Proteins and RNA in Mouse L Cell Core Nucleoli and Nucleolar Matrix†

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ABSTRACT: When intact nucleoli were prepared in the presence of enough leupeptin and phenylmethanesulfonyl fluoride to inhibit protease action, electrophoretic patterns of their constituent proteins were reproducible and very similar for L, HeLa, CHO, and rat hepatoma cells. "Core nucleoli", defined as that nucleolar fraction which remains after extensive DNase I action, had a protein composition similar to that of crude intact nucleoli, but were enriched for snRNA U3. Core nucleolar proteins included all of the histones, ribosomal proteins, and phosphorylated proteins with mobilities corresponding to 110 (protein C23) and 160 kilodaltons (kDa). The presence of protein C23 and of lamins A and C in nucleoli and core nucleoli was further verified by reaction with specific antibodies after one- or two-dimensional electrophoresis. A class of higher molecular weight proteins, ranging from 70 to >200 kDa by mobility, was observed. It included at least 25 specific proteins, almost all of them highly acidic (pI < 3.5). Treatment of core nucleoli with ethylenediaminetetraacetic acid/hypotonic buffer solubilized 30–35% of the small and large molecular weight proteins. In contrast, washing core nucleoli with 2 M NaCl selectively released U3 snRNA, 95% of the ribosomal RNA, and about half of the proteins, including C23 and most of the histones, ribosomal proteins, and other lower molecular weight proteins. The fraction remaining insoluble, "nucleolar matrix", was enriched for proteins of 34 and 57 kDa, lamins A and C, and most higher molecular weight proteins, as well as a portion of ribosomal spacer DNA.

In mammalian cell nucleoli, sites of ribosome biosynthesis are localized at the chromosomal nucleolar organizer region. It has, however, been difficult to precisely define the nucleolus, since this organelle has no limiting membrane. Nucleoli prepared by standard methods retain the full cellular content of preribosomes, ribosomal DNA (rDNA), specific snRNAs, and characteristic proteins (Reddy & Busch, 1981), but large amounts of perinucleolar chromatin, nucleoplasm, and even some cytoplasmic components are also seen (Busch & Smetana, 1970).

Recently we have suggested that treatments with DNase I digest most of the extranucleolar DNA from nucleoli prepared by standard procedures (Bolla et al., 1985). This also removes the transcribed portion of rDNA and possibly some perinucleolar DNA that may be of importance in nucleolar function. However, the nontranscribed spacer portions of rDNA are largely retained in these "core nucleoli" in a form highly protected against nuclease action. These nontranscribed spacer (NTS) segments of rDNA contain a number of repetitive sequences (Arnheim et al., 1980; Kominami et al., 1981; Kuehn & Arnheim, 1983; Mroczka et al., 1984), including tracts of poly(pyrimidine-purine) (Thomas et al., 1985) which border the transcription unit, and as such may be involved in nucleolar organization or regulation of rDNA transcription (Thomas et al., 1985).

Here we have optimized the preparation of core nucleoli in order to study their content of non-DNA components. Certain nucleolar components, including, for example, U3 snRNA (Epstein et al., 1984; Reddy & Busch, 1981), are enriched in the core nucleoli compared to fractions not treated with DNase I. Most of the nucleolar proteins remain in core nucleoli but can be solubilized by treatment with either hypotonic buffer or 2 M NaCl. The fraction that remains insoluble in 2 M NaCl, operationally defined as "nucleolar matrix", is highly enriched for some nonhistone chromosomal proteins.

Experimental Procedures

The optimal preparation of core nucleoli and nucleolar matrix requires actively growing cells, adequate levels of the protease inhibitors phenylmethanesulfonyl fluoride (PMSF) and leupeptin, and sufficient volumes of washing buffers to permit the uniform release of proteins during extraction procedures.

Preparation of Nucleoli and DNase-Treated Nucleoli. Nucleoli were prepared by the method of Bolla et al. (1985) with modifications as follows.

All procedures were carried out at 4 °C except where otherwise noted. Mouse L cells were cultured either in 150-mm tissue culture dishes or in spinner flasks to a density of 8.8×10^8 cells/L. Cells were washed three times with phosphate-buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl). The cells were lysed in 15 mL of buffer A (150 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris HCl, pH 7.4) containing 0.35% (v/v) Triton X-100, 1 mM PMSF, and 0.2 mM leupeptin and homogenized in a Dounce homogenizer (Pestle B). [When protease inhibitors were not included in preparative buffers, nucleolar proteins showed a random and irreproducible pattern of fragmentation. For example, the C23 protein (see text), detected by a specific antibody, was recovered in fragments (as reported by Bugler et al. (1982)). The same concentrations of PMSF and leupeptin were used throughout the preparative procedures.] Sodium deoxycholate was then added to a final concentration of 0.3% (w/v), and the suspension was mixed by vortexing twice for 1 min.

