed for both the 110,000- and 94,000-mol-wt antigens in such a way that the nucleolar interior was no longer stained but instead increased intensity accumulated at the nucleolar periphery and in the surrounding nucleoplasm (Fig. 4, *e* and *f*). No major effect was observed for anti-40,000 antibodies. We conclude from this result that the 110,000- and 94,000-mol-wt polypeptides are either directly or indirectly associated with components of the ribosomal transcription units and are released upon transcription arrest.

We have confirmed the sites for the 110,000- and the 94,000-mol-wt antigens in the interior of the nucleoli using the indirect immunoperoxidase technique and electron microscopy. In both cases nucleoli were marked with an osmophilic precipitate rather homogenous for 110,000-mol-wt antigens but slightly more patchy for 94,000-mol-wt antigens (Fig. 5). The staining pattern for 40,000-mol-wt antigens is remarkably different in that the antibodies predominantly bind to the peripheral zone of nucleoli and enhance an often well-defined granular substructure. To study the nucleolar distribution of the three antigens at higher resolution, labeling thin sections with antibody gold complexes would seem most appropriate. However, the embedding procedure must have

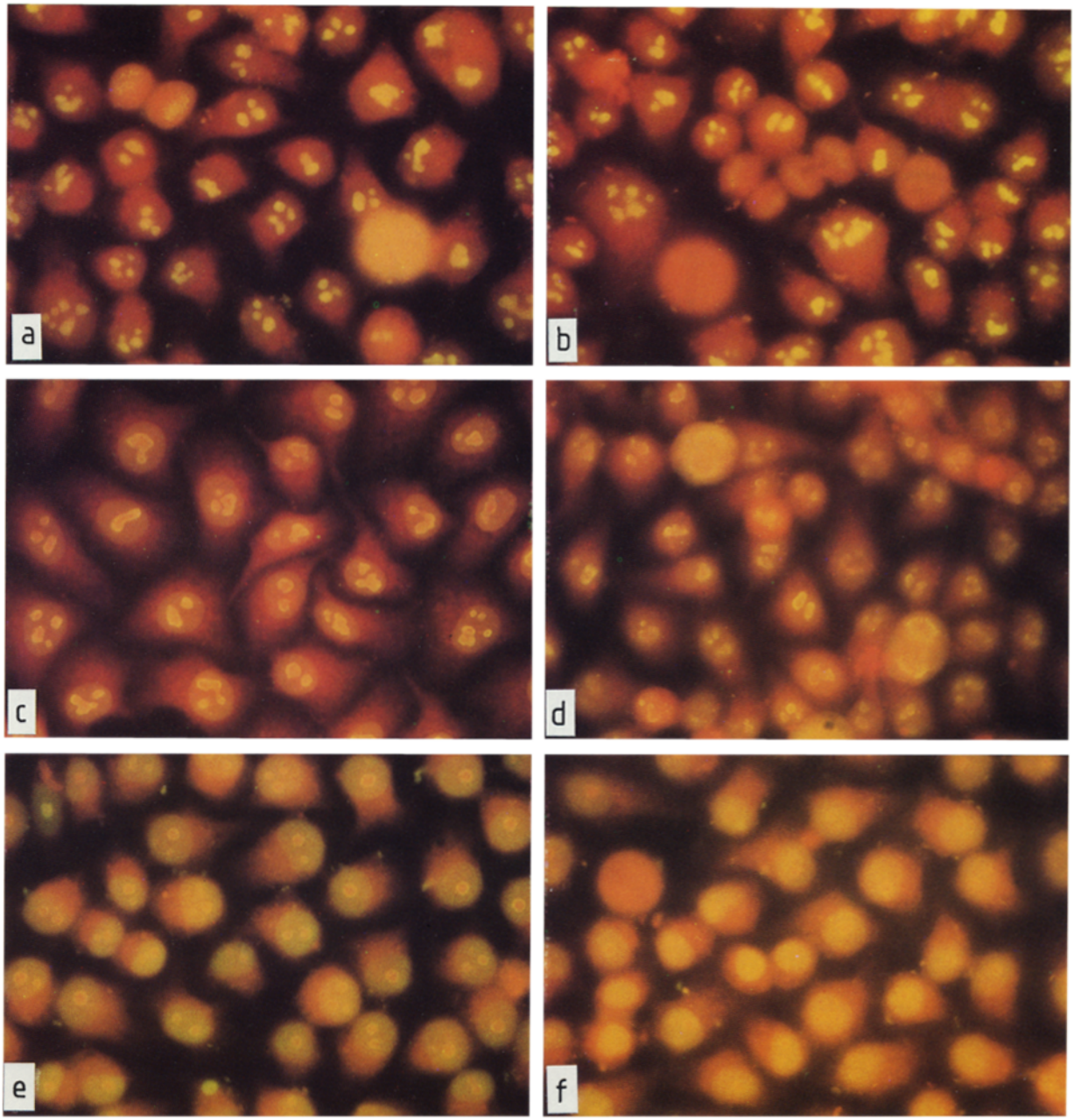

FIGURE 4 Immunofluorescence micrographs of HeLa cell monolayers. (*a*) Anti-110,000; (*b*) anti-94,000; (*c* and *d*) anti-40,000; (*e*) anti-110,000 after treatment with 0.1 μg/ml actinomycin D for 1 h; (*f*) anti-94,000 after treatment with 0.1 μg/ml actinomycin D for 1 h. The FITC signal appears yellow; the Evan's Blue stain appears red.

