Nucleolus-like Morphology Produced During the In Vitro Reassociation of Nucleolar Components

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Abstract. Nucleoli, the sites of rRNA synthesis, rRNA processing, and the assembly of ribosomes, are dynamic organelles that, in most cells, disperse and reform during mitosis. The mechanisms that regulate nucleolar formation are unknown as is the relationship between nucleolar morphology and the pathway of ribosome biogenesis. In this report we describe the in vitro formation of nucleolus-like particles (NLPs) from soluble extracts of nucleoli. NLPs, which reached sizes comparable to nucleoli (1–3 μm), were found to contain 40% of the nucleolar DNA, RNA, and protein.

The ultrastructure of NLPs resembled that of a number of in vivo structures including compact nucleoli, prenucleolar bodies, and pseudonucleoli. The particles were composed of two morphologically distinct regions. The core resembled the dense fibrillar component (DFC) of nucleoli while the cortex resembled the granular component (GC) of nucleoli. The cortex of NLPs contained numerous 15–20 nm osmophilic granules that resembled the preribosomes found in the GC of nucleoli.

The distribution of nucleolar proteins in NLPs also resembled that in nucleoli. BN46/51, a component of the GC of nucleoli, was restricted to the GC-like cortex of NLPs. A mAb that bound to the DFC of nucleoli, bound only to the DFC-like core of NLPs while a second mAb that bound to both the DFC and GC of nucleoli, bound to both the core and cortex of NLPs. Thus solubilized components of nucleoli can reassociate in vitro to produce particles that resemble nucleoli in their size, ultrastructure, and protein distribution.

NUCLEOLI are a ubiquitous feature of eukaryotic cell nuclei. They are the sites of rRNA synthesis, rRNA processing and the assembly of ribosomes (reviewed in 4, 16, 19, 43, 45, 57). Almost all nucleoli share a common set of ultrastructural features and a similar pattern of assembly and disassembly during mitosis. Typically, nucleoli disperse at prophase with some nucleolar components associating with the condensing chromosomes while other components are released to the cytoplasm. During telophase nucleoli reform as the daughter nuclei assemble (reviewed in 3, 9, 25, 33). Reformation of typical nucleoli requires the reactivation of rRNA transcription. If transcription is blocked after mitosis, nucleolar components reassociate in the nucleus into complexes described as prenucleolar bodies or nucleolus-like bodies (39, 49).

The biochemical basis for the assembly and disassembly of nucleoli is not understood. The fact that the phosphorylation state of nucleolin, a nucleolar protein that is associated with pre-rRNA, changes during the mitotic cycle suggests that phosphorylation may play a role in regulating this process (1).

The relationship between nucleolar morphology and nu-cleolar function is also unclear (reviewed in 19, 57). It is generally agreed that the granular component of nucleoli represents a region where ribosomes are assembled and from which they pass out of the nucleolus (18, 19). The presence of 15–20 nm osmophilic granules that resemble cytoplasmic ribosomes is consistent with this view. The location of rDNA and the site of transcription are, however, controversial. The fibrillar centers (FC)[1] are frequently described as the site of rDNA and sometimes as the site of rRNA transcription (reviewed in 43, 57). However, this interpretation has been challenged by Wachtler and his colleagues who find rDNA and rRNA transcription in the DFC (47, 54, 55). Thus, even at this fundamental level, the organization of nucleolar function is unclear.

The role of rRNA in nucleolar morphology is also uncertain. Karpen, Schaefer, and Laird (29) demonstrated that a single rRNA gene inserted into various euchromatic regions of Drosophila chromosomes could give rise to a nucleolus as viewed in the light microscope. This could be interpreted to mean that nucleoli simply represent sites of rRNA synthesis (57).

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1. *Abbreviations used in this paper:* DFC, dense fibrillar component; FC, fibrillar center; GC, granular component; HSS(s), high speed supernatant(s); NLPs, nucleolus-like particles.

There are, however, a number of instances where nucleolar components associate to produce characteristic features of nucleolar morphology in the absence of rRNA synthesis and even in the absence of rDNA (33). Mini cells lacking nucleolar organizer regions contain nucleolus-like bodies (23, 24). Cells of the anucleolate mutant of *Xenopus laevis* contain pseudonucleoli (21, 26). Cells of the early embryo in many species contain structures that resemble nucleoli at a stage before transcription of rRNA has begun (4, 28, 36). Thus, fibrillar centers, the granular component and the dense fibrillar component may reflect an underlying organization independent of rRNA transcription.

The recent report that prenucleolar bodies can form during the assembly of nuclei in extracts of *Xenopus* oocytes suggest that nucleogenesis may be amenable to study in vitro (2). In this report we describe the formation of NLPs in soluble extracts of nucleoli. Our results support the concept that specific protein–protein interactions provide the basis for the morphology of the dense fibrillar component and the granular component of nucleoli. They also provide a system in which the molecular basis for nucleolar morphology can be analyzed in vitro.

Materials and Methods

Cell Culture and Isolation of Nucleoli

Naegleria gruberi strain NB-1 (11) was grown on NM plates with *Klebsiella pneumoniae* as described previously (12). Nucleoli were isolated by lysing cells in Triton X-100 containing buffer (53) or by sonication of isolated nuclei (56).

The growth of amebae in the presence of 40 μg/ml actinomycin D was accomplished by growing suspensions of amebae on washed *K. pneumoniae* as described by Fulton and Guerrini (13).

Nucleoli prepared by sonication were separated from residual cysts and bacteria by banding on gradients of Percoll (Pharmacia Biotechnology, Inc., Piscataway, NJ). Nucleoli from 2–4 × 10⁸ cells were suspended in 3 ml of 70% Percoll in SPMG buffer (62.5 mM sucrose, 25 mM NaH₂PO₄, 2.5 mM MgCl₂, 0.5 mM EGTA, pH 7.2) and centrifuged at 48,000 g for 30 min in a Sorvall SS34 rotor. The white-fluffy band of nucleoli near the top of the gradient was removed with a Pasteur pipette. Nucleoli were washed by dilution to 3 ml with SPMG buffer and centrifugation at 1,500 g for 5 min.

Preparation of Nucleolus-like Particles

Nucleoli were extracted for 30 min in ice-cold 0.4 M NaCl in SPMG buffer at a concentration of 4 × 10⁸/ml. Insoluble material was removed by centrifugation at 15,000 g for 10 min. The extract was dialyzed against SPMG buffer for 1.5 h. In some experiments NaCl extracts of nucleoli were centrifuged at 436,000 g for 60 min before dialysis.

Analysis of DNA, RNA, and Protein

Nucleoli, isolated on Percoll gradients, and the NLPs prepared from them, were analyzed by cold HClO₄ precipitation, extraction of RNA by KOH and extraction of DNA with hot HClO₄ (35). RNA was quantified by absorbance at 260 nm (35), DNA by a modification of the diphenylamine assay (15), and protein by dye binding (Bio-Rad Laboratories, Richmond, CA) with gamma globulin as a standard.

Electron Microscopy

Suspensions of nucleoli and NLPs were fixed by adding an equal volume of ice-cold 2% glutaraldehyde in SPMG. Amebae were fixed by adding an equal volume of ice-cold 2% glutaraldehyde in 100 mM NaH₂PO₄, pH 7.2. After 30 min in ice, samples were pelleted by centrifugation at 15,000 g for 10 min for nucleoli and NLPs or for 2 min at a setting of 7 in a Clinical Centrifuge for amebae. Pellets were washed twice by overlaying with ice-cold 50 mM NaH₂PO₄, pH 7.2 for 15 min. For conventional electron microscopy, samples were postfixed in 1% OsO₄ in PBS in ice for 30 min, dehydrated through ethanol followed by propylene oxide, and embedded in Spurr's resin (48). Silver sections were stained with 1% aqueous uranyl acetate for 10 min followed by Reynold's lead citrate for 5 min (30).

For immunoelectron microscopy, samples were dehydrated through ethanol and embedded in LR White at 43°C for 48–60 h. Gold sections were blocked, washed and incubated with antibodies as described by Wright and Rine (58). Sections were incubated with primary antibodies for 60 min at 37°C. Sections were incubated with secondary antibodies against mouse IgG coupled to 10 nm colloidal gold for 60 min at 37°C. After washing to remove unbound antibody, sections were stained with uranyl acetate and lead citrate as described above.

Primary antibodies were the mouse monoclonals BN5.1, directed against BN46/51 (53), AH6 and DE6. AH6 and DE6 were obtained by immunizing mice with NLPs prepared from a 15,000 g supernatant of nucleoli isolated by detergent lysis (53). Hybridomas were obtained and screened as previously described (53).

Light Microscopy

Amebae and NLPs were fixed in 0.9% formaldehyde as previously described for amebae (53), air dried onto slides, stained with BN5.1, AH6 or DE6 followed by a rhodamine labeled antimouse-IgG second antibody and DAPI at 0.1 μg/ml.

Electrophoresis and Immunoblotting

Proteins were fractionated on 7.5% SDS gels (31) and visualized by staining with the silver reaction (34) or by immunoblotting (52) as described previously (53).

DNA Isolation and Southern Blotting

DNA was isolated from nucleolar fractions after RNase and Proteinase K digestion (44). After digestion with BamHI to linearize the ribosomal plasmid (7), equivalent amounts of DNA were fractionated on 0.7% agarose gels (44) and blotted to GeneScreen Plus (New England Nuclear, Boston, MA). Ribosomal plasmid sequences were identified by hybridization with ribosomal plasmid from *Naegleria gruberi* strain NEG (7) labeled by random priming.

Results

Nucleolus-like Particles Form in Nucleolar Extracts

Amebae of the amebo-flagellate *Naegleria gruberi* have a single, large, central nucleolus (11). Nucleolar fractions can be prepared simply by extracting amebae with nonionic detergent followed by low speed centrifugation. The fragile Naegleria nucleus is disrupted during cell lysis and the pellet is highly enriched in nucleoli (53, 56). When nucleolar pellets are suspended in SPMG buffer containing 0.4 M NaCl, the nucleoli are rapidly dispersed. About 80% of the nucleolar protein is solubilized (Table I) including virtually all of the nucleolar antigen BN46/51 (53). Centrifugation of NaCl extracts at 15,000 g for 10 min removes insoluble nucleolar material including about half of the DNA, Table I.

During characterization of BN46/51 it was observed that NaCl extracts of nucleoli became turbid when they were dialyzed against buffer without NaCl. The turbidity was found to be due to the formation of a large number of 1–3 μm spherical particles (Fig. 1). The size of these particles approached that of intact nucleoli (compare Fig. 2, *a* with *g*, or *b* with *h*). These particles contained both BN46/51 and DNA as judged by fluorescence microscopy (Fig. 1).

