Preribosomal Ribonucleoprotein Particles Are a Major Component of a Nucleolar Matrix Fraction†

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Received July 16, 1985

ABSTRACT: Biochemical and morphological studies were performed on Novikoff hepatoma ascites cell nucleolar matrix fractions prepared by deoxyribonuclease I digestion and high-molarity salt extractions essentially according to a published method [Berezney, R., & Buchholz, L. A. (1981) *Exp. Cell Res. 20*, 4995–5002]. The nucleolar matrix fraction was enriched in polypeptides of molecular mass of 28, 37.5, 40, 70, 72, 110 (protein C23), and 160 kDa, compared to the nuclear fraction in which polypeptides of molecular mass of 31, 33.5, 43.5, 46, 50, 56, and 59 kDa were predominant. About one-fourth of the protein, half of the RNA, and less than 4% of the DNA originally present in the nucleoli remained in the matrix fraction. Addition of single agents such as ethylenediaminetetraacetic acid, ribonuclease A, or mercaptoethanol during preparation had no significant effect on the polypeptide composition of the nucleolar matrix fraction. However, the combination of mercaptoethanol and ribonuclease A caused most of the RNA and protein to be removed, including protein C23 and the 160-kDa polypeptide, with polypeptides in the range of M_r 30 000–50 000 remaining. Electron microscopy of nucleolar matrix fractions revealed the presence of particles similar in size to the granular elements of nucleoli. However, when ribonuclease A and mercaptoethanol were included in the procedure, only amorphous material remained. Many proteins of nucleolar preribosomal RNP particles were also associated with the nucleolar matrix fraction. RNA from the nucleolar matrix fraction was enriched in sequences from 18S and 28S ribosomal RNA. These results indicate that preribosomal RNP particles are major constituents of a nucleolar matrix fraction prepared by the deoxyribonuclease I–high-molarity salt method.

A variety of studies suggest that an underlying protein network contributes to the structure of the cell nucleus. This substructure has been termed the nuclear matrix (Berezney & Coffey, 1974), the nuclear scaffold (Adolph, 1980), the nuclear cage (Cook & Brazell, 1980), or the interphase genome skeleton (Hancock, 1982). In addition to the maintenance of the three-dimensional structure of the nucleus, evidence for a more dynamic role for a nuclear matrix has been presented; e.g., DNA replication appears to take place in association with a nuclear matrix (Berezney & Buchholtz, 1981b; Pardoll et al., 1980), and DNA polymerase has been found tightly associated with nuclear matrix preparations (Smith & Berezney, 1980). In addition, transcriptionally active genes are associated with a nuclear matrix (Jackson et al., 1981; Robinson et al., 1982, 1983) as is heterogeneous nuclear RNA (Ciejek et al., 1982; Herman et al., 1978; Miller et al., 1978; van Eekelen & van Venrooij, 1981) and small nuclear RNAs (Long & Ochs, 1982; Miller et al., 1978). However, the actual polypeptides that constitute a nuclear matrix have not been defined.

Little is known about the nucleolar portion of the putative nuclear matrix although it has been shown that spacers (Bolla et al., 1985) and actively transcribed and rDNA[1] sequences are associated with a nuclear matrix (Randall & Vogelstein, 1980). However, a striking phenomenon is that a residual nucleolus remains when nuclear matrix is prepared by digestion of nuclei with deoxyribonuclease I and extraction with high-molarity salt without the aid of ribonuclease (Berezney & Buchholtz, 1981a; Bouvier et al., 1980). Thus, the three-dimensional structure of the nucleolus appears to be dependent to a large extent on intact ribonucleoprotein particles and/or nuclear matrix, and to a lesser extent on the chromatin component of the nucleolus.

At this point the protein constituents of the putative nucleolar matrix are only partially identified. Franke et al. (1981) have reported the presence of a predominant high molecular weight polypeptide (M_r 145 000) in the nucleolar skeleton or matrix of *Xenopus* oocyte nucleoli. Olson & Thompson (1983) found a protein of somewhat higher molecular weight (160 000) and a polypeptide that comigrated with nucleolar protein C23 (M_r 110 000) in the Novikoff hepatoma ascites cell nucleolar matrix fraction. The presence of protein C23 in the nuclear matrix (chromatin-depleted nuclei) was confirmed by Long & Ochs (1982) using immunocytochemical methods.

In view of the uncertainty regarding the existence and/or the nature of the nucleolar matrix, these studies were undertaken to (1) compare the polypeptide compositions of nucleolar and whole nuclear matrix fractions prepared under the same conditions, (2) determine whether RNA or RNP particles are significant constituents of the nucleolar matrix fraction, and (3) identify protein components of the nucleolar

† This work was supported in part by Grant GM28349 to M.O.J.O. from the National Institutes of Health and a Pharmaceutical Manufacturers Association Inc. postdoctoral fellowship to L.M.-C.