Nuclei were isolated by centrifugation through buffer A containing 0.5 M sucrose at 1500*g* for 10 min. The nuclear pellet was resuspended in 10 volumes of buffer A and sonicated (40 W, Branson sonifier) until no intact nuclei were observed

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microscopically. DNase I (Biomedical Cooper DPRF, 2026 units/mg with less than 0.01% RNase activity) was added to a final concentration of 50 μg/mL (or occasionally other concentrations; see text). After incubation at 37 °C for 30 min, nucleoli were collected by sedimentation through a step gradient containing 2 mL each of 0.25 and 0.8 M sucrose and were suspended in 5 volumes of a hypotonic buffer [1 mM Tris HCl, pH 8.5, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA)] for 10 min on ice with occasional mixing. The buffer concentration was then restored to that of buffer A by the addition of an equal volume of twice-concentrated buffer A, and treatment with DNase I was repeated. The final sediment fraction represents core nucleoli. When nucleoli were isolated from sonicated nuclei by centrifugation through a sucrose step gradient without the first DNase I treatment, many nucleoli remained in the supernatant fraction trapped within a chromatin network. The DNase I treatment of a nuclear sonicate before centrifugation then led to a 50%–60% increase in the yield of nucleoli, as determined by estimation of rDNA content.

In some experiments DNase I treatment of the nuclear sonicate was substituted with 80 A_{260} units/mL micrococcal nuclease (Sigma) for 20 min on ice in a buffer containing 25 mM KCl, 0.9 mM $MgCl_2$, 0.9 mM $CaCl_2$, 0.14 mM spermidine, and 1 mM Tris HCl, pH 7.6. The action of micrococcal nuclease was stopped by the addition of EDTA to 25 mM.

Fractionation of Core Nucleoli with Hypotonic Buffer or 2 M NaCl Buffer. Core nucleoli, prepared by using DNase I as above, were recovered by centrifugation at 100*g* for 10 min, incubated in 10 volumes of 0.1 mM EDTA, 1 mM Tris HCl, pH 8.5 on ice for 2 h and collected at 100*g* for 10 min. This treatment is called "washing with hypotonic buffer" in the text. In other experiments, the core nucleoli were extracted with 10 volumes of 2 M NaCl buffer (10 mM Tris HCl, pH 8.5, 10 mM $MgCl_2$, 0.2 mM EDTA, and 2 M NaCl) for 20 min on ice. The fraction insoluble in this buffer, operationally defined as "nucleolar matrix", was collected by centrifugation at 100*g* (or at higher *g* force; see text) for 10 min.

Preparation of Ribosomes, Chromatin, and RNA. Cytoplasmic ribosomes, prepared from mouse L cells by the method of Blobel and Potter (1967), were used to obtain ribosomal proteins.

Chromatin was prepared essentially as described by Marushige and Bonner (1966). RNA was isolated from cytoplasm, from nucleoplasm, and from nucleoli and nucleolar subfractions by the method of Bowman et al. (1981).

Labeling of Cellular Components. Phosphoproteins were labeled in cells cultured in tissue culture dishes. At a cell density of (2–2.5) × 10^6 cells/150-mm-diameter tissue culture dish, the growth medium was replaced with 25 mL of phosphate-free Earle's MEM containing 5% phosphate-free fetal calf serum, and 0.1 mCi [^{32}P]orthophosphate was added for 16 h.

To label nucleic acids, cells were cultured in 150-mm tissue culture dishes to approximately 3 × 10^6 cells/plate, and either [^{3}H]thymidine (0.02 μCi/mL) or [^{3}H]uridine (0.05 μCi/mL) was added for 16 h.

Gel Electrophoresis of Protein and RNA. Protein electrophoresis was carried out in discontinuous sodium dodecyl sulfate (SDS) gel system (PAGE) with 10% or 12% polyacrylamide (Laemmli, 1970). Molecular weight standards included lysozyme, β-lactoglobulin, α-chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase *b*, and myosin (H chain). After electrophoresis, proteins were stained with either 0.25% Coomassie Brilliant Blue or with silver (Wray et al., 1981). To detect the presence or absence of C23 or lamin C, the contents of the gel were electroblotted to activated 2-aminophenyl thioether (APT) paper and treated with appropriate antibodies followed by the binding of ^{125}I protein A (Amersham, as in Symington et al., 1981). Antibodies to C23 and lamins A and C were respectively generous gifts from Dr. M. Olson, University of Mississippi Medical Center, and Dr. L. Gerace, Johns Hopkins University. The two-dimensional protein electrophoresis was done by the nonequilibrium pH gradient electrophoresis method of O'Farrell et al. (1977) using 2% ampholines, pH 3.5–10 (LKB).

Small nuclear RNAs were separated in 10% polyacrylamide gel at a constant voltage of 150 V for 20 h (Bolla et al., 1985). The RNAs were stained with 0.5 μg/mL ethidium bromide and the gels photographed. Photographic negatives of protein or RNA gels were analyzed by densitometry.