altered the epitopes on all three nucleolar proteins as labeling above background levels was never observed. Neither did labeling isolated nuclei or nucleoli with antibody gold complexes before embedding yield satisfactory results because the dense chromatin network appeared to inhibit diffusion of the immunogold particles.

Solubility of Nucleolar Antigens

Proteins which resist extraction with high salt may participate in DNA folding and serve skeletal functions (10, 11, 13). None of the three nucleolar antigens under investigation are contained in insoluble material after 2 M NaCl extraction (Table I). Therefore, they do not appear to be components of a nucleolar scaffold.

The 94,000-mol-wt antigen was insoluble in a lysis mixture containing 100 mM NaCl, but was readily solubilized when RNase-digested nuclei were extracted under the same conditions (Table I). Similarly, for the 110,000-mol-wt antigen, predigestion of nucleoli with RNase but not with DNase drastically increased the release of this protein in lysis buffer that contained 400 mM NaCl (Fig. 6). Only salt concentrations higher than 400 mM rendered the 40,000-mol-wt peripheral component fully soluble and no major effect on extractability was observed with nucleases.

We take the RNase dependent solubility as evidence that the two polypeptides of 94,000- and 110,000-mol-wt are components of the ribonucleo-protein fibers or interact with the latter. This is consistent with the earlier conclusion, drawn from the actinomycin D–induced changes in immunofluores-