When the particles were examined in the electron microscope they were found to be composed of two concentric layers (Fig. 2, *a–c*). The outer layer resembled the GC of

Table I. The Composition of Nucleoli and Nucleolus-like Particles

Sample	DNA	RNA	Protein
		μg per 1 × 10⁸ nucleoli or equivalent	
Total nucleoli*	12.3 ± 4.9	105.3 ± 11.5	396.6 ± 132.2
		Extracts prepared by centrifugation at 15,000 g	
NaCl insoluble*	6.6 ± 4.8 (53.3)‡	39.2 ± 6.5 (37.2)	87.3 ± 23.9 (22.0)
NLPs*	5.0 ± 1.4 (40.9)	42.8 ± 7.1 (40.7)	167.9 ± 90.2 (42.3)
NLP supernatant*	0.1 ± 0.1 (0.8)	20.5 ± 4.3 (19.4)	52.6 ± 50.0 (13.3)
		Extracts prepared by centrifugation at 436,000 g	
NaCl insoluble§	9.7 ± 0.3 (78.9)	70.0 ± 8.8 (66.5)	149.5 ± 29.4 (37.7)
NLPs	0 (0)	12.2 (11.6)	40.5 (10.2)
NLP supernatant	0.1 (0.1)	15.2 (14.4)	55.4 (14.0)

Nucleoli, prepared by sonication of isolated nuclei followed by banding on a Percoll gradient, were dissociated in 0.4 M NaCl-SPMG buffer. NaCl insoluble material was removed by centrifugation for 10 min at 15,000 g or 60 min at 430,000 g. The supernatants were dialyzed against SPMG to produce NLPs. NLPs were pelleted by centrifugation at 15,000 g for 10 min. The supernatants from sedimentation of the NLPs are designated NLP supernatants. DNA, RNA, and protein were isolated by HClO₄ precipitation and analyzed as described under Methods.
* Average of three or more separate preparations plus and minus SD. The variability of these data is partially a reflection of the difficulty in estimating the number of nucleoli recovered from Percoll gradients, this procedure produces extensive clumping, and variability in the extent of sonication. The low values for NaCl insoluble material from 436,000 g pellets reflects the difficulty in recovering this material.
‡ Percent of total nucleoli.
§ The average of two separate preparations for NaCl insoluble material and the value for one preparation for NLPs and NLP supernatant.

nucleoli (Fig. 2, h). It consisted of 15–20 nm granules embedded in a loose fibrous matrix. The core of the particles was composed of a relatively homogeneous, osmophilic, finely granular material that resembled the DFC of nucleoli, Fig. 2 h.

The resemblance between the particles and nucleoli was even more apparent when they were compared to nucleoli isolated by detergent lysis. Naegleria nucleoli fixed in situ show a compact structure in which the DFC is visible as irregular islands surrounded by the GC while the FC are pres-
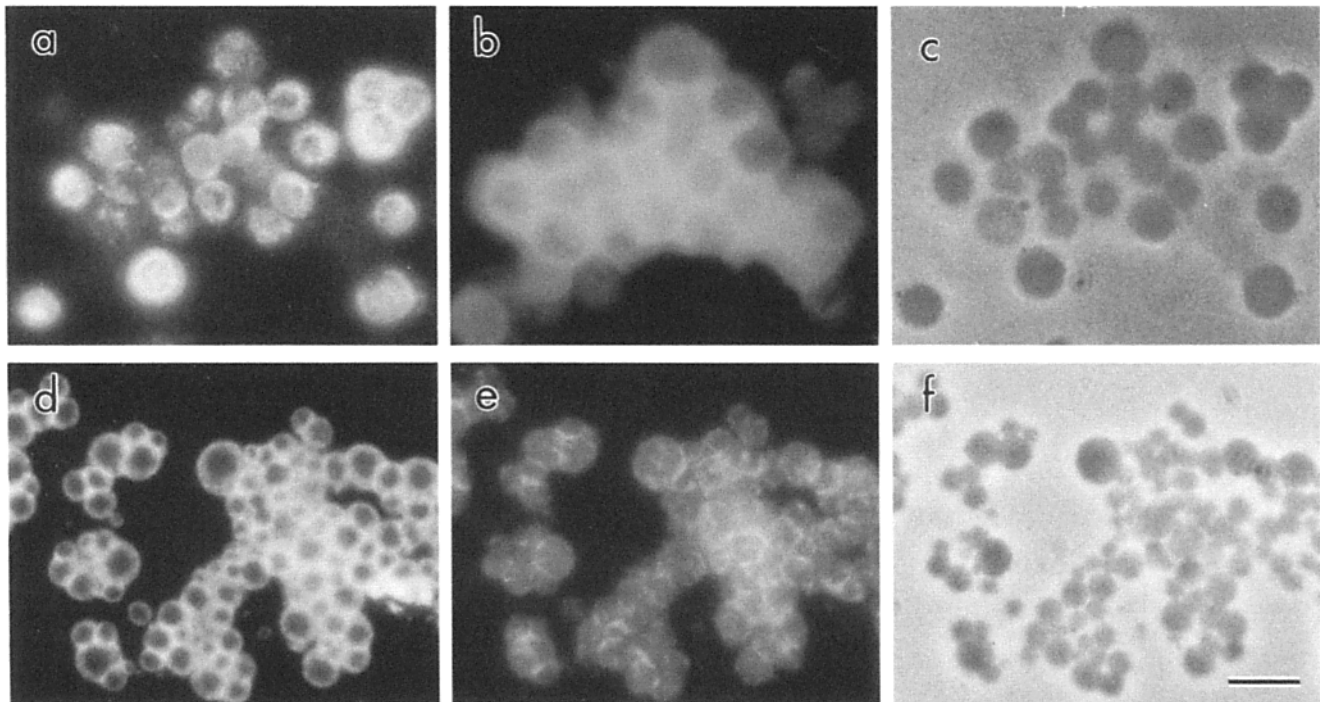

Figure 1. Phase contrast and fluorescence microscopy of nucleoli and particles formed in vitro. Nucleoli were isolated from Naegleria amebae by detergent lysis. Isolated nucleoli were extracted with 0.4 M NaCl in SPMG buffer and the insoluble material was removed by centrifugation at 15,000 g for 10 min. The NaCl soluble material was dialyzed against SPMG buffer without NaCl for 1.5 h to produce nucleolus-like particles (NLPs). Nucleoli and NLPs were fixed in buffer with formaldehyde (MgCF) (53), dried onto slides, stained with BN5.1, a mAb against the nucleolar protein BN46/51, and then with a rhodamine conjugated antibody against mouse IgG followed by the DNA specific dye DAPI. (a, b and c) Isolated nucleoli. (d, e, and f) Particles formed in vitro. (a and d) Binding of BN5.1. (b and e) DAPI staining. (c and f) Phase contrast. All parts are the same magnification. Bar in f, 5 μm.

Figure 2.