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[1] Abbreviations: DNase I, deoxyribonuclease I; DRB, 5,6-dichloro-1-β-ribofuranosylbenzimidazole; DTT, dithiothreitol; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; NP-40, Nonidet P-40; PBS, phosphate-buffered saline (0.15 M NaCl and 0.01 M sodium phosphate, pH 7.2); PCA, perchloric acid; PMSF, phenylmethanesulfonyl fluoride; RNase A, ribonuclease A; RNP, ribonucleoprotein; SDS, sodium dodecyl sulfate; SSC, standard saline citrate containing 0.15 M NaCl and 0.017 M sodium citrate (pH 7.0); TCA, trichloroacetic acid; rDNA, DNA derived from a gene for preribosomal 45S RNA; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

0006-2960/86/0425-0484$01.50/0

matrix fraction. It was found that the nucleolar and nuclear matrix fractions differ significantly in their polypeptide compositions and that ribosomal RNA is a major component of the nucleolar matrix fraction. Futhermore, several of the nucleolar matrix fraction polypeptides appear to be derived from RNP elements with only a few polypeptides in the M_r 30000–50000 range remaining after removal of RNA. These studies suggest that preribosomal RNP elements are major constituents of the nucleolar matrix fraction prepared by DNase I digestion and high-molarity salt extraction.

Experimental Procedures

Animals, Cells, and Cell Fractions. Novikoff hepatoma ascites cells, grown in male Sprague-Dawley rats (locally bred in The University of Mississippi Medical Center Animal Facilities), were harvested 6 days after transplantation. The Novikoff hepatoma cells were used for preparing nuclei and nucleoli. Nuclei were prepared by the Nonidet P-40 (NP-40) detergent method (James et al., 1977). Nucleoli were isolated by sonication with the magnesium–sucrose method essentially according to the method of Rothblum et al. (1977) with the exception that 0.5 mM dithiothreitol (DTT) was added to all solutions. Nucleolar preparations were monitored by light microscopy and were essentially free of extranucleolar contamination. Nucleoli that were not used immediately were stored in a buffer containing 50% glycerol, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), and 10 mM Tris (pH 8.0).

Preparation of Nuclear and Nucleolar Matrix Fractions. Nuclei or nucleoli were extracted essentially according to the method used for rat liver by Berezney & Buchholtz (1981b) with minor modifications. Briefly, nuclei or nucleoli were suspended at a concentration of 20 A_{260} units/mL in a digestion buffer containing 0.2 M sucrose, 50 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.4 mM PMSF, and 5 mM PIPES buffer (pH 7.5). Nuclei or nucleoli were washed 2 times in the digestion buffer, centrifuging after each wash for 10 min at 10000*g*. After resuspension at the original concentration, digestion was carried out with deoxyribonuclease I (DNase I) at a concentration of 10 μg/mL at 0–4 °C for 30 min. The above procedure is designated as the standard method. In some experiments ribonuclease A (RNase A) at 100 μg/mL was added to the digestion mixture. The digest was centrifuged as above, and the pellet was subjected to three extractions with a high-salt buffer (HS) containing 2 M NaCl, 0.2 mM $MgCl_2$, 0.4 mM PMSF, 0.4 mM EPNP (Lindsey et al., 1981), and 10 mM Tris (pH 7.4). In some experiments, either 1% 2-mercaptoethanol or 10 mM EDTA was included in the HS washes. The pellet was extracted once with a low-salt–Triton buffer (TR) containing 0.2 mM $MgCl_2$, 0.4 mM PMSF, 0.4 mM EPNP, 1% Triton X-100, and 10 mM Tris (pH 7.4) and twice with a low-salt buffer (LM) that did not contain Triton X-100.

Preparation of Nucleolar Preribosomal RNP Particles. Nucleoli were extracted essentially according to the procedure of Auger-Buendia & Longuet (1978) by suspension at a concentration of 20 A_{260} units/mL at 15 °C for 20 min in a buffer containing 10 mM NaCl, 10 mM DTT, 100 μg/mL yeast RNA, and 10 mM Tris (pH 8.0). The mixture was centrifuged at 10000*g* for 10 min, and the supernatant solution was used for sucrose density gradient centrifugation. In some experiments the pellet after the RNP extraction was used to prepare the nucleolar matrix fraction as described above. The RNP extract was applied in 0.4-mL aliquots to linear sucrose gradients (10–40%) and centrifuged for 2 h at 41 000 rpm in a Beckman SW41 rotor. The gradients were fractionated into 0.8-mL fractions with an Isco fractionator and analyzed by Laemmli-type polyacrylamide gel electrophoresis.

Analytical Methods. Polyacrylamide gel electrophoresis was run in SDS according to the method of Laemmli (1970) using 10, 12.5 or 15% polyacrylamide. For dilute samples such as sucrose gradient fractions or extracts of nuclei or nucleoli, the protein was precipitated with 10% TCA after addition of 20 μg/mL poly(adenylic acid) as carrier, and the precipitates were washed with ethanol and ethanol–ether as described by Valenzuela et al. (1976) prior to dissolution in sample buffer.

DNA was determined by the diphenylamine method essentially according to Burton (1968), scaling down all reagents and solvents. RNA was determined by the orcinol method (Hurlbert et al., 1954). Protein was quantitated by using Coomassie blue dye with the Bio-Rad kit (Bradford, 1976).

Electron Microscopy. Pellets of nucleoli or nucleolar matrix fractions were fixed in 2% glutaraldehyde in PBS at 4 °C and processed as previously described (Marshall et al., 1984). The thin sections were stained by floating the grids on a saturated solution of uranyl acetate for 5 min at room temperature.