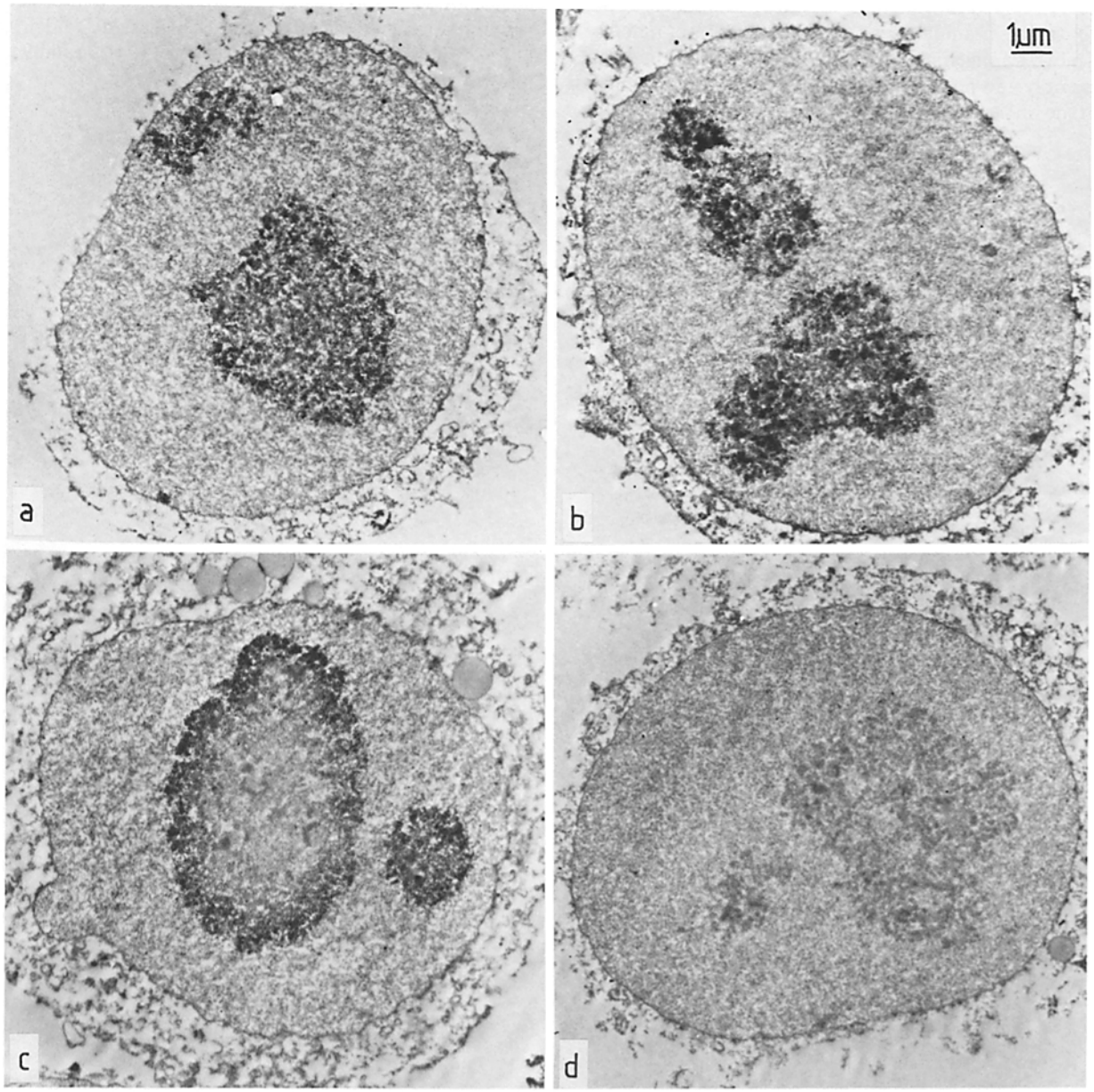

FIGURE 5 Electron micrographs of immunoperoxidase-labeled HeLa cells. (a) Anti-110,000; (*b*) anti-94,000; (c) anti-40,000; (*d*) medium.

cence pattern, that these two antigens are associated with the ribosomal transcription units.

DISCUSSION

Over 100 nucleolar proteins of mammalian cell origin have been identified (19, 21, 22, 23). Many of these have been assigned to different groups of nucleolar proteins as a function of their solubility in increasing ionic strength. Vague correlations between solubility and function of these proteins have been made (25). Only a few have been purified to homogeneity and further characterized (21).