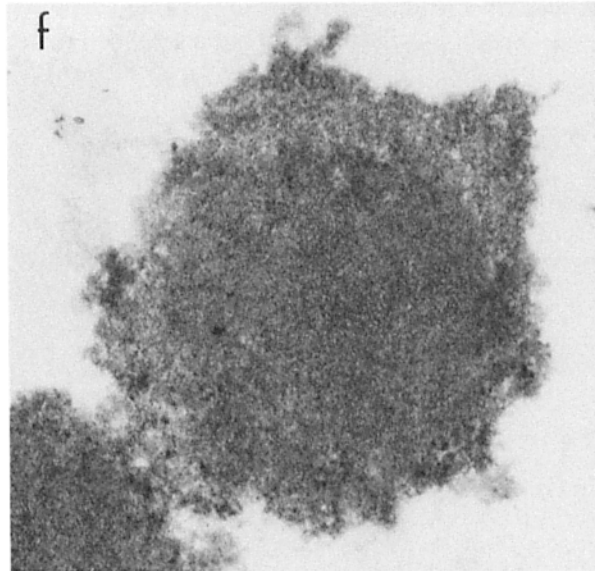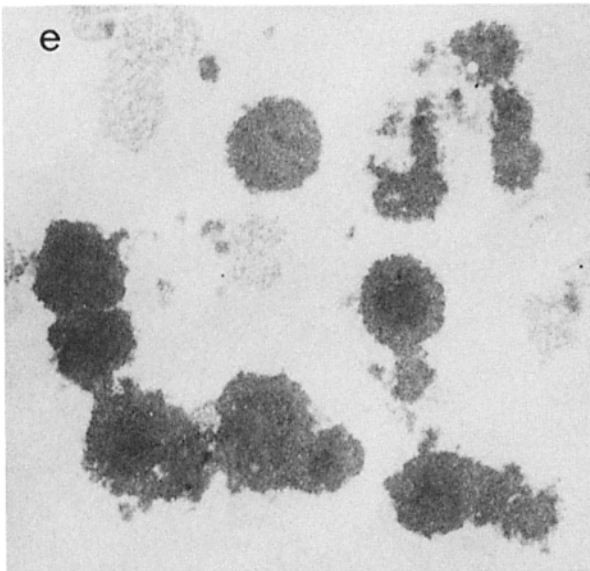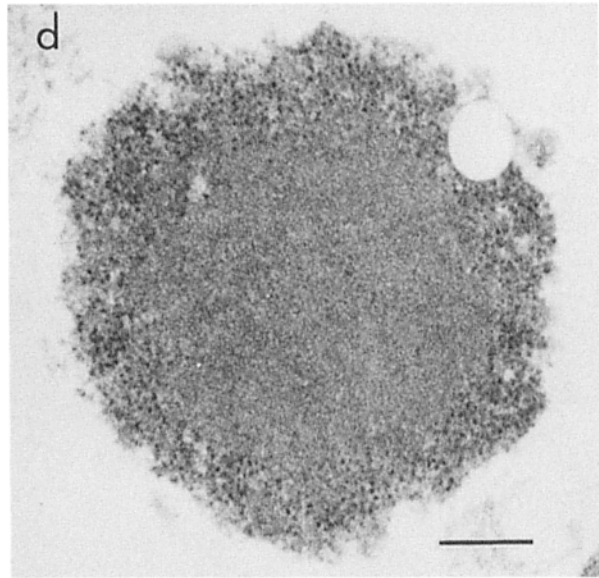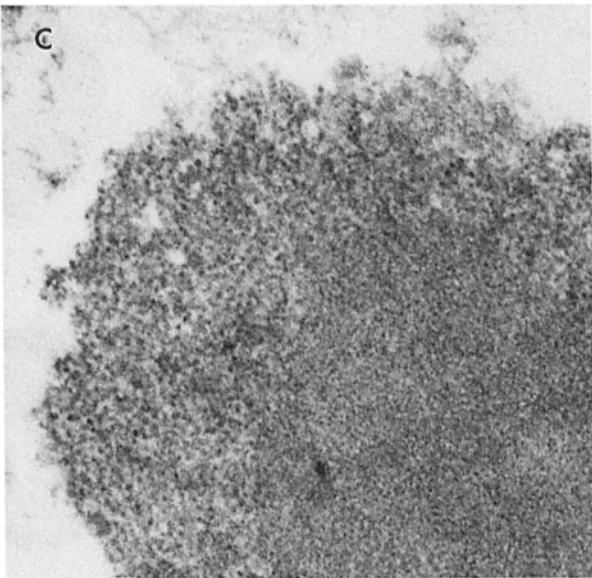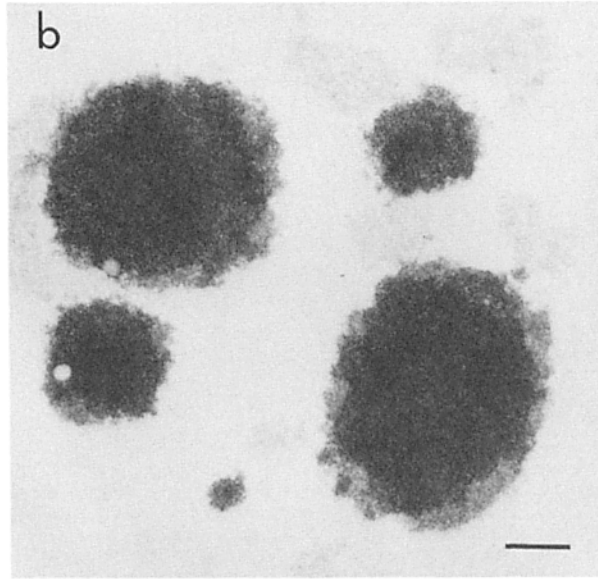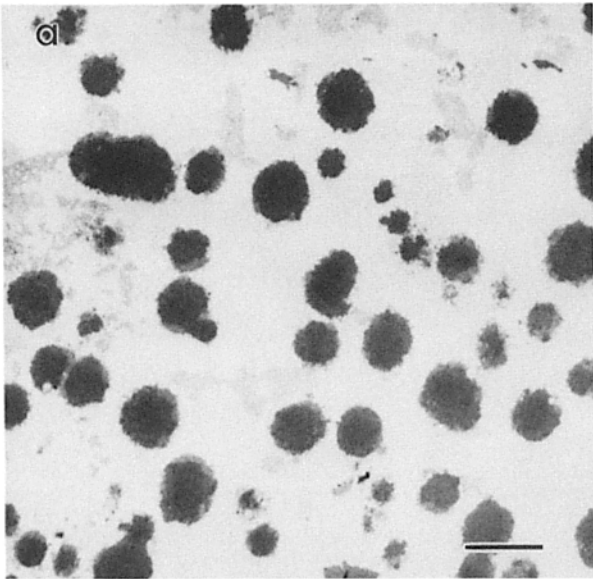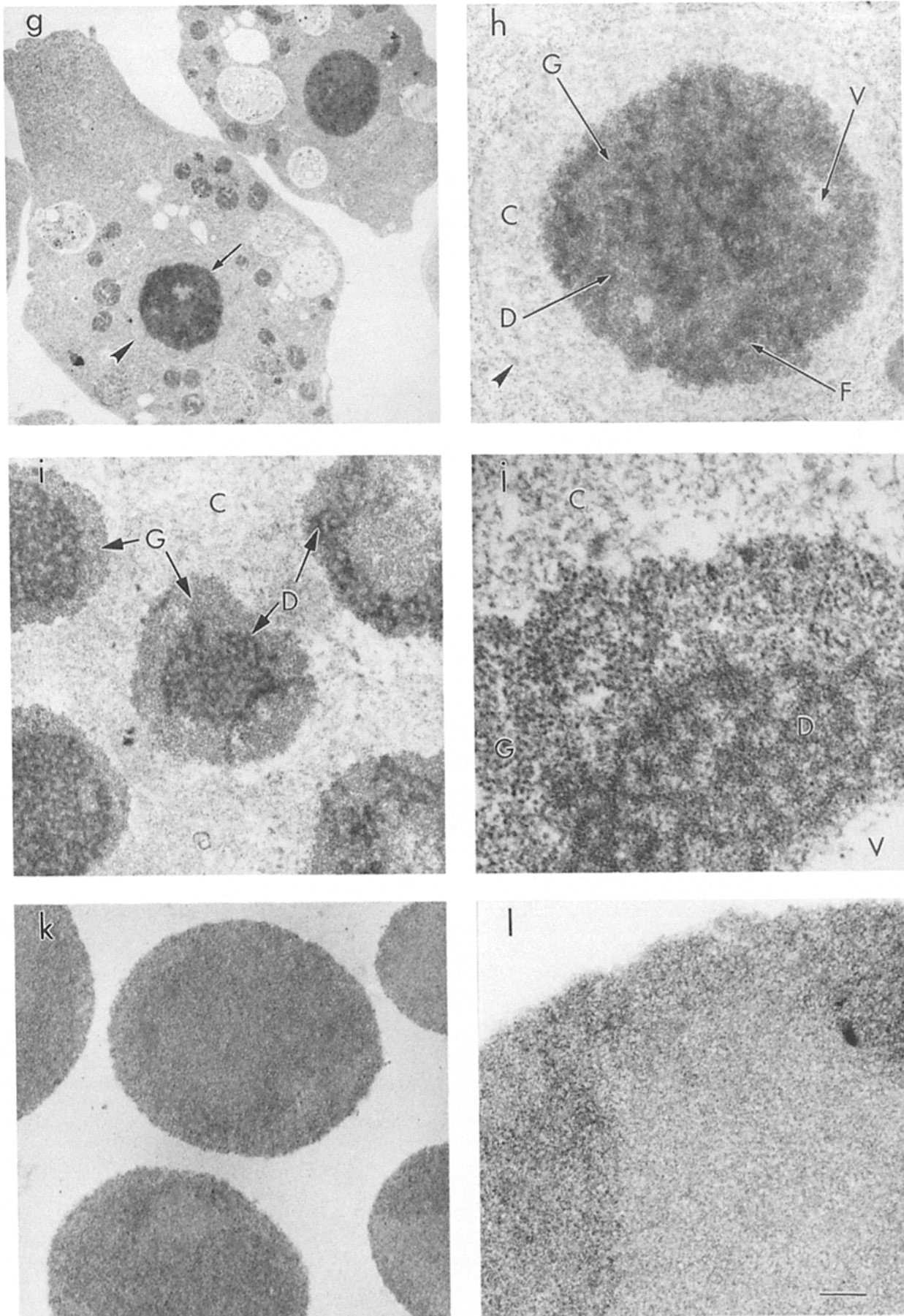Figure 2.

ent as small patches within the DFC, Fig. 2 *h*. Isolated nucleoli show a sharper distinction between the GC and the DFC, presumably because of material lost during the isolation procedure (see for example Fig. 11.7 in Busch and Smetana [4]). The GC of isolated nucleoli, Fig. 2, *j*, was very similar to the cortex of the particles (Fig. 2, *c*) and the DFC of isolated nucleoli had the same fine granular, osmophilic structure as the core of the particles (cf., Fig. 2, *c* and *j*).

In isolated Naegleria nucleoli, the DFC is more evident as a nucleolonema (27) distributed in a layer through the GC. In peripheral sections the DFC has a lace-like arrangement while in more central sections the DFC is seen as a band with a chain-like organization, Fig. 2 *i*. The DFC appears to exist as a shell of dense strands surrounded by the GC on both sides.

In isolated nucleoli, the GC frequently surrounds a large, central electron lucent region (Fig. 2 *j*). This central space is not seen in electron micrographs of intact nucleoli but nucleoli do contain one or more electron lucent vacuoles which may give rise to this feature on extraction, Fig. 2 *g* and *h*. In contrast to isolated nucleoli, the core of the particles formed in vitro was homogeneous. However, the particles did contain clear vacuole-like inclusions ranging from a few nm up to 0.5 μm in diameter, usually in the periphery (Fig. 2, *b* and *d*).

There is a striking resemblance in both size and ultrastructure between the particles formed in vitro and a variety of structures described in vivo. For example, the particles resemble the classical definition of a compact nucleolus (see Fig. 16 in Fawcett [10] and the discussion, p. 440, in Busch and Smetana [4]). The in vitro particles also resemble the extrachromosomal nucleoli of amphibian oocytes (21, 26, 32) and a variety of in vivo structures referred to as nucleolus-like bodies (39, 40, 49, 51), nucleolus-like "blobs" (23), or prenucleolar bodies (17, 37, 50). For convenience, we have adopted the terminology used by Swift and Stevens (51) and Stevens and Prescott (49). We will refer to the in vitro particles as nucleolus-like particles or NLPs.

In the experiments described above, NLPs were prepared by dialysis of 15,000-g supernatants. However, NLPs were also formed if NaCl extracts were subjected to high speed centrifugation (436,000 *g* for 60 min) before dialysis. (Based on the clearance factor for the rotor used, this should be sufficient to pellet material greater than ~8S.) The size of NLPs prepared from high speed supernatants (HSSs) was re-

duced, perhaps reflecting the decrease in protein concentration (see below).

The ultrastructure of the NLPs prepared from HSSs differed from that of NLPs prepared from 15,000 g supernatants (Fig. 2, *k* and *l*). The particles were composed of two regions that differed slightly in their staining intensity. Both regions lacked the 15–20 nm granules seen in NLPs prepared from 15,000 g supernatants. The two regions were both granular and organized in irregular patches with the more densely staining material usually found at the surface as a crescent shaped region when seen in section.

NLPs Contain At Least Three Nucleolar Proteins

SDS gel electrophoresis demonstrated that the protein composition of NLPs was complex. All of the major polypeptides in the 0.4 M NaCl extract of nucleoli appeared to be represented in NLPs prepared from 15,000 g supernatants (Fig. 3, lane 2). The protein composition of NLPs prepared from HSSs, although still complex, was simplified compared to nucleoli or NLPs from a 15,000 g supernatant (Fig. 3, lane 3).

To follow individual polypeptides, mAbs were prepared against NLPs. Two cell lines, AH6 and DE6, were identified that produced antibodies that bound to Naegleria nucleoli. As illustrated in Fig. 4, DE6 binding was confined to the nucleolus but AH6 showed a low level of binding to the nucleoplasm as well. AH6 identified polypeptides of 125 kD, 74 ± 2 kD, 60 ± 2 kD, 35 kD and 29 kD present in both isolated nucleoli and in NLPs (Fig. 3, lanes *10* and *11*). The 74-kD and 60-kD polypeptides gave M_r values that varied slightly from one preparation to another. The above values represent the average of 11 determinations (± SD) as measured by silver staining and immunoblotting. The reason for the variability of these polypeptides is unknown. DE6 bound to polypeptides of 44 ± 2 kD and 31 ± 2 kD present in both fractions (Fig. 3, lanes *7* and *8*).

NLPs prepared from HSSs were significantly enriched in both the 46- and 51-kD subunits of BN46/51 (Fig. 3, lane *6*). They were also enriched in the 44-kD form of the DE6 antigen relative to the 31-kD form (Fig. 3, lane *9*). The 125-kD form of the AH6 antigen, a relatively minor component of nucleoli, was also enriched in HSS NLPs (Fig. 3, lane *12*).