Table I: Macromolecular Contents of Nucleolar Fractions[a]

	protein		DNA	RNA
	μg/10^6 cells	%	(%)	(%)
whole cells	136	100	100	100
nuclei	33.2	24.4	100	9.5
DNase treated				
nucleoli	8.9	6.5	3.7	6.7
core nucleoli	3.3	2.4	2.3	2.4
nucleolar matrix	0.32	0.24	0.2	1.1
micrococcal nuclease treated				
core nucleoli	4.5	3.3	23	1.7
nucleolar matrix	0.75	0.55	0.5	0.17

[a] Fractions were prepared as in Experimental Procedures. Protein content was determined on each fraction with the Folin reagent. DNA and RNA were estimated by first labeling the cells for 18 h with [^{3}H] precursors (Experimental Procedures) and then preparing the fractions and measuring the percent of total acid-insoluble counts/min in each fraction. The results are given for one experiment; replicate experiments agree to within ±20% of each value.

Table II: Protein Content of Nucleoli Isolated after DNase I Treatments

	DNase I (μg/mL)[a]			
protein	20	50	100	200
nucleoli (μg/10^6 cells)	23.4	8.9	12.2	10.8
core nucleoli (μg/10^6 cells)	6.3	3.3	8.6	9.6
nucleolar matrix (μg/10^6 cells)	4.3	0.32	4.0	2.2

[a] In each case, nucleoli were treated twice with the indicated levels of DNase I to yield core nucleoli. These were then fractionated with 2 M NaCl and centrifuged 5 min at 100*g* to obtain the nucleolar matrix (see Experimental Procedures). Comparative data from a single experiment.

Results

Core nucleoli contain a "protected" rDNA NTS fraction, as well as ribosomal precursor RNAs, specific nucleolar proteins, and snRNAs; and nucleolar matrix components can be separated from many nonmatrix ones. The macromolecular content of various fractions characterized below is summarized in Table I.

Proteins of Core Nucleoli. When nucleoli were treated with 20 or 50 μg/mL DNase I, the protein content decreased, but when higher levels of DNase I were used to prepare core nucleoli, the resulting fractions unexpectedly contained *more* protein (Table II). A standard preparation with 50 μg/mL DNase I was therefore adopted. It reproducibly yielded core nucleoli with a minimum protein content, and nucleolar preparations using micrococcal nuclease instead of DNase I

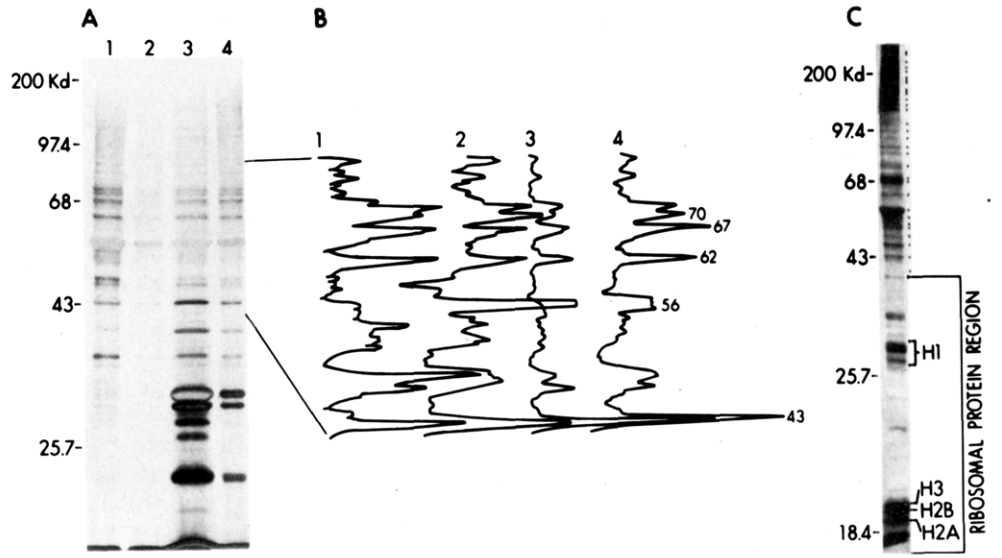

FIGURE 1: Proteins associated with core nucleoli and nucleolar matrix. A: 10% SDS-PAGE with a 5% stacking gel for 18 h at 85 V, with marker protein positions as indicated. (Lane 1) Proteins from nucleolar matrix sedimented at 2000*g* after 2 M NaCl treatment. (Lane 2) Proteins from nucleolar matrix sedimented at 100*g* after 2 M NaCl treatment. (Lane 3) proteins solubilized from core nucleoli by 2 M NaCl. (Lane 4) proteins associated with unfractionated core nucleoli. Each lane contains 75 μg of protein, starting from core nucleoli prepared with 50 μg of DNase I. B: Densitometric tracings of gel lanes 1–4. Densitometry patterns were normalized to the content of the band at 43 kDa, which showed the same content per microgram protein in core nucleoli and the subfractions. Enhancement of the lamin protein peaks (70, 67, and 62 kDa) is evident by comparison of lanes 1 and 2 vs. lane 3. C: Nucleolar matrix proteins separated on a higher resolution polyacrylamide gel (10% SDS-PAGE at 85 V for 72 h). Dots mark the protein bands of apparent high molecular weight enriched in the nucleolar matrix preparation. The ribosomal protein region and the histones are indicated.

yielded nucleoli with a very similar protein content (see below).