RNA Extraction and Dot Blot Hybridization. RNA was extracted from nucleoli or nucleolar matrix fractions by the guanidine hydrochloride method of Cheley & Anderson (1984) and applied to nitrocellulose filters in a Schleicher & Schuell Minifold apparatus in the presence of equal volumes of 15% formaldehyde and 20× SSC. Plasmids containing rDNA inserts were originally obtained from Dr. Motohiro Fuke of the Phillips Petroleum Co., Bartlesville, OK, and were amplified and purified as previously described (Olson et al., 1983). The plasmids were labeled by nick translation (Rigby et al., 1977) with [α-^{32}P]dCTP (obtained from ICN). Before hybridization, the filters were treated with 4× SSC at 25 °C for 30 min, followed by a prewash for 3 h at 37 °C in a solution containing 4× SSC, 30% formamide, 10× Denhardt's solution [Denhardt's solution consists of 2% Ficoll, 2% poly(vinylpyrrolidone), and 2% bovine serum albumin], and 0.1% SDS. The filters were then washed for 3 h at 37 °C in a prehybridization mix consisting of the above prewash solution plus 0.1% sodium pyrophosphate. Hybridization was carried out in sealed bags containing the filters and 2 mL of hybridization mix (same as the prehybridization mix plus heat-denatured radioactive probe. The sealed bags were incubated in a shaking water bath for 36 h at 37 °C. After hybridization, the filters were washed in three changes of prehybridization mix, each for 1 h at 37 °C, followed by a brief wash in 2× SSC at room temperature. The filters were then blotted to near dryness and placed in cassettes to expose Kodak XAR-5 X-ray film. The relative densities of the dots on the film were quantitated with a Transidyne densitometer.

Results

In most of the studies described here, nuclei or nucleoli were depleted of chromatin components by DNase I followed by multiple extraction with solutions containing high-molarity salt, Triton X-100, and low-molarity salt essentially according to the method of Berezney & Buchholtz (1981a,b), with minor modifications. Although the published procedure included low-molarity salt extractions following the DNase I digestion, preliminary experiments showed that elimination of these did not result in significant differences in the polypeptide composition of the final product. Therefore, in this study, all matrix fractions were prepared with the exclusion of the initial low-salt wash. The residue after all extractions is operationally defined as the nuclear or nucleolar "matrix fraction". Since the structures and constituents of the nuclear or nucleolar

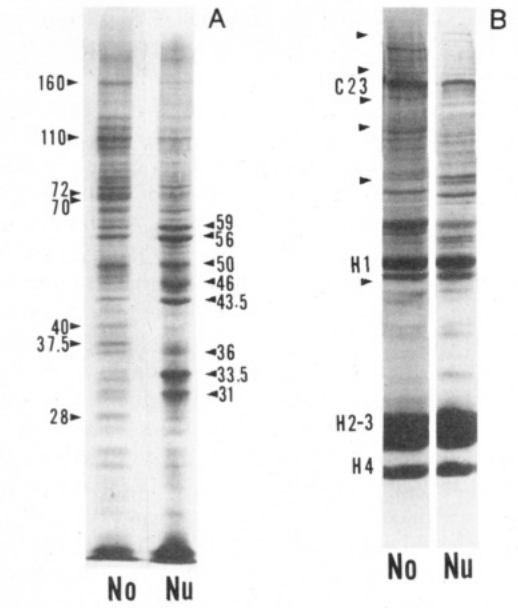

FIGURE 1: Polypeptide compositions of nuclear and nucleolar matrix fractions and of high-molarity salt extracts. Equal amounts (20 A_{260} units) of nuclei (Nu) or nucleoli (No) were treated as described under Experimental Procedures, and the residual or high salt extracted fractions were solubilized in SDS sample buffer and run on Laemmli-type gels containing 12.5% polyacrylamide. (A) Matrix fractions. Each lane represents 25% of the respective matrix fraction from a given preparation. The estimated molecular weights of various polypeptides are given on the right and left. The numbers on the left designate polypeptides enriched in the nucleolar matrix fraction. Numbers on the right indicate polypeptides enriched in the nuclear matrix fraction. (B) Polypeptides removed by high-molarity salt during preparation of nuclear and nucleolar matrix fractions. The high-salt (HS) extract contained 80–90% of the total protein removed and was representative of all of the polypeptides extracted during the procedure. Combined HS supernatants were run on Laemmli-type gels containing 12.5% polyacrylamide. Pointers indicate migration of molecular weight standards (from top): myosin (205 000), β-galactosidase (116 000), phosphorylase B (97 400), bovine serum albumin (66 000), ovalbumin (45 000), and carbonic anhydrase (29 000). Histones are designated by H1, H2-3, and H4. The position of protein C23 is indicated.