In contrast to BN5.1, the mAb that identified BN46/51 (53), both AH6 and DE6 also bound to nucleoli in *Saccharomyces cerevisiae* and mammalian cells (data not shown). The DE6 antigen in yeast is a polypeptide of ~36 kD. Based on

Figure 2. The ultrastructure of Naegleria amebae, nucleoli, isolated nucleoli and particles formed in vitro. Amebae, nucleoli isolated by detergent lysis of amebae and particles produced by dialysis of 0.4 M NaCl extracts of nucleoli were fixed in glutaraldehyde, postfixed with OsO₄, and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate. (*a, b*, and *c*) Particles prepared by dialysis of a 15,000 g nucleolar supernatant against buffer without NaCl. Note the central core of dense material and the peripheral region containing 15–20 nm granules. (*d*) A particle prepared from an extract treated with DNase before dialysis. (*e* and *f*) particles prepared from an extract treated with RNase before dialysis. Note the greatly decreased number of 15–20 nm granules. (*g*) Amebae. Note the large central nucleolus in each cell (*arrow*) surrounded by a rim of nucleoplasm. The nuclear membrane is indicated by the arrowhead. (*h*) A nucleolus fixed in situ. Note the granular component (*G*), dense fibrillar component (*D*), fibrillar centers (*F*), vacuoles (*V*), and the surrounding nucleoplasm containing chromatin (*C*). The location of the nuclear membrane is indicated by the arrowhead. (*i* and *j*) isolated nucleoli. Note the arrangement of the DFC (*D*), the GC (*G*) and the chromatin (*C*) still associated with nucleoli isolated by detergent lysis. (*k* and *l*) Particles prepared by dialysis of a 436,000 g nucleolar supernatant against buffer without NaCl. Note the absence of 15–20 nm particles and the concentration of more electron dense material at the periphery. *a* and *g* are at the same magnification. *b, e, h, i*, and *k* are at the same magnification. *c, d, f*, and *j* are at the same magnification. Bars: (*a*) 2 μm; (*b*) 0.5 μm; (*d*) 0.2 μm; (*l*) 0.1 μm.
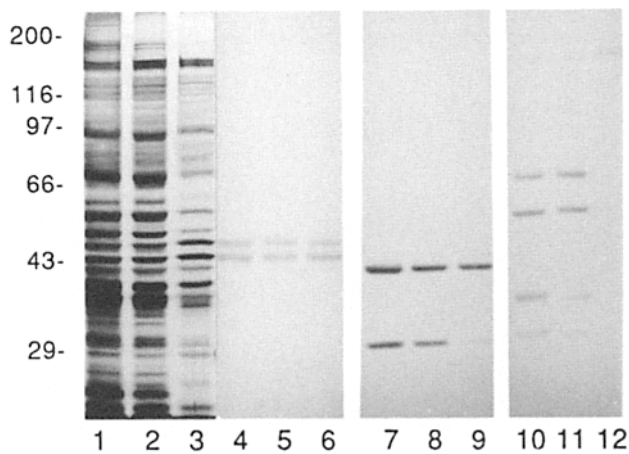
Figure 3. The protein composition of isolated nucleoli and NLPs. Proteins from nucleoli isolated by the detergent lysis method (lanes *1, 4, 7*, and *10*); NLPs pepared from a 15,000 g supernatant (lanes *2, 5, 8,* and *11*); and NLPs prepared from a 436,000 g supernatant (lanes *3, 6, 9,* and *12*) were fractionated by SDS-PAGE. Proteins were visualized by the silver staining method (lanes *1-3*) or by immunoblotting (lanes *4-12*) using the mAbs BN5.1 (lanes *4-6*), DE6 (lanes *7-9*) or AH6 (lanes *10-12*). Numbers to the left indicate the position of size markers in kilodaltons.

the binding of DE6 to purified NOP1 (fibrillarin) (22, 46), the yeast antigen has been identified as fibrillarin. In mammalian cells, DE6 bound to a single polypeptide of 32 kD that did not comigrate with mammalian fibrillarin at 34 kD (6, 38) (Trimbur and Walsh, manuscript in preparation). In Naegleria, the DE6 antigen appears to be homologous with fibrillarin based on the reaction of affinity purified antigen with a series of well characterized anti-fibrillarin monoclonal antibodies (6) (unpublished observations).

AH6 bound to three polypeptides in whole cell extracts of yeast, 96, 72, and 58 kD, while it bound to only a 72-kD polypeptide in mammalian cells (Trimbur and Walsh, manuscript in preparation). Based on its size, the AH6 antigen does not appear to be any previously reported yeast or mammalian nucleolar protein.

The Protein Distribution in Nucleolus-like Particles Resembles that in Nucleoli

The distribution of BN46/51 and the AH6 and DE6 antigens in nucleoli and NLPs was examined by immunoelectron microscopy. BN46/51 was found concentrated in the peripheral, granular part and in isolated islands of more granular material in the interior of particles from a 15,000 g supernatant (Fig. 5 *b*). These particles were also strongly labeled with the DE6 antibody but the antigen was restricted to the central core of the particles (Fig. 5 *c*). In contrast, AH6 gave a lower level of binding throughout these particles (Fig. 5 *d*).

The distribution of the three nucleolar proteins in nucleoli fixed in situ was similar to that in NLPs from a 15,000 g supernatant. In nucleoli, BN46/51 was found throughout the nucleolus in the GC and concentrated along the borders of the DFC rather than in the DFC itself (Fig. 5 *f*). (In retrospect, this distribution is evident in the fluorescence micrographs [Fig. 1] in which BN46/51 is seen throughout isolated nucleoli but concentrated on the periphery of NLPs). The DE6 antigen, in contrast, was concentrated in the DFC (Fig. 5 *g*). The AH6 antigen was found at a lower concentration
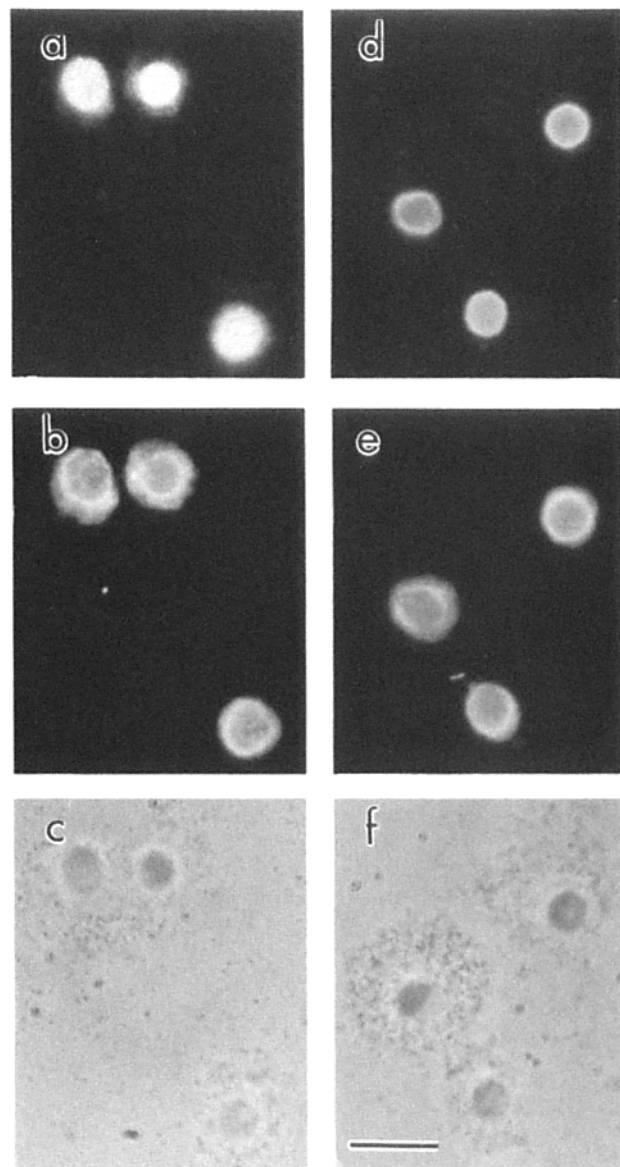

Figure 4. Binding of mAbs prepared against NLPs to Naegleria nucleoli. Amebae were fixed with formaldehyde (MgCF) (53) and stained with either AH6 (*a-c*) or with DE6 (*d-f*) followed by DAPI. (*a* and *d*) Antibody staining; (*b* and *e*) DAPI staining; and (*c* and *f*) phase contrast. Note that while DE6 is confined to the nucleolus, AH6 shows some binding to the nucleoplasm. All parts are the same magnification. Bar in *f*, 10 μm.

throughout the nucleolus and to a limited extent in the nucleoplasm (Fig. 5 *h*). Note that while there is a similarity between the organization of the nucleoplasm (*arrowhead* in Fig. 5 *f*) and nucleolus (*arrow* in Fig. 5 *f*) and the cortex and core of NLPs (Fig. 5 *b*), these are not homologous structures. Both the core and cortex of NLPs are derived from nucleolar components.