When the maximal concentrations of protease inhibitors were included in all preparative buffers, core nucleolar proteins showed a very characteristic PAGE pattern. One-dimensional analyses of core nucleolar proteins and nucleolar proteins extracted with 2 M NaCl proteins are shown in Figures 1 and 2. Essentially identical PAGE patterns were obtained with fractions from mouse liver, cultured Chinese hamster ovary cells, mouse myeloma cells, human HeLa cells, and rat hepatoma cells (data not shown). This facilitated analysis of the nucleolar fractions, since a number of proteins could be identified by comparing their mobility, intensity, immunological reactivity, and degree of phosphorylation with earlier studies and with proteins from purified chromatin and ribosomes. Figure 1 indicates the positions of histones H1, H2a and b, and H3, identified by their mobilities and relative intensities compared to those of histones isolated from chromatin (Olson & Thompson, 1983). Ribosomal proteins were tentatively identified by direct comparison of their mobilities to proteins purified from cytoplasmic ribosomes. All of the ribosomal proteins were included along with the histones in the bracketed region (Figure 1C). Another group of core nucleolar proteins ranged mainly from 43 to 200 kDa in molecular weight (indicated by dots, Figure 1C). Specific proteins were further characterized as follows.

Previous studies of nucleolar proteins have not displayed PAGE separation of proteins larger than 100000 kDa in detail, but for the smaller proteins (Figures 1–3), the pattern in both one- and two-dimensional gels corresponded in general to those reported for Novikoff rat hepatoma (Lischwe et al., 1979, 1982; Olson & Thompson, 1983). In the region of histone H1, two protein bands are seen in all cell types examined except for rat hepatoma, which shows three bands at that position. Also, both proteins C23 and B23, previously identified as the most prominent nonhistone, nonribosomal proteins in the preparations of Busch and co-workers (Lischwe et al., 1979, 1981; Mamrack et al., 1979), are present, but in much less dominant amounts in the core nucleoli.

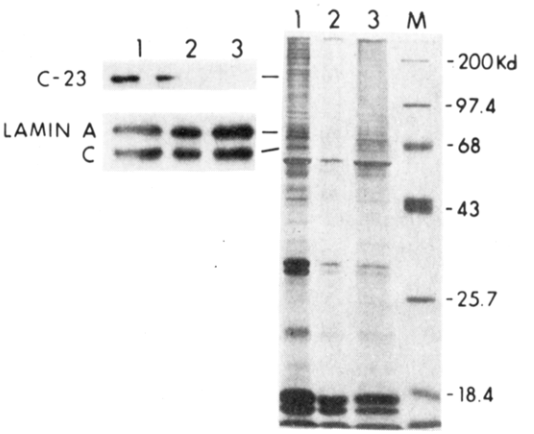

FIGURE 2: Identification of proteins from nucleolar fractions including core nucleoli and nucleolar matrix. Proteins from core nucleoli (lane 1), proteins solubilized from core nucleoli by 2 M NaCl (lane 2), and proteins remaining in a nucleolar matrix fraction (lane 3) were separated by SDS-PAGE on a 10% gel and were electroblotted to APT paper. After blotting, the gel was stained with silver (at right). Replicate blots were then reacted with antibody to protein C23 or to lamins A and C, followed by [^{125}I] protein A (30 μCi/mg; 0.5 μCi/assay). Blots were then autoradiographed at −70 °C for 18 h; relevant portions of the blots are shown at the left.

The most highly phosphorylated protein species in rat hepatoma nucleoli, protein C23, was identified as a silver-stained or Coomassie Blue stained species or by immunological reaction in the mouse L cell core nucleoli (Figure 2C). This identification is consistent with previous analyses (Olson & Thompson, 1983).

Core nucleoli prepared by digestion with micrococcal nuclease showed essentially the same content of protein and a PAGE pattern identical with core nucleoli prepared with DNase I. It is notable, however, that the content of protein C23 was significantly higher in preparations made with micrococcal nuclease and was in fact comparable to that seen in rat hepatoma nucleoli isolated by standard procedures [data not shown; cf. Olson and Thompson (1983)]. The presence