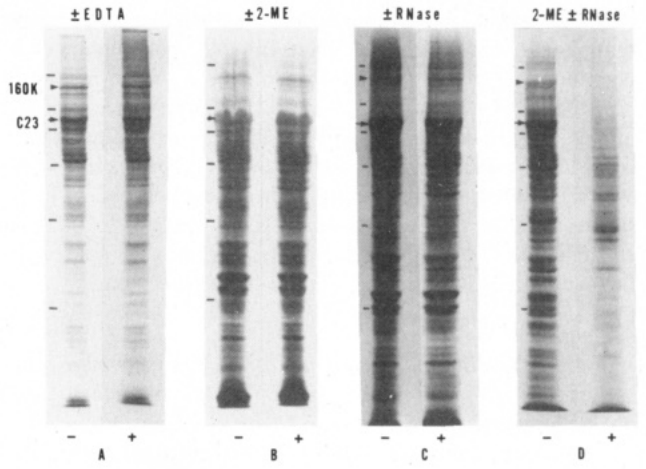

FIGURE 2: Effects of various treatments on the polypeptide composition of the nucleolar matrix fraction. The nucleolar matrix fractions was prepared as in Figure 1 except that various agents were added (+) or omitted (–) during the procedure. Matrix fractions were run on 7.5% Laemmli-type gels as in Figure 1. (A) EDTA (5 mM) was either included or omitted in all extractions. (B) The HS extraction was done in the presence or absence of 1% 2-mercaptoethanol. (C) The digestion was done with or without ribonuclease A. (D) Extraction buffers contained 2-mercaptoethanol, but digestions were done with or without ribonuclease A. Molecular weight standards (small bars) are the same as in Figure 1.

matrix are poorly defined, we prefer to use the former term rather than the latter.

To compare the polypeptide compositions of the chromatin-depleted fractions, nuclei and nucleoli from Novikoff hepatoma ascites cells were subjected to the digestion and extraction procedure under identical conditions, and the insoluble matrix fractions as well as the supernatant fractions were analyzed by Laemmli-type polyacrylamide gel electrophoresis. Although a complete characterization of this fraction should include two-dimensional electrophoretic analyses, the insoluble nature of the matrix fraction did not permit us to achieve adequate separation in the isoelectric focusing dimension. Therefore, all of the studies reported here utilize one-dimensional gel electrophoresis. When the matrix fractions were compared, a large number of polypeptides of identical mobility were found in both nuclei and nucleoli although a number of them were enriched in either the nuclear or nucleolar matrix fraction (Figure 1A). The nucleolar matrix fraction was enriched in polypeptides having molecular masses of 28, 37.5, 40, 70, 72, 110, and 160 kdaltons (kDa). The 110-kDa polypeptide comigrates with protein C23, which was found previously in nucleolar matrix fractions (Long & Ochs, 1982) as has the 160-kDa polypeptide (Olson & Thompson, 1983). An antibody prepared against protein C23 (Olson et al., 1981) reacts with the 110-kDa polypeptide on immunoblots (data not shown), further supporting the presence of protein C23 in the matrix fraction. Polypeptides of molecular mass 31, 33.5, 43.5, 46, 50, 56, and 59 kDa were enriched in the nuclear matrix fraction.

In the process of obtaining the matrix fractions, the vast bulk of the soluble protein was extracted in the high-molarity salt wash with only small amounts of protein present in the DNase I, Triton X-100, or final low-salt supernatants. Figure 1B indicates that most polypeptides in the high-salt supernatant were common to both nuclei and nucleoli with histones being the predominant polypeptides. However, as expected, protein C23 that was previously localized to the nucleolus (Olson et al., 1981; Lischwe et al., 1981) was enriched in the nucleolar high-salt fraction. Repeated extractions beyond the numbers specified under Experimental Procedures section did not result in further removal of significant amounts of protein, suggesting that the extraction procedure had a relatively high degree of selectivity for nonmatrix fraction components.

The large number of polypeptides in the nucleolar matrix fraction suggested that components other than strictly nucleoskeletal elements might be present; therefore, experiments were initiated to determine whether alteration of digestion or extraction conditions would change the polypeptide profile. Identical paired samples of nucleoli were subjected to the standard matrix fraction preparation except that various agents (EDTA, ribonuclease, or mercaptoethanol) were either added or omitted during the procedure. Figure 2A shows that addition of 5 mM EDTA to all of the extractants had little or no effect on the polypeptide profile of the matrix fraction. Similarly, the inclusion of 1% 2-mercaptoethanol in the high-molarity salt extractions (Figure 2B) or ribonuclease A (100 μm/mL) in the digestion (Figure 2C) had no significant effect on the distribution of polypeptides in this fraction. However, the combined addition of RNAse A in the digestion stage and 1% mercaptoethanol in the 2 M NaCl extraction buffer resulted in a major qualitative and quantitative reduction in the polypeptide content of the matrix fraction (Figure 2D). Notably absent were the higher molecular mass polypeptides including protein C23 and the 160-kDa polypeptide. The polypeptides remaining were largely in the range of 30–50 kDa. The requirement of ribonuclease digestion for removal of the high molecular weight polypeptides suggests