In NLPs from HSSs, BN46/51 was concentrated in the more central, less intensely staining regions of the particles that was similar to the cortex of NLPs from 15,000 g supernatants, Fig. 5 *j*. The DE6 antigen, in contrast, was concentrated in the more densely staining peripheral, crescent shaped regions of the particles that resembled the core of
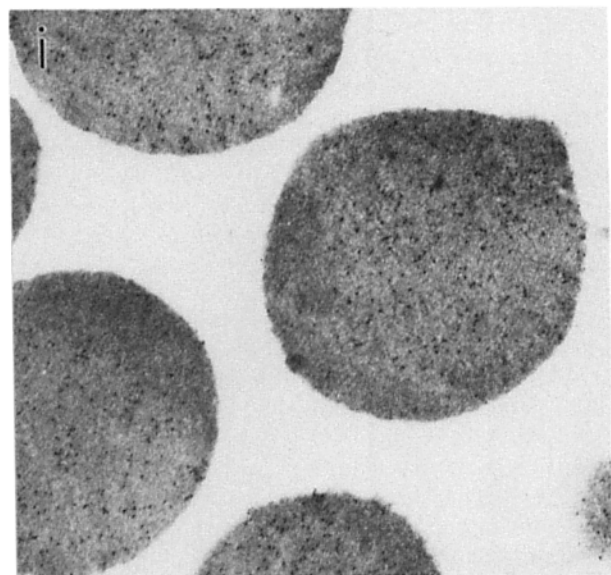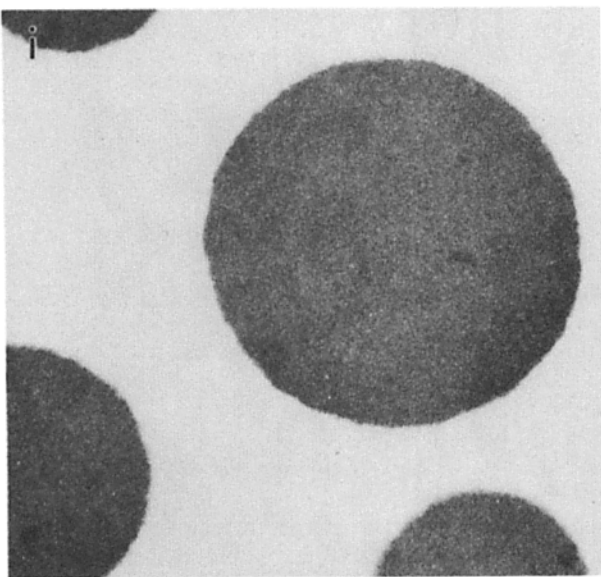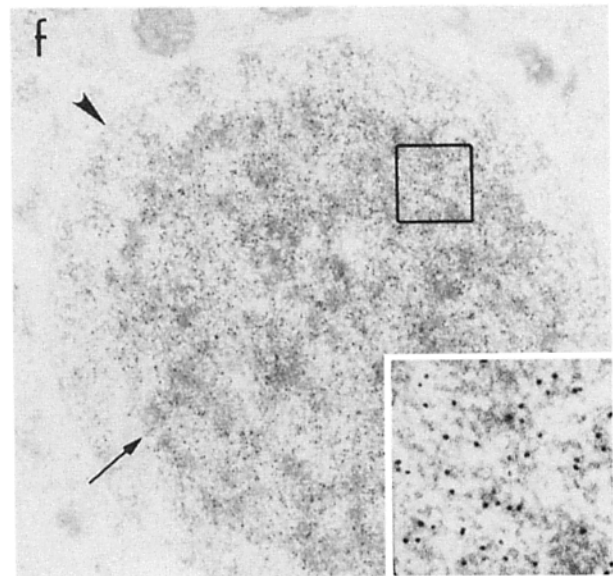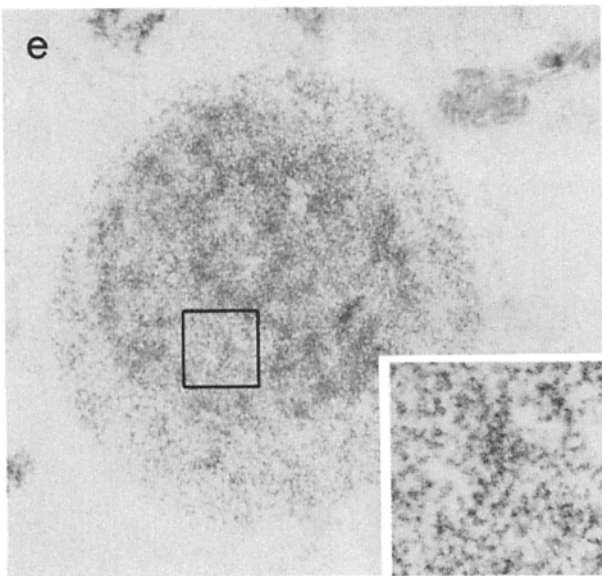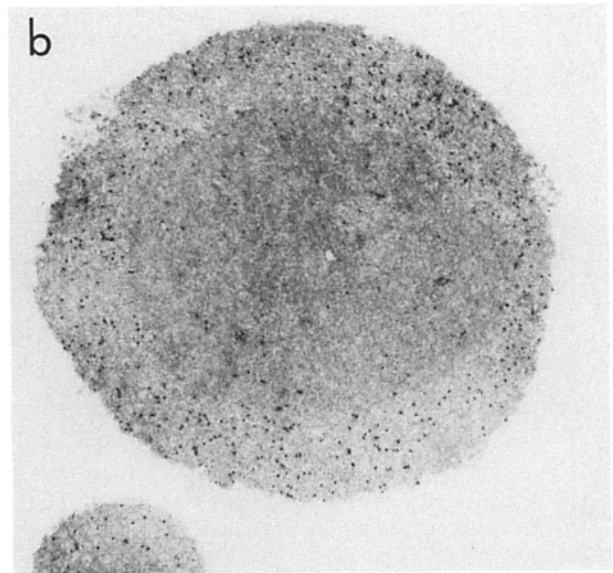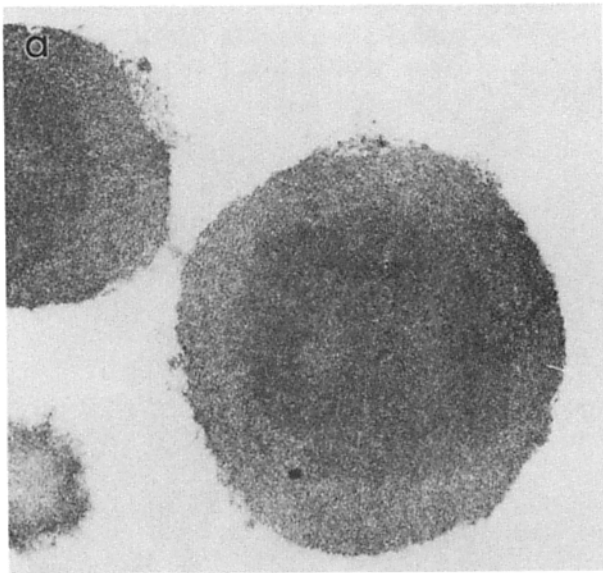
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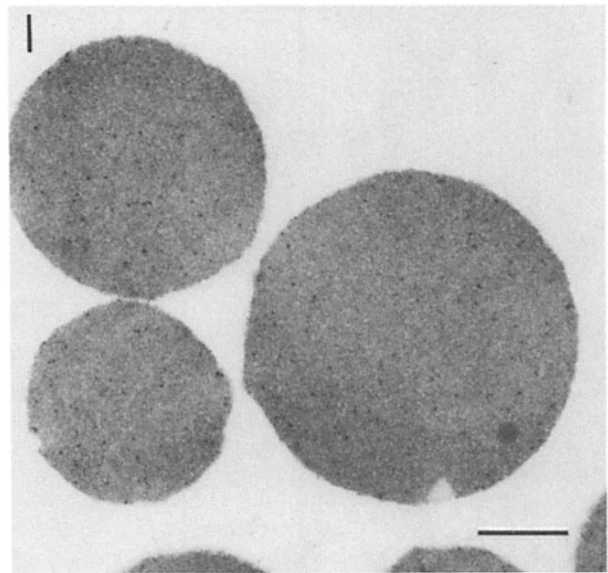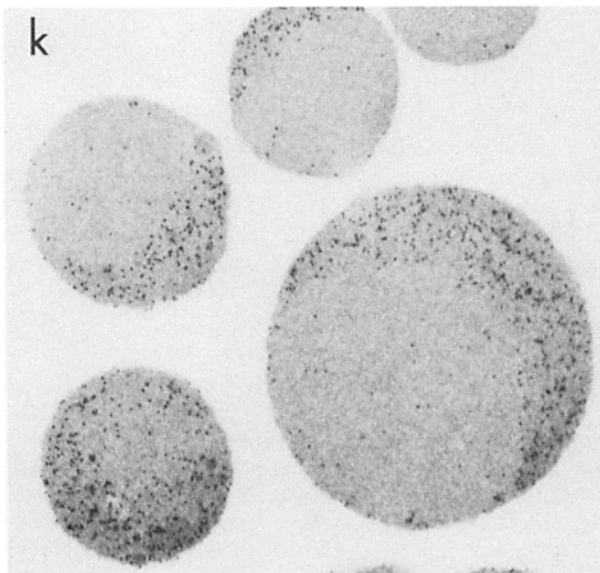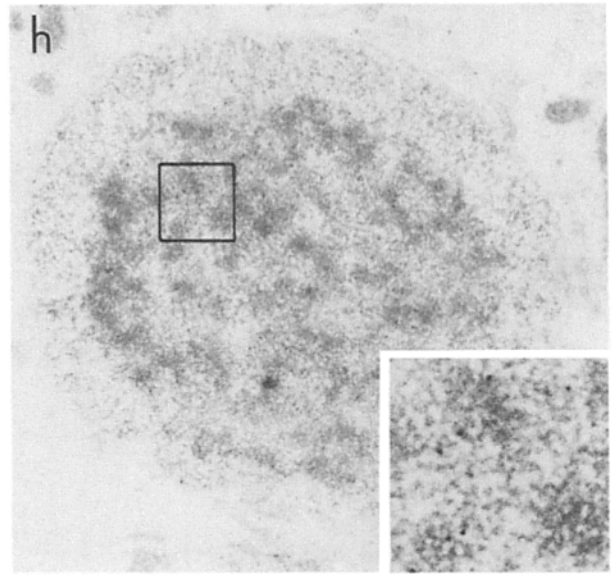
Figure 5.

Figure 6. The binding of the mAbs BN5.1, AH6, and DE6 to nucleoli segregated by treatment with actinomycin D. Amebae were grown in 40 μg/ml actinomycin D. At this concentration growth is slowed but not stopped (16). Amebae were fixed, embedded in LR White and incubated with antibodies as described in Fig. 5. (*a*) Control, no first antibody. (*b*) BN5.1. (*c*) DE6. (*d*) AH6.