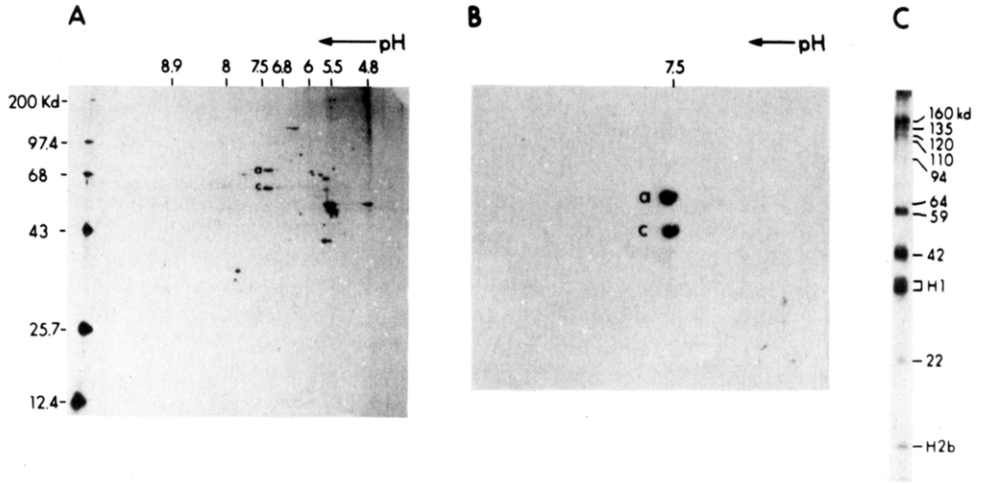

FIGURE 3: Two-dimensional gel electrophoresis of proteins of the nucleolar matrix fraction (A); identification of lamins A and C by immunoblotting of the gel (B); and identification of some phosphorylated proteins in core nucleoli (C). The nucleolar matrix proteins were isolated as described in Experimental Procedures. Proteins were separated in the first dimension by nonequilibrium pH gradient electrophoresis in 5% tube gels containing ampholines for the 3.5–10 range (arrow indicates direction of electrophoresis). The second dimension was in 10% SDS–polyacrylamide gels with a 4% stacking gel. Marker sizes are indicated. A: Silver-stained gel. Positions of lamins A and C are indicated. B: Gel was blotted to APT paper and reacted with antibodies to lamins A and C followed by the binding of [^{125}I] protein A (30 μCi/mg; 0.5 μCi/assay). Autoradiography was at 70 °C for 18 h, a = lamin A, c = lamin C. C: Nucleolar matrix proteins labeled with [^{32}P]. Mouse L cells were labeled overnight with [^{32}P] and core nucleoli isolated and treated with 50 μg/mL of RNase A. The proteins remaining in the core nucleolar fraction were then separated by electrophoresis on a 12% SDS–polyacrylamide gel. Autoradiography was at room temperature for 12 h. The position of histones H1 and H2b and of the molecular weights of other major phosphorylated proteins are indicated.

of lamins A and C was confirmed by reaction with a specific antibody for mammalian lamins A and C (Gerace & Blobel, 1980). As expected, two bands, 68 and 70 kDa in apparent mobility (Lebel & Raymond, 1984; Gerace & Blobel, 1980), were seen (Figures 2 and 3). The numbers and PAGE mobilities of major phosphorylated proteins were consistent with previous results (see below).

Fractionation with Hypotonic Buffer or 2 M NaCl. Many solubilizing reagents and fractionation methods failed to further fractionate core nucleoli. Metrizamide density gradients (Higashinakagawa et al., 1979), percoll fractionation of nuclei prepared by polyamine separation (Kistler et al., 1984), and treatment with 3,5-diiodosalicylic acid (LIS) (Mirkovitch et al., 1984), Nonidet P-40, sodium deoxycholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), or heparin (Mirault and Scherrer, 1972) gave little or no release of proteins into a form nonsedimentable at 3000*g* in 5 min from the core nucleolar fraction.

In contrast to the other methods, fractionation by washing core nucleoli with buffer A containing 2 M NaCl, a treatment used by several groups to define "nuclear matrix" (Berezney & Coffey, 1977; Capco et al., 1982; Mirkovitch et al., 1984), invariably produced two distinct fractions. After the addition of 2 M NaCl, about 10% of the core nucleolar protein sedimented at 100*g* in 5 min (see Table II). Another 40% of the nucleolar protein was sedimented by centrifugation at 1500*g* for 10 min. The PAGE patterns of proteins from these two successive sedimentable fractions were essentially indistinguishable (Figure 1, lanes 1 and 2; Figure 2, lane 3).

In contrast, the fraction of proteins that remained soluble after 2 M NaCl extraction showed a very different protein composition (Figure 1, lane 3; Figure 2, lane 2). Compared to the insoluble nucleolar matrix, the soluble phase was highly enriched for histones, ribosomal proteins, and nucleolar proteins C23 and B23 (Figure 1, lane 3; Figure 2, lane 2). To facilitate quantitative comparisons between the fractions, a portion of each lane containing a well-resolved molecular weight range of proteins was traced on a densitometer. The densitometer patterns were normalized to the content of the band at 43 kDa, which distributes equally, per microgram of protein, in the nucleolar matrix and soluble fractions.

The enrichment of protein C23 in the soluble phase was confirmed by immunological assays. Proteins, separated on a replicate gel to that of Figure 2, were transferred to APT paper and probed with radioactive antibody specific to protein C23; the protein found in unfractionated core nucleoli (lane 1) is quantitatively found in the soluble phase (lane 2) and absent from the nucleolar matrix (lane 3).

A number of proteins are specifically enriched in the nucleolar matrix. By inspection of Figure 1A, these include a 34-kDa protein, which may be the same as the nucleolar protein thought to bind to small nuclear RNA U3 (Lischwe et al., 1985); a 57-kDa protein; and lamins A and C (70 and 67 kDa). Although lamins still appeared as protein bands and by Western blotting (Figure 2) in both soluble and matrix fractions, they were significantly enriched in the matrix.