Using the "shotgun" monoclonal antibody approach, we have identified three nucleolar antigens and have determined their molecular weights, cellular distributions, nuclease dependent solubilities, and actinomycin D–induced rearrangements. Both the 94,000- and 40,000-mol-wt antigens are significantly enriched in the polyamine nucleolar fraction. In contrast, the major stained band at 110,000 apparent molecular weight appears not to be enriched in isolated nucleoli. The antigen recognized by hybridoma H10-C10-D10 may therefore be represented by a minor stained band at the same location on the gel. This is supported by the observation that immunoblots of the upper gradient band fraction show only trace amounts of this protein. Antibodies to all three antigens strongly light up nucleoli by fluorescence light microscopy. None of the three antigens appears to be part of mature ribosomes since the interphase cytoplasm shows no fluorescence signal and immunoblots of isolated HeLa polysomes were negative. However, this does not exclude the possibility that the antigens may have lost their antigenic determinants due to proteolytic cleavage associated with the ribosome maturation.

At higher resolution using indirect immunoperoxidase labeling and electron microscopy, the 110,000- and 94,000-mol-wt antigens were localized in the nucleolar interior,

whereas the 40,000-mol-wt antigen was preferentially accumulated at the granular periphery. We propose that the 40,000-mol-wt antigen may be involved in pre-ribosome particle assembly and possibly in the transport of pre-ribosomes through the nucleoplasm to the nuclear envelope. The results from electron microscopy, the actinomycin D-induced change of the fluorescence pattern, and the RNase-dependent solubility lead us to the conclusion that the 110,000- and 94,000-mol-wt proteins are components of, or interact with, the ribonucleoprotein fibers. Consistent with this, it has been suggested earlier that the more soluble nucleolar proteins appear to be involved in synthesis and processing of pre-ribosomal RNA (25).

Another protein with 110,000 apparent molecular weight has been isolated from nucleoli (15, 29) and designated C23. Its presence in nucleoli and nucleolar organizer regions on chromosomes has been demonstrated by immunochemical methods (14). It has been suggested that C23 binds specifically to ribosomal DNA sequences and is part of the transcriptional complex (18). We have no evidence for DNA binding specificity of our 110,000-mol-wt antigen, and have failed to label nucleolar organizer regions using immunomicroscopy techniques. Therefore we can not comment on the possible identity of these proteins.

HeLa mitotic cell cytoplasm stains with anti-110,000 and anti-40,000 antibodies. This suggests that the 110,000- and 40,000-mol-wt antigens are solubilized in the cytoplasm dur-

TABLE I
Solubility of Nucleolar Antigens

	anti-110,000		anti-94,000		anti-40,000	
solution 4	P++		P++		P++	
100 mM	P++		P++		P++	
DNase/100 mM	P++		P++		P++	
RNase/100 mM	P++			S++	P++	
400 mM	P+	S+		S++	P+	S+
DNase/400 mM	P+	S+		S++	P+	S+
RNase/400 mM		S++		S++	P+	S+
2 M		S++		S++		S++

Immunoblots of extracted polyamine nucleoli like those represented in Fig. 6 were analysed; the results for the three nucleolar antigens are summarized in this table. (*P++*) Insoluble; (*S++*) soluble; (*P+* and *S+*) partially soluble. Concentration values are for NaCl.