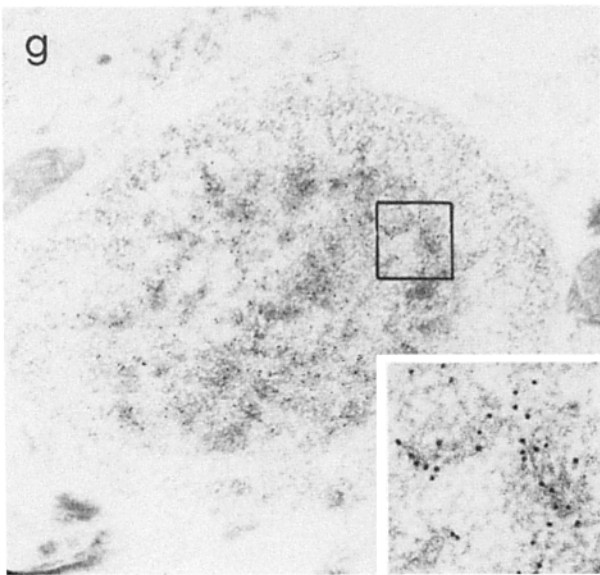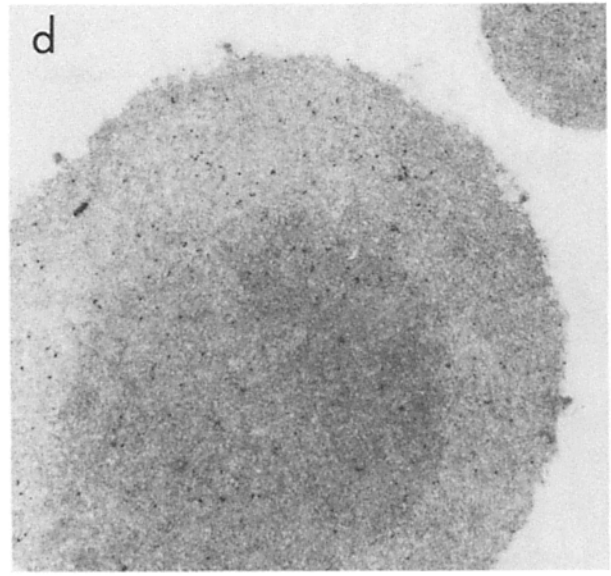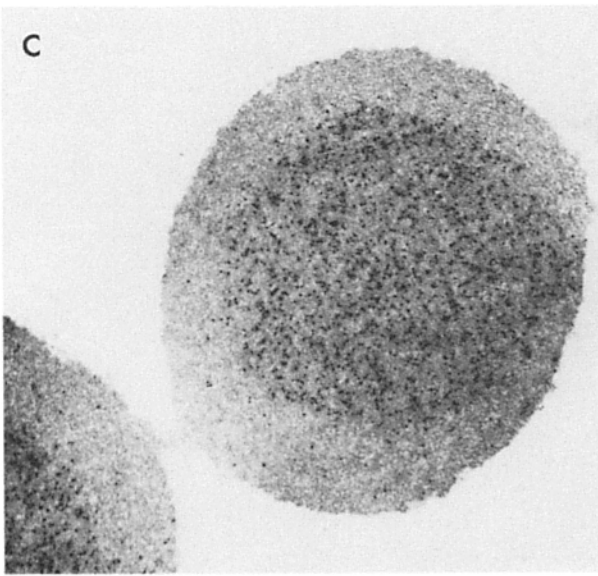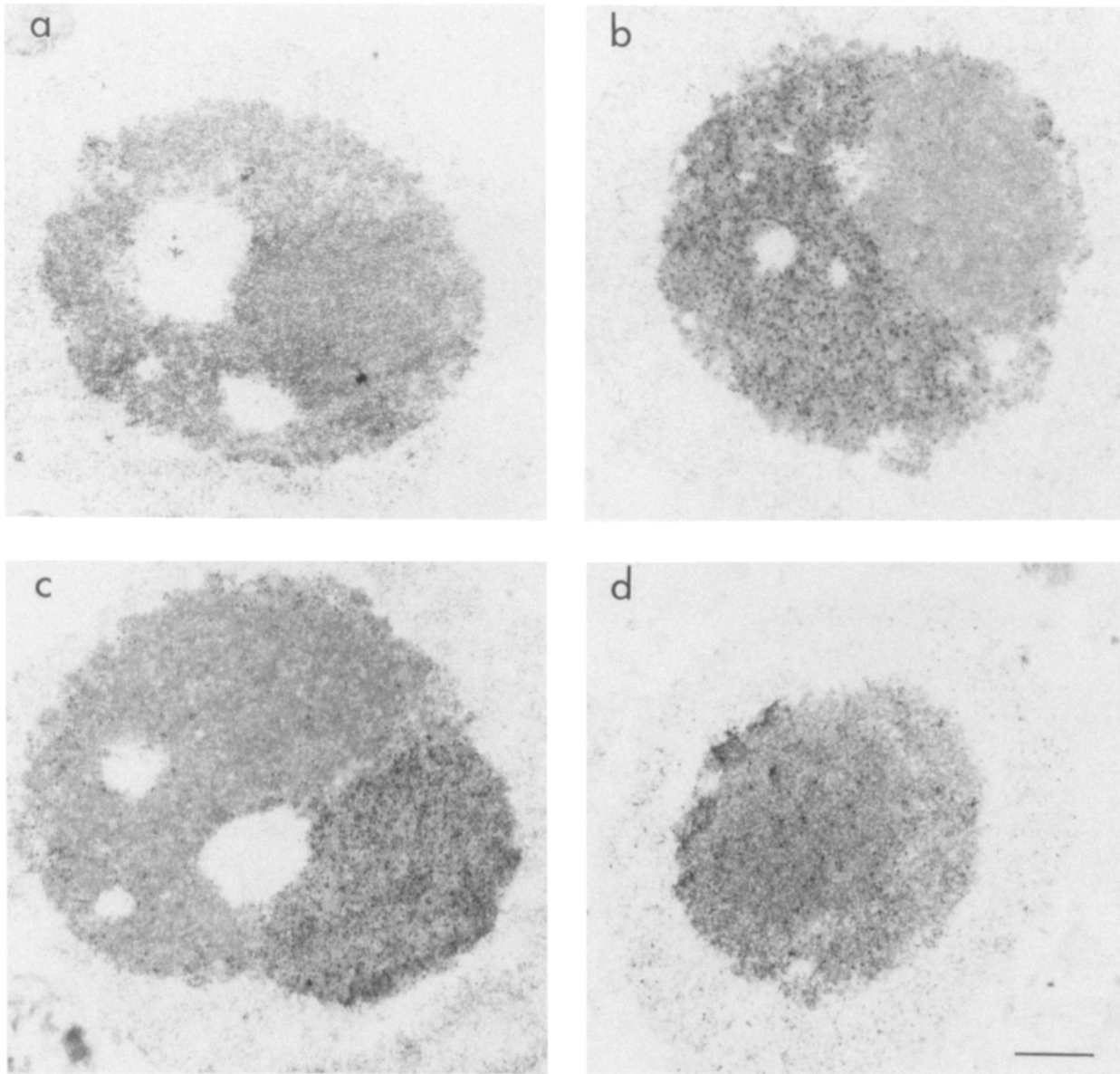NLPs from the low speed supernatants (Fig. 5 *k*). As in the case of NLPs from a low speed supernatant, the AH6 antigen was found throughout the particles from HSSs (Fig. 5 *l*).

To clarify the distribution of BN46/51 in nucleoli, amebae were grown in actinomycin D to inhibit rRNA synthesis (14).

As in other organisms this produces a segregation of the nucleolar components in which the GC and DFC are concentrated in distinct subregions (4) (Fig. 6 *a*). In the nucleoli of actinomycin D treated cells, BN46/51 was found concentrated in the GC (Fig. 6 *b*) just as it is in NLPs prepared from

Figure 5. The binding of the mAbs BN5.1, AH6, and DE6 to Naegleria nuclei and NLPs. NLPs were prepared from 15,000 and 436,000 g supernatants as described in the text. Amebae and NLPs were fixed with glutaraldehyde and embedded in LR White as described under Materials and Methods. Sections were incubated with the mAb BN5.1, AH6, or DE6 followed by a second antibody labeled with 10 nm colloidal gold particles. (*a–d*) Particles prepared from a 15,000-g supernatant. (*a*) Control, no first antibody. (*b*) BN5.1. (*c*) DE6. (*d*) AH6. (*e–h*) Naegleria nuclei fixed in situ. (*e*) Control, no first antibody. (*f*) BN5.1. (*g*) DE6. (*h*) AH6. In *f*, the location of the nucleolus is indicated by an arrow and the nuclear membrane is indicated by an arrowhead, cf. Fig. 2, *h*. (*i–l*) Particles prepared from a 436,000-g supernatant. (*i*) Control, no first antibody. (*j*) BN5.1. (*k*) DE6. (*l*) AH6. *a–l* are at the same magnification. The inserts in *e* through *h* are a threefold magnification of the areas indicated by the black squares. Bar in *l*, 0.5 μm.

a 15,000 g supernatant (Fig. 5 b). The DE6 antigen was found exclusively in the DFC of drug treated cells (Fig. 6 c) just as it is concentrated in the DFC-like core of NLPs (Fig. 5 c). After growth in actinomycin D the AH6 antigen was found distributed in both the GC and the DFC of nucleoli (Fig. 6 d) as it is in NLPs (Fig. 5 d).

The morphological distinction between the DFC and the GC of segregated nucleoli prepared for immunoelectron microscopy is difficult to see in Fig. 6 because the photographs were printed to show the gold particles. To confirm that BN5.1 and DE6 were binding to distinct compartments, we have carried out double label immunofluorescence of segregated nucleoli. These experiments gave the same results as shown in Fig. 6 (data not shown).

Factors Affecting Nucleolus-like Particles Formation

Divalent cations were not required for formation of NLPs. However, eliminating magnesium ions and adding EDTA during the dialysis reduced the size of the particles from 1–3 μm to 0.5–1 μm. Calcium ions at 0.5 mM could be substituted for magnesium ions with little effect on the size and no effect on the protein composition of the particles (data not shown). Dialysis of the extract against SPMG buffer with 200 or 250 mM NaCl resulted in particle formation but particles were not formed after dialysis against SPMG with 300 mM NaCl. Particle diameter showed an approximately linear relationship to the protein concentration in the nucleolar extract in the range of 1–4 mg/ml. Lowering the protein concentration from 3.5 mg/ml to 0.4 mg/ml reduced the average diameter of the particles from 2.8 to 1.1 μm. The particles formed at the highest protein concentrations examined were comparable in size to intact nucleoli, ~4 μm (Fig. 2 g).

The Composition of Nucleolus-like Particles

Quantitative analysis of NLPs prepared from detergent extracts of whole amebae was not feasible because the nucleoli used to extract BN46/51 contained adhering nuclear components as well as some Naegleria cysts and some of the bacteria used as a food source for the amebae. To provide a more homogeneous preparation of nucleoli as starting material, nucleoli were prepared by sonication of isolated nuclei (56). This isolation technique has been shown to produce homogeneous nucleoli as judged microscopically. It has also been shown to provide a quantitative separation of nucleolar RNA from nucleoplasmic RNA as judged by both steady state and pulse labeling (56). Isolation of nuclei minimizes contamination with cytoplasmic components while brief sonication removes much of the nuclear DNA leaving the nucleolus intact. Small numbers of contaminating bacteria and Naegleria cysts were removed by banding the isolated nucleoli in gradients of Percoll.

When Percoll purified nucleoli were suspended in 0.4 M NaCl SPMG, 78% of the protein, 63% of the RNA and 47% of the DNA were present in the supernatant after centrifugation at 15,000 g for 10 min (Table I). After dialyzing the supernatant into buffer without NaCl, 42% of the nucleolar protein, 41% of the RNA, and 41% of the DNA were sedimentable under these same conditions.