The most extensive group of proteins enriched in the nucleolar matrix are those included at high molecular weights (>70 kDa). In a somewhat more resolving PAGE analysis (10% SDS-PAGE gel, 35 cm long) shown in Figure 1C, dots have been placed at the positions of more than 25 protein bands enriched in the matrix fraction.

A characteristic separation of nucleolar matrix proteins was seen by two-dimensional gel electrophoresis (Figure 3A). The group of low-mobility proteins (apparently M_r > 70 kDa) included highly acidic proteins having an apparent p*I* of 4.8 or less, as well as a 160 kDa/p*I* 5.5 protein and a 110 kDa/p*I* 6.8 protein. The p*I* of the highly acidic proteins was found to be less than 3.5 in additional two-dimensional gels by using the isoelectric focusing method of O'Farrell (1975) with ampholines in the range of 2.5–5. The major proteins at $M_r \leq$ 70 kDa include lamins A and C [70 kDa/p*I* 7.5 and 60 kDa/p*I* 7.5, respectively (Gerace & Blobel, 1980)], as well as 43 kDa/p*I* 5.8, 36 kDa/p*I* 7.9, and 34 kDa/p*I* 7.9 proteins. The locations and identity of lamins A and C in nucleolar matrix were verified with a specific antibody test (Figure 3B). Probing the same gel with radioactive antibody specific for protein C23 gave no reaction, again consistent with the finding that C23 is quantitatively released from the nucleolar matrix by the 2 M NaCl extraction.

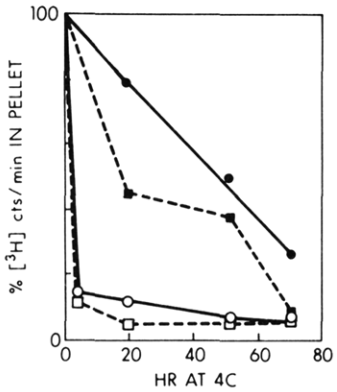

FIGURE 4: DNA and RNA content of core nucleoli from cells labeled with [^{3}H]thymidine or [^{3}H]uridine. Release of DNA and RNA from core nucleoli by incubation in hypotonic buffer. Core nucleoli were prepared from cells labeled with [^{3}H]thymidine (○ and ●) or [^{3}H]uridine (□ and ■) by treatment twice with 50 μg/mL of DNase I (filled symbols) or with 80 A_{260} units of micrococcal nuclease (open symbols).

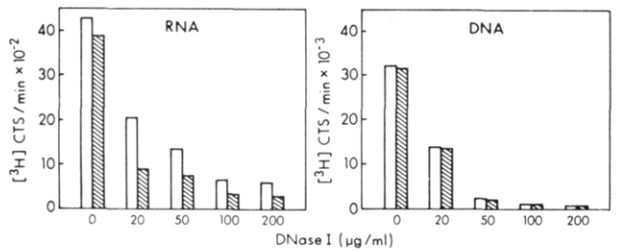

FIGURE 5: DNA and RNA concentration in core nucleoli as a function of the concentration of DNase I in the presence and absence of PMSF and leupeptin. Core nucleoli were prepared by two incubations with the indicated concentrations of DNase I with (open bar) or without (hatched bar) added protease inhibitors.

We considered the possibility that the very high net acidity of the high molecular weight proteins resulted from extensive phosphorylation. However, Figure 3C shows that only a few nucleolar matrix proteins were phosphorylated with ^{32}P in vivo. One prominent phosphorylated protein, 160 kDa, was certainly that detected earlier in rat hepatoma preparations (Olson & Thompson, 1983; it is probably the prominent spot at that molecular weight and p*I* in Figure 3C). In addition, protein with the mobility expected for histones H1 and H2b, as well as some ribosomal proteins, showed levels of ^{32}P-labeling comparable to those observed by Olson et al. (1974). Most of the proteins with very low p*I* showed little if any phosphorylation.

The acidic behavior of these proteins remained unchanged after extensive treatment with RNase or with endo-β-*N*-acetylglucosaminidase F (work in progress), so that their acidity is unlikely to arise from glycosylation or bound RNA.

A second method of washing core nucleoli (EDTA–hypotonic salt solutions at 4 °C for 2 h, essentially as described by Busch and co-workers, Rothblum et al., 1977; Daskal et al., 1978) also solubilized some of the proteins (25.1% ± 4.3%, N = 4) and a fraction of the RNA (Figure 4). With this method, the dissociation was much more complete when core nucleoli were prepared by micrococal nuclease treatment. Within 1 h, 87.8% ± 2.9% (N = 4) of the protein and a comparable amount of the DNA and RNA of the core nucleoli (Figure 4) were no longer sedimentable by centrifugation for 10 min at 3000*g*. The soluble protein pattern and the matrix-associated proteins, however, showed very similar PAGE patterns in this case (data not shown).

RNA Content of Nucleoli. Concentrations of DNA and RNA in core nucleoli decrease depending on the amount of DNase I used for preparation (Table I; Figure 5; and Bolla et al., 1985). The amount of RNA was consistently 30%–50% less in the absence of protease inhibitors (Figure 5), which were included in the standard preparations.