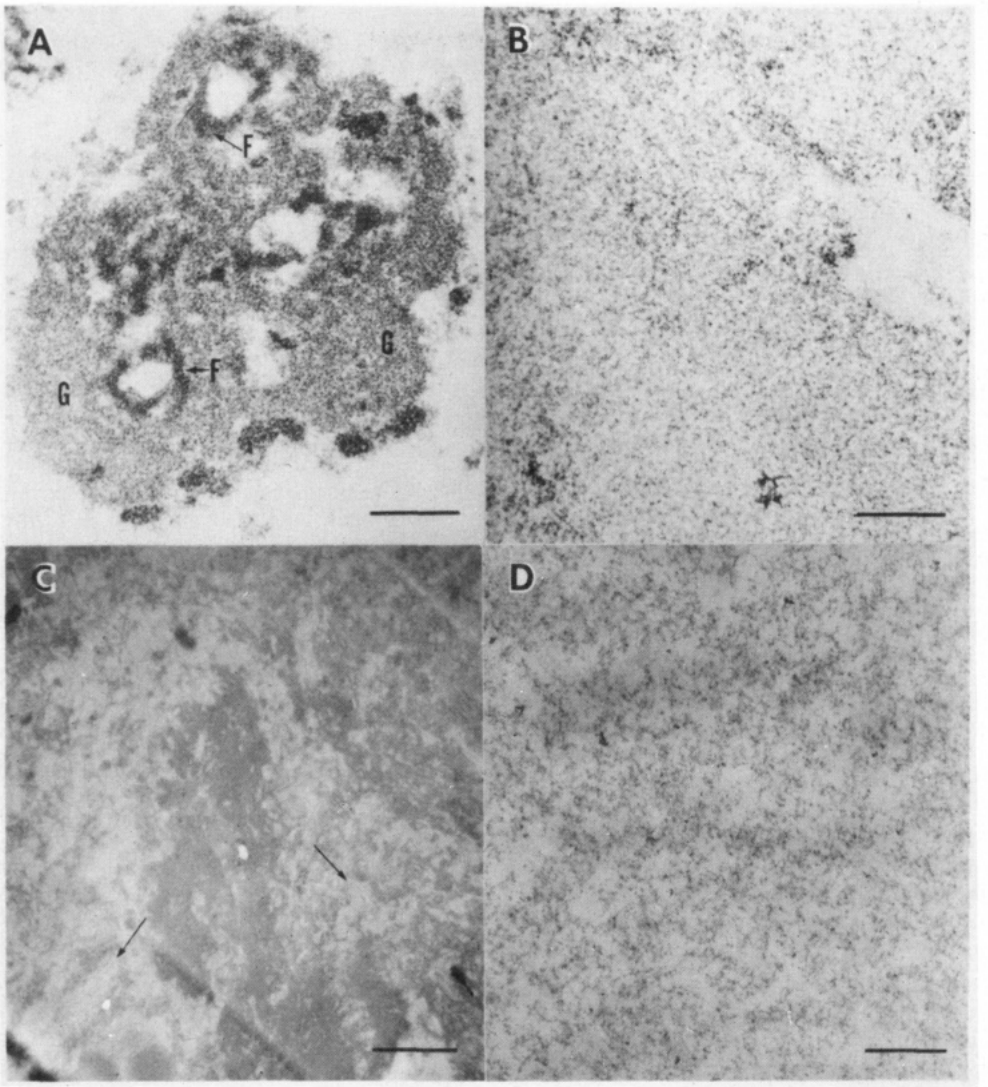

FIGURE 3: Electron microscopy of nucleoli and nucleolar matrix fractions. (A) Isolated nucleoli. Granular (G) and fibrillar (F) regions are indicated. (B) Nucleolar matrix fraction as prepared in Figures 1 and 2. Note the presence of particles similar in size to those found in the granular regions of (A). (C) Nucleolar matrix fraction prepared with the aid of RNase A and mercaptoethanol as in Figure 2D. Arrows in (C) indicate regions containing filamentous material. (D) Preribosomal 60S RNP fraction prepared by sucrose gradient ultracentrifugation of the nucleolar extract (see Figure 4). For all photographs, magnification is 14000×. Bar = 0.5 μM.

that they are RNP-associated.

To determine the extent of RNP association with this fraction, further analyses for protein, DNA, and RNA were performed on nucleoli and nucleolar matrix fractions prepared either by the standard procedure or with the aid of RNase and mercaptoethanol. Table I indicates that the matrix fraction prepared by the standard method typically contained approximately half of the RNA and one-fourth of the protein originally present in the nucleoli, thereby confirming the presence of RNP-like material in this fraction. However, when the matrix was prepared by the RNase/mercaptoethanol procedure, the protein content was significantly reduced and the amount of RNA was only about 2.5% of that originally present in nucleoli. Thus, in nucleolar matrix fractions prepared by the modified procedure the removal of proteins is accompanied by the removal of most of the RNA and vice versa.

The nucleolar matrix fractions were further analyzed for the presence of RNP-like components by transmission electron microscopy. Nucleoli from which the matrix fraction was derived showed the typical pattern of fibrillar centers surrounded by dense fibrillar components, with punctate granular regions scattered throughout the nucleolus (Figure 3A). In comparison, the standard nucleolar matrix fraction (Figure 3B) had lost most identifiable features of nucleoli with the exception of the small particulate material that resembled the granular components of nucleoli in size and shape. In contrast, in nucleolar matrix fractions prepared with the aid of RNase and mercaptoethanol, the RNP-like particles were notably absent (Figure 3C). Except for a few strands of filamentous material, this final nucleolar matrix fraction was generally amorphous. Thus, as visualized by electron microscopy, RNP particles appear to be associated with the nucleolar matrix fraction prepared by the standard method.