HSSs contained ~25% of the nucleolar RNA and protein but very little of the nucleolar DNA (Table I). Approxi-
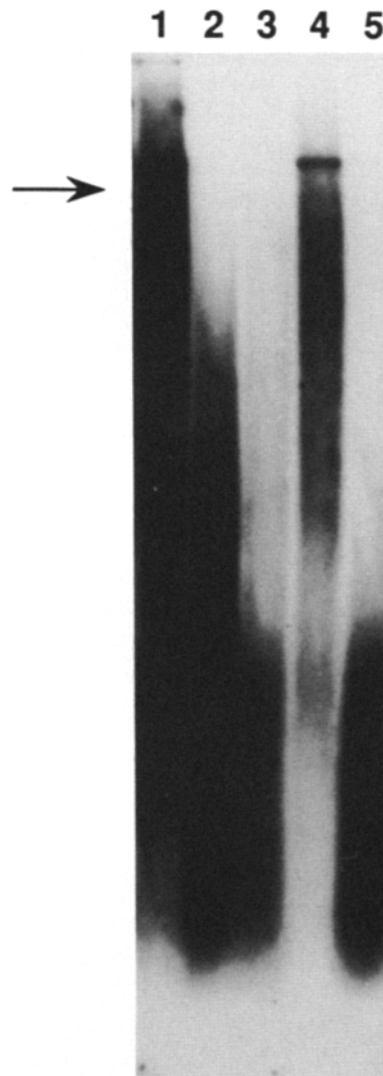

Figure 7. The distribution of the Naegleria ribosomal plasmid in nucleolar extracts and NLPs. Nucleoli, prepared by sonication of isolated nuclei followed by banding in Percoll, were dissociated in 0.4 M NaCl SPMG buffer followed by centrifugation at 15,000 g for 10 min. NLPs, prepared by dialysis of the 0.4 M NaCl supernatant against SPMG, were collected by centrifugation at 15,000 g for 10 min. DNA was extracted from equivalent samples of nucleoli (lane 1); NLPs (lane 2); the nucleoplasm released by sonication of nuclei (lane 3); the NaCl insoluble fraction of nucleoli (lane 4); and the supernatant after dialysis and sedimentation of the NLPs (lane 5). The DNA samples were digested with Bam HI to linearize the ribosomal plasmid, fractionated on an agarose gel and transferred to Gene Screen Plus. Ribosomal plasmid sequences were located by hybridization with the cloned ribosomal plasmid from Naegleria strain NEG which is 14 kbp in length (7). The ribosomal plasmid in Naegleria strain NB-1, used in the current work, is 17 kbp (8). The arrow indicates the location of a 14-kbp plasmid marker in an adjacent lane as visualized by staining with ethidium bromide.

mately 10% of the nucleolar RNA and protein were incorporated into these particles (Table I).

The Role of DNA and RNA in NLP Formation

Treatment of 15,000 g extracts with 1 mg/ml DNase I for 30

min at 10°C before dialysis did not prevent particle assembly but it did eliminate DAPI staining. DNase treatment also greatly reduced the DNA content of the extract as judged by ethidium bromide staining of extracts fractionated on agarose gels. However, the distribution of the major proteins in the NLPs was not affected (data not shown). DNase treatment of the extracts also did not significantly alter the structure of the NLPs as seen in the electron microscope (Fig. 2 d).

The ribosomal genes of Naegleria are carried exclusively on 3,000–5,000 copies of a circular plasmid (7). To follow the fate of these plasmids during NLP formation, the 14 kbp plasmid cloned from Naegleria strain NEG (7) was used to probe Southern blots of DNA extracted from the particles. A significant amount of low molecular weight, heterogeneous DNA complementary to this probe was present in the reassembled particles, however most of the intact plasmid sequences were found in the NaCl insoluble fraction that pelleted at 15,000 g (Fig. 7).

Treatment of the nucleolar extract with RNase also did not block formation of NLPs but it did reduce the size of the particles, Fig. 2e and f. This is not surprising as RNA makes up about 20% of both nucleoli and NLPs while DNA contributed only about 2% to NLPs, Table I. RNase treatment also altered the structure of the particles as seen in the electron microscope, Fig. 2e and f. These NLPs still showed a dense central component and a less dense peripheral region but the latter lacked most of the 15–20 nm granules present in particles prepared from untreated extracts. This is consistent with the usual identification of these granules as preribosomal particles (18, 19).

Nucleolus-like Particles Can Be Repeatedly Assembled and Disassembled

To evaluate the consistency of the protein composition of NLPs prepared from HSSs, particles were subjected to three cycles of assembly and disassembly. After dialysis, HSS NLPs were pelleted by centrifugation at 15,000 g and resuspended in one half the original volume of SPMG buffer containing 0.4 M NaCl. This completely dissociated the particles as judged by light microscopy and the failure to sediment at 15,000 g. The dissociated particles were again dialyzed against SPMG and the NLPs recovered by centrifugation. The same process was then carried out for a third time. As illustrated in Fig. 8, the protein composition of the particles remained constant through three cycles of assembly and disassembly. In this case, the higher protein concentrations on the gel make it possible to identify eleven major polypeptides in these particles.

Discussion

The NLPs that form when NaCl extracts of Naegleria nucleoli are dialyzed into low salt buffer resemble nucleoli in their size, protein composition, and ultrastructure. The resemblance between NLPs and compact nucleoli is striking. NLPs also resemble a variety of other in vivo structures. These include prenucleolar bodies, pseudonucleoli, and nucleolus-like bodies. This suggests that, for the first time, some aspects of the molecular basis for nucleolar morphology may be amenable to analysis in vitro.
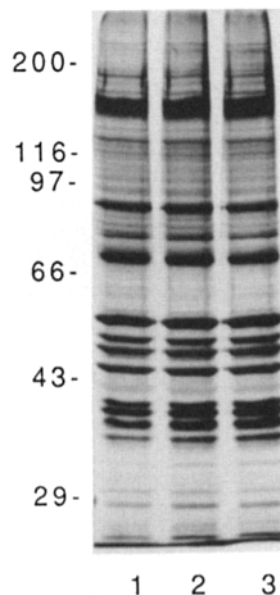

Figure 8. The protein composition of NLPs during repeated cycles of assembly and disassembly. NLPs were prepared by dialysis of a 436,000-g supernatant and then pelleted by centrifugation at 15,000 g for 10 min. The NLP pellet was resuspended in one half the original volume of 0.4 M NaCl SPMG and dialyzed against SPMG a second time. The NLPs resulting from the second dialysis were dissociated in one-quarter the original volume of 0.4 M NaCl and the extract dialyzed for a third time. Equal volumes from each cycle of NLPs were fractionated by SDS PAGE and the proteins visualized by the silver staining method. (Lane 1) NLPs from the first cycle; (lane 2) NLPs from the second cycle; (lane 3) NLPs from the third cycle. Numbers to the left indicate the position of size markers in kilodaltons. The arrows to the right indicate, from the top down, the position of the 51-kD and 46-kD subunits of BN46/51 and the 43-kD DE6 antigen, which stains poorly with silver but is enriched in these particles as shown in Fig. 3.

The morphological resemblance between the GC of nucleoli and the cortex of NLPs is marked. This is due, in part, to the presence of 15–20 nm osmophilic granules in both structures. These granules are usually described as preribosomes (18, 19) and this identification is supported by their absence when NLPs are prepared from either RNase treated extracts or from high-speed supernatants. Overall, the cortex of NLPs resembles the periphery of compact nucleoli and the periphery of extra chromosomal nucleoli of *Xenopus* oocytes.

The concentration of the nucleolar protein BN46/51 in the cortex of NLPs is also consistent with GC-like morphology. In nucleoli, BN46/51 was found along the interface between the GC and DFC as well as in the GC proper. The U3 small nucleolar RNA shows a similar concentration along this interface but the functional basis for this is unknown (41). Because of this distribution, it was difficult to assign BN46/51 to a single compartment based on normal nucleoli. However in nucleoli in which the DFC and GC were segregated, BN46/51 was restricted to the GC, suggesting that it is primarily if not exclusively a component of the GC. Nonetheless, assignment of BN46/51 to the GC must remain tentative until we better understand the molecular basis for nucleolar segregation. This process has been widely observed and used to help assign nucleolar components to specific subregions (4, 19, 41) but it is unclear to what extent various nucleolar components may be rearranged by treatment with the drug.

The DE6 antigen showed an unambiguous association with the DFC of nucleoli. The gold particles were found directly over the electron dense fibers of the DFC and they

Table II. Nucleolar Antigens in Nucleoli and Nucleolus-like Particles

Antibody	Location in nucleoli	Location in NLPs
BN5.1*	Granular component (GC)	Cortex (GC-like)
DE6‡	Dense fibrillar component (DFC)	Core (DFC-like)
AH6§	Granular component and dense fibrillar component	Cortex and core

* The BN5.1 antibody identifies the BN46/51 nucleolar protein (53).
‡ The DE6 antibody recognizes a protein in Naegleria that shares multiple epitopes with fibrillarin.
§ The AH6 antibody also binds to a small amount of material in the nucleoplasm.

were not found in the GC. In examining many of these images we found that this pattern was readily recognized as being different from that of BN46/51 where the gold particles were clustered along the interface between the DFC and GC and were not found directly over the electron dense DFC. In NLPs, the DE6 antigen was restricted to the core of the particles. This region showed a fine granular morphology and more intense osmium binding, features characteristic of the DFC of nucleoli. In segregated nucleoli the DE6 antigen was also restricted to the DFC. The distribution of nucleolar antigens in nucleoli and NLPs is summarized in Table II.

The restriction of the DE6 antigen to the DFC is consistent with its identification as the Naegleria homologue of fibrillarin, a nucleolar protein found in the DFC. We tested affinity purified DE6 antigen with four mAbs against fibrillarin prepared by Christensen (5). All four mAb bound to the 44-kD form of the DE6 antigen (Trimbur and Walsh, manuscript in preparation). The 31-kD form of the DE6 antigen did not react with these mAb and so its relationship to fibrillarin is uncertain.

The AH6 antigen is primarily, although not exclusively, nucleolar. This is most evident from a comparison of the pattern with DE6 as seen by fluorescence microscopy. When examined by electron microscopy, AH6 was found in both the GC and the DFC of normal and segregated nucleoli. In NLPs, AH6 was associated with both the DFC-like core and the GC-like cortex. Thus, for all three nucleolar antigens examined, the distribution in NLPs resembled that seen in nucleoli.

DNA does not appear to be essential for the formation of NLPs. Treatment of extracts with DNase did not block the formation of particles. The effectiveness of the DNase digestion was confirmed by DAPI staining and DNA extraction. The fact that most of the intact ribosomal plasmid remained in the pellet after extraction of nucleoli with 0.4 M NaCl is consistent with this conclusion. The plasmid sequences that were present in the extracts were of low molecular weight and presumably represent material degraded during the fractionation. It is interesting that almost all of this low molecular-weight material was incorporated into NLPs but the basis for this is not understood.

The role of RNA in NLP formation is less certain. Treatment of extracts with RNase resulted in the loss of the 15–20-nm putative preribosomal particles from NLPs. However, the effectiveness of the RNase digestion and the actual RNA sequences present in NLPs is still under investigation. The fact that some RNA is still incorporated into NLPs prepared from high speed supernatants is interesting but the nature of

this low molecular weight material is unknown. The possibility that it may represent small nucleolar RNAs is being examined.

There is a marked resemblance between the NLPs prepared from high speed supernatants that lack both rDNA and most of the nucleolar RNA and the pseudonucleoli seen in later developmental stages of the anucleolate mutant of *Xenopus*, cf., Fig. 29 *B* of Hay (20) with Fig. 2, *k* and *l*. At these later stages of development, the mutant larvae have exhausted the maternal store of rRNA. The spherical pseudonucleoli consist of a large fibrillar region with crescent shaped surface patches of osmophilic material. Thus, the interactions between the nucleolar components present in high speed supernatants may provide a model for the interactions seen in vivo when cells are unable to synthesize rRNA.

The data available so far suggest that the formation of NLPs is the result of protein–protein interactions. The rapid formation of these particles as the salt concentration is lowered from 300 to 250 mM and the absence of a significant temperature effect on particle formation (unpublished observations) suggests that this process may be driven by the free energy produced when bound ions are released from the solubilized components (42). We are attempting to test this hypothesis and to identify the components that are essential for particle formation. This may make it possible to begin to define the molecular basis for nucleolar morphology.