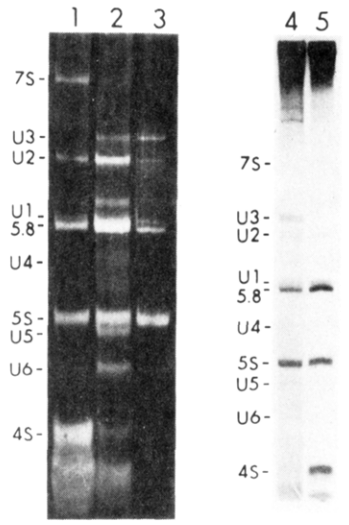

FIGURE 6: Small nuclear RNAs from L-cell cytoplasm, nuclei, nucleoplasm, and core nucleoli. Small nuclear RNAs were separated from total RNA by electrophoresis in 10% polyacrylamide gels containing 6 M urea. At left, gel stained with ethidium bromide: (lane 1) snRNA in 215 μg total of cytoplasmic RNA; (lane 2) snRNA in 490 μg of nucleoplasmic RNA; (lane 3) snRNA in 120 μg of core nucleolar RNA. At right, fluorogram (Bonner & Laskey, 1974) of snRNAs isolated from core nucleoli (lane 4; 100 μg) or cytoplasm (lane 5; 299 μg) of mouse L cells labeled with [^{3}H]uridine and separated by electrophoresis on 10% polyacrylamide/6 M urea gels. Note absence of snRNAs U1, U4, and U6 and 7S RNA in core nucleoli.

Core nucleoli retained intact pre-rRNA species (Bolla et al., 1985) and nucleolar snRNAs. Figure 6 compares the electrophoresis of snRNAs from cytoplasm, nucleoplasm freed of nucleoli by centrifugation, and core nucleoli. The levels in the cytoplasm (lanes 1 and 5) should be increased about 25-fold to compare the relative amounts of different species with nuclear fractions. Thus, about 95% of the cellular tRNA, 5S rRNA, and 5.8S rRNA were found in the cytoplasm, as expected. Certain other prominent species of snRNA, including 7S and tRNA species, were undetectable in core nucleoli (lanes 3 and 4), whereas U3 snRNA, considered to be nucleolar-specific (Epstein et al., 1984; Reddy & Busch, 1981), was indeed enriched as compared to cytoplasmic (lane 1) or nucleoplasmic (lane 2) RNA. Some preparations of snRNAs from crude nucleoli (before DNase I treatment) showed patterns similar to Figure 6, lanes 3 and 4 (cf. also Epstein et al., 1984), but, in general, crude nucleoli contained variable levels of predominantly nucleoplasmic species (U1, U2, and U4 snRNAs, for example). These were selectively reduced in core nucleoli compared to U3 snRNA. We estimate that 60%–80% of the cellular U3 snRNA was associated with the core nucleolar fraction.

Washing of core nucleoli with 2 M NaCl released essentially all the complete pre-rRNA and rRNA chains along with about 80% of the U3 snRNA to the soluble fraction, as indicated by gel electrophoresis (data not shown).

Discussion

DNase I treatment during the preparation of nucleoli by standard procedures removed the transcribed portion of rDNA, making studies of transcription impossible. Nevertheless, these preparations have some advantages for the study of nucleolar organization. First, the yields of nucleoli were as much as 60%

greater than by sonication alone, an advantage especially when cultured cells are being used. Second, most of the components identified as being enriched in nucleoli (Olson & Thompson, 1983; Lischwe et al., 1981; Epstein et al., 1984) were selectively retained and enriched further in core nucleoli—as were the segments of nontranscribed spacer rDNA protected against DNase I action (Thomas et al., 1985). Third, a number of nonnucleolar components were sharply reduced or totally eliminated in the core nucleolar preparations.

Preparations with or without protease inhibitors added were equivalent for the study of the protected fraction of DNA, but without protease inhibitors present throughout the procedure, proteins were appreciably degraded and the content of RNA was decreased. The DNA content of core nucleoli prepared with micrococcal nuclease or DNase I was very comparable (Bolla et al., 1985). However, the dissociation of micrococcal nuclease prepared core nucleoli in hypotonic buffer was much more complete. This could facilitate the isolation of large amounts of nucleolar-associated proteins, including the putative nucleolar matrix proteins, in a soluble form. Also, preparations made with micrococcal nuclease retained much higher levels of protein C23 than did core nucleoli prepared with DNase I. C23 may bind either to perinucleolar chromatin or to the nontranscribed region of rDNA (Olson et al., 1983) or to both. Further characterization of the DNA fraction is required to determine the binding site for C23 and whether C23 binding sites are selectively digested by DNase I compared to micrococcal nuclease.

Although the steps employed here were adapted to mouse L cells, this method gave very comparable results with a number of other cultured cells, and we infer that the composition of core nucleoli is probably similar in a variety of mammalian cells.