Additional experiments were performed to compare the polypeptide compositions of the nucleolar matrix fraction with nucleolar RNP particles. Preribosomal RNP particles were extracted from nucleoli by the method of Auger-Buendia & Longuet (1980) and fractionated by sucrose density gradient centrifugation. The centrifugation produced a major peak sedimenting at approximately 60S, presumably corresponding to the 60S preribosomal RNP particle. Analysis of the polypeptides from fractions across the gradient indicated that proteins B23, C23, and 160K were present in the RNP region but that the bulk of protein C23 was not associated with 60S RNP particles and sedimented near the top of the gradient

Table I: Compositions of Nucleoli and Nucleolar Matrix Fractions[a]

	nucleoli [μg (%)][b]	nucleolar matrix fraction [μg (%)]	nucleolar matrix fraction + RNase [μg (%)]
protein[c]	730 (100)	193 (26)	109 (15)
DNA[d]	776 (100)	29 (3.7)	13 (1.7)
RNA[e]	1050 (100)	525 (50)	25 (2.4)

[a] Results of analysis of representative preparations of nucleoli and nucleolar matrix fractions. "Nucleolar matrix fraction" refers to samples prepared by the standard DNase I–high-molarity salt extraction method. "Nucleolar matrix fraction + RNase" designates samples prepared by inclusion of RNase A during the DNase I digestion stage and 1% mercaptoethanol in the high-molarity salt washes. All values are the average of triplicate determinations. The values for each determination did not vary by more than 5%. Various preparations differed by as much as 20% in their ratios of protein, RNA, or DNA to total nucleolar protein. [b] The percentages are based on the nucleolar value being 100%. [c] Protein determinations were done by the Coomassie blue dye method as described under Experimental Procedures. Nucleoli or nucleolar matrix fractions were solubilized by boiling in 5% SDS and then dialyzing the aliquots against 0.05% SDS prior to the protein determinations. [d] DNA was determined by the diphenylamine procedure as described under Experimental Procedures. Samples were washed twice with cold 0.4 N perchloric acid (PCA) and then solubilized by treatment with 0.4 N PCA at 100 °C for 30 min prior to analyses. Rat nuclear DNA treated as above was used as a standard. [e] RNA was determined by the orcinol method as described under Experimental Procedures. The RNA was solubilized as in footnote *d*. Yeast tRNA (Sigma) was used as a standard.

(Figure 4). As expected, the 60S RNP region of the gradient contained a large number of polypeptides, many of which are presumed to be ribosomal proteins. Figure 4 (lanes M1 and 11–15) also reveals that many of the polypeptides of the nucleolar matrix fraction had mobilities identical with RNP-associated polypeptides, e.g., 160K, several bands migrating below C23, four to five polypeptides in the 50–70-kDa range, and a number of lower molecular weight polypeptides near the bottom of the gel. Thus, a high proportion of the polypeptides found in the 60S preribosomal RNP particles were also associated with the nucleolar matrix fraction.

For comparison with the matrix fraction, the extracted 60S RNP particle gradient fraction was examined by electron microscopy. Figure 3D shows that the 60S RNP fraction is morphologically similar to the matrix fraction (Figure 3B). Although the 60S RNP fraction appears more diffuse and has a wider range of particle sizes, similar size particles are found in both B and D of Figure 3, further supporting the presence of RNP elements in the nucleolar matrix fraction.

To determine whether prior extraction of the RNP particles affected the polypeptide composition of the nucleolar matrix fraction, the standard matrix fraction was compared with matrix fractions prepared from nucleoli in which the RNP particles had been previously extracted. In this case, no major qualitative or quantitative differences were seen between the two matrix preparations (Figure 4, lanes M1 and M2). This suggests that there are two classes of RNP components on the basis of their extractabilities: one that is readily solubilized and another that is associated with the nucleolar matrix fraction and is not extractable under the standard RNP extraction conditions.

To establish that the RNP particles associated with the nucleolar matrix fraction are indeed preribosomal in nature, the nucleolar matrix fraction was analyzed for ribosomal RNA sequences by dot blot hybridization. Nucleolar and nucleolar matrix fraction RNA at identical concentrations were bound to nitrocellulose filters that were hybridized against ^{32}P-labeled plasmids containing cloned segments of a gene for preribosomal