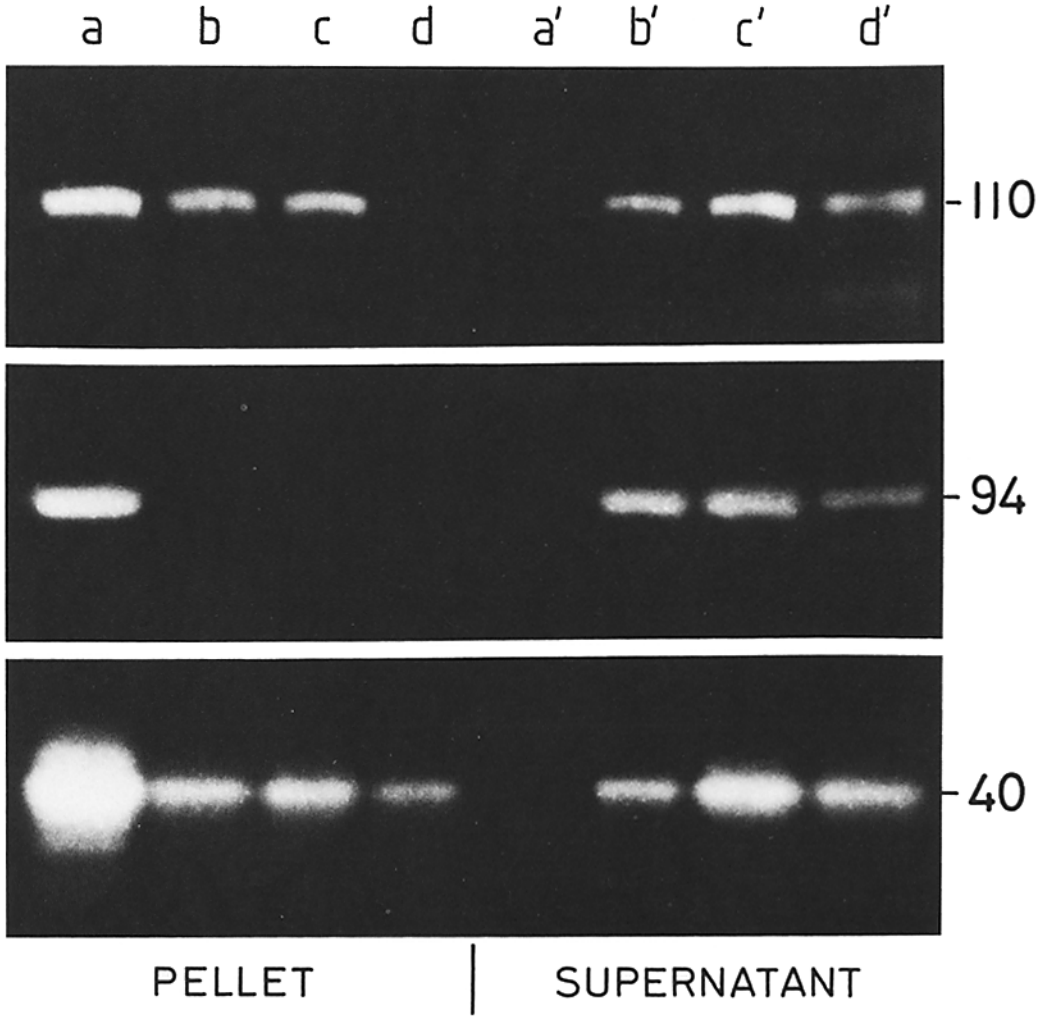

FIGURE 6 Solubility of nucleolar antigens. Polyamine nucleoli untreated or predigested with DNase I or RNase A were extracted with lysis buffer containing 400 mM NaCl. After centrifugation, proteins in the pellets and supernatants were separated by SDS PAGE. Each lane contained material derived from 0.5 A^{260} U of nucleoli. The immunoblots are shown for each of the three nucleolar antigens. (*a* and *a'*) Control in sol. 4. No antigen appears in the supernatant; (*b* and *b'*) extraction in lysis buffer containing 400 mM NaCl without predigestion; (*c* and *c'*) extraction with 400 mM NaCl after digestion with 40 μg/ml DNase I; (*d* and *d'*) extraction with 400 mM NaCl after digestion with 10 μg/ml RNase A.

ing mitosis, and that they partition into the daughter cells upon division. In contrast, we have never observed cytoplasmic fluorescence in mitotic cells using antibodies directed against the 94,000-mol-wt antigen although this protein is the most abundant in polyamine nucleoli. This protein may be modified during mitosis such that the antigenic site is no longer accessible to antibodies. Alternatively, it may be degraded and synthesised *de novo* during the reassembly of nucleoli in the daughter cells.

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