The fractionation of core nucleoli with 2 M NaCl extraction can be operationally useful, but the relation of the fractions obtained to the structure of nucleoli in vivo remains unclear. The 2 M NaCl insoluble fraction almost certainly corresponds to the nucleolar subfraction of the "nuclear matrix". Nuclear matrix has been suggested to be an organizing ground substance to which other nuclear components are directly or indirectly attached (Berezney & Coffey, 1977; Capco et al., 1982). Consistent with this notion are, first, the relative enrichment of a subclass of nucleolar proteins in the nucleolar matrix (Figure 1–3). These proteins include a 110-kDa protein [which shows properties distinct from these reported for protein C23 and topioisomerase I (Lischwe et al., 1979; Durban et al. 1981)]; a 43 kDa/p*I* 5.8 protein; and a 36 kDa/p*I* 7.9 protein. In addition, a 34 kDa/p*I* 7.9 protein, which may correspond to the fibrillarin described by Lischwe et al. (1985) as the protein which binds snRNA U3, was observed. Second, one proposed constituent of the nuclear matrix is lamin, and Western blot analysis with antilamin antibody shows that this protein is present in the nucleolar matrix fraction as well. In addition, the 70-kDa heat shock protein, which has been detected in nucleoli with specific antibody (Lowe & Moran, 1984; Pelham, 1984; Munro & Pelham, 1984), was also present in the core nucleoli (work in progress). Third, dot-blot hybridization and pulse-chase studies or nucleolar RNA were consistent with the idea that nascent RNA chains are selectively bound to nucleolar "matrix" fractions (cf. Jackson et al., 1982; Ciejek et al., 1983). In dot-blot hybridization analyses, DNA fragments corresponding to all portions of the rDNA transcription unit were found in the fraction solubilized from core nucleoli by 2 M NaCl, but the RNA sequences corresponding to the initial transcribed spacer portion of the 45S pre-rRNA (clone 3 of Bowman et al., 1983) were enriched 4-fold in the 10% of the total RNA that remained bound to the nucleolar matrix (work in progress; Bolla et al., 1985).

Also consistent with an organized structure for core nucleoli and nucleolar matrix, these fractions appeared in the electron microscope as a network of fibrils with a diameter of about 10–50 nm (data not shown; cf. Capco et al., 1982).

It is difficult, however, to exclude the alternative that the matrix residue does not underlie the nucleolar structure and does not exist in vivo, but forms only in the conditions of high salt extraction used for its preparation. This possibility seems more complicated. However, it is suggestive that washing with low salt buffer and EDTA produced far less protein fractionation and that the nucleolar matrix contained larger more insoluble and highly negatively charged proteins which would indeed be more easily salted out of solution. It should be possible to analyze the interactions involved in detail when the extracted matrix proteins are further characterized.

The nucleolar matrix proteins which include so many highly acidic, high molecular weight species were unanticipated. The reason for the extremely acidic nature of these proteins remains uncertain, but we have found that in spite of their net acidity they bind very strongly to DNA–sepharose columns (work in progress). One possibility to be tested is that these proteins show the high content of sequences of dimethylarginine and glycine characteristic of several nucleolar proteins (Lischwe et al., 1985). Basic and acidic portions within each large protein might then be differentially involved in binding to DNA and to other proteins in the nucleolar structure.

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Processivity of T4 Endonuclease V Is Sensitive to NaCl Concentration†

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ABSTRACT: We previously reported that endonuclease V of bacteriophage T4 reacts processively with pyrimidine dimers in UV-irradiated DNA, tending to react with all of the dimers on one DNA molecule before reacting with any dimers on another DNA molecule [Lloyd, R. S., Hanawalt, P. C., & Dodson, M. L. (1980) *Nucleic Acids Res. 8*, 5113–5127]. In this paper we show that this processivity depends upon salt concentration: it can be detected in 10 mM NaCl but not, by our methods, in 100 mM NaCl. In addition, we show that endonuclease V binds to unirradiated DNA in 10 mM NaCl but not in 100 mM NaCl. We conclude that T4 endonuclease V binds to pyrimidine dimers in a two-step process in 10 mM NaCl. It first binds electrostatically to undamaged sections of DNA, and it remains bound during the second step in which it "searches" for pyrimidine dimers. Our conclusion is analogous to the expanded target theory developed for *Lac* repressor [Berg, O. G., Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry 20*, 6929–6948].

T4 endonuclease V, coded by the *denV* gene of bacteriophage T4, comprises a DNA glycosylase that specifically recognizes pyrimidine dimers in DNA and an AP[1] endonuclease that recognizes apurinic and apyrimidinic sites. DNA containing pyrimidine dimers is incised by the sequential action of the glycosylase and the AP endonuclease (Gordon & Haseltine, 1980; Radany & Friedberg, 1980; Seawell et al., 1980; Nakabeppu & Sekiguchi, 1981).

Previously we reported that when limiting amounts of endonuclease V were incubated under appropriate conditions with superhelical DNA containing several pyrimidine dimers per molecule, partial digests contained a mixture of intact, unnicked DNA molecules and completely digested molecules

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[1] Abbreviations: AP, apurinic or apyrimidinic; N-AAAF, 2-[*N*-(acetoxyacetyl)amino]fluorene; 4-NQO, 4-nitroquinoline 1-oxide.

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