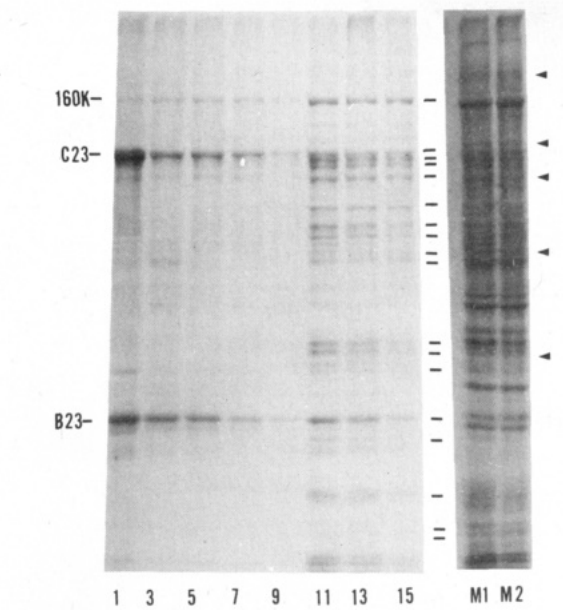

FIGURE 4: Polypeptides associated with nucleolar RNP particles and nucleolar matrix fractions. Nucleoli were extracted with a buffer containing 10 mM NaCl, 10 mM dithiothreitol, and 10 mM Tris (pH 7.5). The extract was fractionated on a 10–40% sucrose gradient in a SW-41 rotor. Nuclear matrix and gradient fractions were applied to a Laemmli-type gel containing 10% acrylamide. Numbers indicate combined gradient fractions (1 designates fractions 1 and 2; 3 contains tubes 3 and 4; etc.). Lanes 11–15 contain the polypeptides from the 60S RNP fraction. Small bars between lanes 15 and M1 indicate polypeptides common to both the 60S RNP particles and the nucleolar matrix fraction. M1, nucleolar matrix fraction prepared by the standard method as in Figure 2; M2, nucleolar matrix fraction prepared from nucleoli from which RNP particles had previously been extracted. Pointers at right indicate migration of high molecular weight standards as in Figure 1.

RNA (Olson et al., 1983). Figure 5 indicates that the RNA of the nucleolar matrix fraction hybridized with either plasmid pDF8 (containing sequences from about half of the 18S rRNA coding region) or plasmid pDF4 (containing most of the coding region of 28S rRNA). For both plasmids, on a microgram basis, the extent of hybridization for whole nucleolar RNA was about 50% of that of the nucleolar matrix fraction RNA. This indicates that ribosomal RNA sequences are present in the nucleolar matrix fraction and suggests that these sequences are enriched in the matrix fraction. To ensure that the labeled plasmids were not hybridizing to contaminating DNA, hybridization was also performed with ^{32}P-labeled plasmid pKW1, which contains DNA from the nontranscribed spacer region of the rDNA (Olson et al., 1983). The spacer region should not contain sequences present in the nucleolar RNA but should contain sequences present in the rDNA. When plasmid pKW1 was used as a probe against either the nucleolar RNA or the nucleolar matrix fraction RNA, no significant hybridization was observed, indicating that DNA contamination was minimal.

Discussion

Although residual nucleoli have been observed in nuclear matrices prepared by a number of workers (Berezney & Buchholtz, 1981a; Berezney & Coffey, 1974, 1977; Capco et al., 1982; Fisher et al., 1982), there has been little, if any, attempt to characterize these structures or the nucleolar portion of the nuclear matrix. The data presented here indicate that the matrix fraction prepared from isolated nucleoli by the standard DNase I–high-molarity salt extraction procedure is highly complex in its polypeptide composition. This complexity appears to be due largely to the presence of RNP particles.