References

1. Belenguer, P., M. Caizergues-Ferrer, J.-C. Labbé, M. Dorée, and F. Amalric. 1990. Mitosis-specific phosphorylation of nucleolin by p34^{cdc2} protein kinase. *Mol. Cell. Biol.* 10:3607–3618.
2. Bell, P., M.-C. Dabauvalle, and U. Scheer. 1992. In vitro assembly of prenucleolar bodies in *Xenopus* egg extract. *J. Cell Biol.* 118:1297–1304.
3. Benavente, R. 1991. Postmitotic nuclear reorganization events analyzed in

living cells. *Chromosoma (Berlin).* 100:215–220.

4. Busch, H., and K. Smetana. 1970. The Nucleolus. Academic Press, New York. 626 pp.

5. Christensen, M. E., and N. Banker. 1992. Mapping of monoclonal antibody epitopes in the nucleolar protein fibrillarin (B-36) of *Physarum polycephalum. Cell Biol. Intl. Rep.* 16:1119–1131.

6. Christensen, M. E., J. Moloo, J. L. Swischuk, and M. E. Schelling. 1986. Characterization of the nucleolar protein, B-36, using monoclonal antibodies. *Exp. Cell Res.* 166:77–93.

7. Clark, C. G., and G. A. M. Cross. 1987. rRNA genes of *Naegleria gruberi* are carried exclusively on a 14-kilobase-pair plasmid. *Mol. Cell. Biol.* 7:3027–3031.

8. Clark, C. G., and G. A. M. Cross. 1988. Circular ribosomal RNA genes are a general feature of schizopyrenid amoebae. *J. Protozool.* 35:326–329.

9. De La Torre, C., and G. Gimenez-Martin. 1982. The nucleolar cycle. *In* The Nucleolus. E. G. Jordan and C. A. Cullis, editors. Cambridge University Press, Cambridge. 153–178.

10. Fawcett, D. W. 1966. An Atlas of Fine Structure: The Cell, Its Organelles and Inclusions. W.B. Saunders Co., Philadelphia. 448 pp.

11. Fulton, C. 1970. Amebo-flagellates as research partners: the laboratory biology of *Naegleria* and *Tetramitus. Methods Cell Physiol.* 4:341–476.

12. Fulton, C., and A. D. Dingle. 1967. Appearance of the flagellate phenotype in populations of *Naegleria* amebae. *Dev. Biol.* 15:165–191.

13. Fulton, C., and A. M. Guerrini. 1969. Mitotic synchrony in Naegleria amebae. *Exp. Cell Res.* 56:194–200.

14. Fulton, C., and C. Walsh. 1980. Cell differentiation and flagellar elongation in *Naegleria gruberi. J. Cell Biol.* 85:346–360.

15. Gendimenico, G. J., P. L. Bouquin, and K. M. Tramposch. 1988. Diphenylamine-colorimetric method for DNA assay: A shortened procedure by incubating samples at 50°C. *Anal. Biochem.* 173:45–48.

16. Gerbi, S. A., R. Savino, B. Stebbins-Boaz, C. Jeppesen, and R. Rivera-Leon. 1990. A role for U3 small nuclear ribonucleoprotein in the nucleolus? *In* The Ribosome: Structure, Function, and Evolution. A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner, editors. American Society for Microbiology, Washington, DC. 452–469.

17. Giménez-Martín, G., C. De La Torre, M. E. Fernández-Gómez, and A. González-Fernández. 1974. Experimental analysis of nucleolar reorganization. *J. Cell Biol.* 60:502–507.

18. Goessens, G. 1984. Nucleolar Structure. *Int. Rev. Cytol.* 87:107–158.

19. Hadjiolov, A. A. 1985. The Nucleolus and Ribosome Biogenesis. Springer-Verlag, New York. 268 pp.

20. Hay, E. D. 1968. Structure and function of the nucleolus in developing cells. *In* The Nucleus. A. J. Dalton, and F. Haguenau, editors. Academic Press, New York. 1–79.

21. Hay, E. D., and J. B. Gurdon. 1967. Fine structure of the nucleolus in normal and mutant *Xenopus* embryos. *J. Cell Sci.* 2:151–162.

22. Henríquez, R., G. Blobel, and J. P. Aris. 1990. Isolation and sequencing of *NOP1.* A yeast gene encoding a nucleolar protein homologous to a human autoimmune antigen. *J. Biol. Chem.* 265:2209–2215.

23. Hernandez-Verdun, D., M. Bouteille, T. Ege, and N. R. Ringertz. 1979. Fine structure of nucleoli in micronucleated cells. *Exp. Cell Res.* 124:223–235.

24. Hernandez-Verdun, D., M. Robert-Nicoud, G. Geraud, and C. Masson. 1991. Behavior of nucleolar proteins in nuclei lacking ribosomal genes: A study of confocal laser scanning microscopy. *J. Cell Sci.* 98:99–105.

25. Jiménez-García, L. F., L. I. Rothblum, H. Busch, and R. L. Ochs. 1989. Nucleologenesis: use of non-isotopic *in situ* hybridization and immunocytochemistry to compare the location of rDNA and nucleolar proteins during mitosis. *Biol. Cell.* 65:239–246.

26. Jones, K. W. 1965. The roles of the nucleolus in the formation of ribosomes. *J. Ultrastruct. Res.* 13:257–262.

27. Jordan, E. G. 1984. Nucleolar nomenclature. *J. Cell Sci.* 67:217–220.

28. Karasaki, S. 1968. The ultrastructure and RNA metabolism of nucleoli in early sea urchin embryos. *Exp. Cell Res.* 52:13–26.

29. Karpen, G. H., J. E. Schaefer, and C. D. Laird. 1988. A *Drosophila* rRNA gene located in euchromatin is active in transcription and nucleolus formation. *Genes Dev.* 2:1745–1763.

30. Knight, D. P. 1977. Cytological staining methods in electron microscopy. *In* Practical Methods in Electron Microscopy. A. M. Glauert, editor. North-Holland, Amsterdam. 25–76.

31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

32. Miller, O. L., Jr. 1966. Structure and composition of peripheral nucleoli of salamander oocytes. *In* International Symposium on the Nucleolus, Its Structures and Function. W. S. Vincent, and O. L. Miller, Jr., editors. National Cancer Institute Monograph No. 23, Washington, DC. 53–65.

33. Morcillo, G., and C. De La Torre. 1979. Mapping nucleologenesis in relation to transcription. *Biol. Cell.* 36:1–6.

34. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307–310.

35. Munro, H. N., and A. Fleck. 1966. The determination of nucleic acids. *Methods Biochem. Anal.* 14:113–176.

36. Naus, C. G., and G. M. Kidder. 1982. Regulation of expression of the ribosomal RNA cistrons in *Ilyanassa* embryos: Nucleolus-like bodies and nucleologenesis. *J. Exp. Zool.* 219:51–66.

37. Ochs, R. L., M. A. Lischwe, E. Shen, R. E. Carroll, and H. Busch. 1985. Nucleogenesis: composition and fate of prenucleolar bodies. *Chromosoma (Berlin).* 92:330–336.

38. Ochs, R. L., M. A. Lischwe, W. H. Spohn, and H. Busch. 1985. Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. *Biol. Cell.* 54:123–134.

39. Phillips, D. M., and S. G. Phillips. 1973. Reproduction of postmitotic nucleoli by preformed RNA. II. Ultrastructure. *J. Cell Biol.* 58:54–63.

40. Phillips, S. G. 1972. Repopulation of the postmitotic nucleolus by preformed RNA. *J. Cell Biol.* 53:611–623.

41. Puvion-Dutilleul, F., S. Mazan, M. Nicoloso, E. Pichard, J.-P. Bachellerie, and E. Puvion. 1992. Alterations of nucleolar ultrastructure and ribosome biogenesis by actinomycin D. Implications for U3 snRNP function. *Eur. J. Cell Biol.* 58:149–162.

42. Record, M. T., Jr., C. F. Anderson, and T. M. Lohman. 1978. Thermodynamic analysis of ion effects on the binding and conformational equilibria of proteins and nucleic acids: the roles of ion association or release, screening, and ion effects on water activity. *Quart. Rev. Biophys.* 11:103–178.

43. Reeder, R. H. 1990. rRNA synthesis in the nucleolus. *Trends Genet.* 6:390–395.

44. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

45. Scheer, U., and R. Benavente. 1990. Functional and dynamic aspects of the mammalian nucleolus. *BioEssays.* 12:14–21.

46. Schimmang, T., D. Tollervey, H. Kern, R. Frank, and E. C. Hurt. 1989. A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4015–4024.

47. Schwarzacher, H. G., and F. Wachtler. 1991. The functional significance of nucleolar structures. *Ann. Genet.* 34:151–160.

48. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31–43.

49. Stevens, A. R., and D. M. Prescott. 1971. Reformation of nucleolus-like bodies in the absence of postmitotic RNA synthesis. *J. Cell Biol.* 48:443–454.

50. Stevens, B. J. 1965. The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J. Cell Biol.* 24:349–368.

51. Swift, H., and B. J. Stevens. 1966. Nucleolar-chromosomal interactions in the microspores of maize. *In* International Symposium on the Nucleolus, Its Structure and Function. W. S. Vincent, and O. L. Miller, Jr., editors. National Cancer Institute Monograph No. 23, Washington, DC. 145–165.

52. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

53. Trimbur, G. M., and C. J. Walsh. 1992. BN46/51, new nucleolar protein, binds to the basal body region in *Naegleria gruberi* flagellates. *J. Cell Sci.* 103:167–181.

54. Wachtler, F., M. Hartung, M. Devictor, J. Wiegant, A. Stahl, and H. G. Schwarzacher. 1989. Ribosomal DNA is located and transcribed in the dense fibrillar component of human Sertoli nucleoli. *Exp. Cell Res.* 184:61–71.

55. Wachtler, F., C. Schöfer, W. Mosgöller, K. Weipoltshammer, H. G. Schwarzacher, M. Guichaoua, M. Hartung, A. Stahl, J. L. Bergé-Lefranc, I. Gonzalez, and J. Sylvester. 1992. Human ribosomal RNA gene repeats are localized in the dense fibrillar component of nucleoli: Light and electron microscopic *in situ* hybridization in human Sertoli cells. *Exp. Cell Res.* 198:135–143.

56. Walsh, C., and C. Fulton. 1973. Transcription during cell differentiation in *Naegleria gruberi.* Preferential synthesis of messenger RNA. *Biochim. Biophys. Acta.* 312:51–71.

57. Warner, J. R. 1990. The nucleolus and ribosome formation. *Curr. Opin. Cell Biol.* 2:521–527.

58. Wright, R. and J. Rine. 1989. Transmission electron microscopy and immunocytochemical studies of yeast: analysis of HMG-CoA reductase overproduction by electron microscopy. *Methods Cell Biol.* 31:473–512.