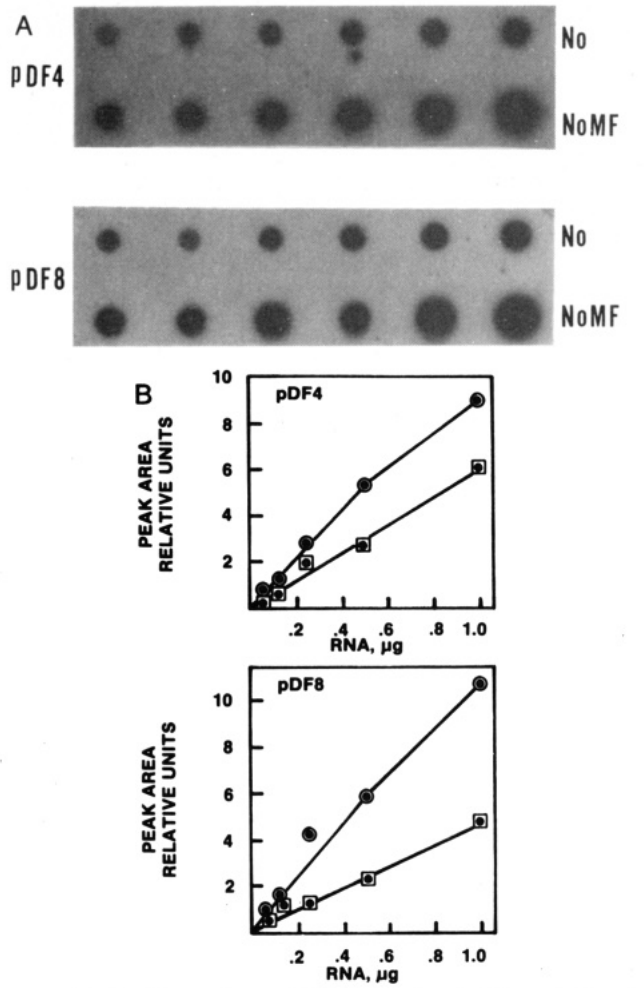

FIGURE 5: Dot blot analyses of RNA associated with nucleoli and nucleolar matrix fraction. RNA was extracted from nucleoli or nucleolar matrix fraction as described in text and applied in various amounts to nitrocellulose filters. The filters were hybridized to ^{32}P-labeled plasmids containing fragments of rDNA. (A) Photograph of autoradiograph after hybridization. pDF4, plasmid containing an rDNA fragment only from the 18S portion of the gene; pDF8, plasmid containing an rDNA fragment containing most of the 28S, transcribed spacer, and 5.8S portions of the gene; No, RNA extracted from whole nucleoli; NoMF, RNA from nucleolar matrix fraction. (B) Plot of relative areas obtained by densitometric scanning of the autoradiographs in (A) (only the linear range was plotted). (⊡) Nucleoli; (⊙) nucleolar matrix fraction.

Approximately half of the RNA present in the nucleolus remains in the nucleolar matrix fraction, and by hybridization studies, it was shown that compared to whole nucleolar RNA the matrix fraction RNA is enriched in sequences from 18S and 28S ribosomal RNA. Thus, preribosomal RNP particles at some stage of assembly and processing appear to be associated with this nucleolar matrix fraction.

The finding of preribosomal RNP particles in the matrix fraction is not entirely unexpected since several classes of RNA have been found attached to nuclear matrix preparations including heterogeneous nuclear (Ciejek et al., 1982; Herman et al., 1978), small nuclear (Long & Ochs, 1982), and ribosomal RNAs (Ciejek et al., 1982; Herlan et al., 1979). The presence of 18S and 28R ribosomal RNA sequences in the nucleolar matrix fraction generally agrees with the reports of Ciejek et al. (1982) and Herlan et al. (1979), which suggest that all precursor RNAs are associated with the nuclear matrix. However, we found that in isolated nucleoli not all RNP particles were tightly associated with the matrix fraction and that a significant portion of the preribosomal RNP particles could be extracted by low ionic strength buffers. If all preribosomal RNP particles are actually matrix associated in vivo, it is possible that the sonication procedure used to prepare nucleoli disrupts matrix–RNP interactions or activates ribonuclease causing a fraction of the RNP particles to become detached, thereby leaving only a portion of the RNP particles in the insoluble residue. The nucleolar matrix fraction prepared after preextraction of preribosomal particles was nearly identical in polypeptide composition with the matrix fraction prepared directly from isolated nucleoli, suggesting that for extraction of the soluble particles ionic strength is not important. It is also interesting that, as judged by electron microscopy, only the granular (more mature) RNP particles were associated with the matrix fraction whereas the fibrillar regions or newly formed RNP elements seemed to be removed by the extractions. Furthermore, the matrix fraction was similar in protein composition to the 60S soluble RNP particles, supporting the idea that the matrix fraction has the 60S particles or granular elements preferentially associated with it. At this point, it is not known whether there are real biochemical differences between the soluble and insoluble classes of 60S particles. It is possible that they differ in their degree of RNA maturation or in protein composition in ways that were not detected by these studies.

The association of preribosomal RNP particles with a matrix fraction that is also known to be enriched in active genes (Hancock, 1982; Jackson et al., 1981; Robinson et al., 1982, 1983) is potentially important in coupling the processes of transcription and ribosome assembly. For purposes of control mechanisms it would seem advantageous for the ribosome assembly system to be in close proximity to the site of transcription so that certain key proteins may either participate in both processes or coordinate the synthesis of preribosomal RNA with the transport of ribosomal proteins from the cytoplasm. This has recently been proposed as a role for protein C23 or an analogous protein by Bourbon et al. (1983).

Kaufman et al. (1981, 1983) showed that the residual nucleolus was not present if reducing agents were used to prevent disulfide formation prior to matrix preparation and if ribonuclease was combined with deoxyribonuclease in the digestion. The findings in this work essentially confirm this. However, we found that by using isolated nucleoli as the starting material the removal of the residual nucleolar structure required ribonuclease at the digestion stage as well as the addition of mercaptoethanol to the high-molarity salt washes even though nucleoli were prepared in the presence of the antioxidant dithiothreitol. Thus, it is not known whether disulfide bridges are important in maintaining the structure of the nucleolus and the association of nucleolar components with the matrix fraction or whether the disulfides are simply formed as an artifact of manipulation of cells and cell fractions. Although disulfide bridges can cross-link the laminar polypeptides (Kaufman et al., 1983), there is no direct evidence for disulfide bridges within the nucleolus or between nucleolar components and the nuclear matrix. It is conceivable that divalent metal ions such as copper or calcium cross-link nucleolar polypeptides as has been observed in the nuclear scaffold (Lewis & Laemmli, 1982) and that reducing agents prevent this interaction. However, this seems unlikely since the addition of EDTA during matrix fraction preparation had no significant effect on the polypeptide composition.

When the nucleolar matrix fraction was prepared with the aid of ribonuclease and mercaptoethanol, a large number of high and low molecular weight polypeptides were removed. Two prominent high molecular weight polypeptides removed

were protein C23 and the 160-kDa polypeptide. This removal by RNase suggests they are RNP-associated and they also are found to some extent in the 60–80S soluble RNP particles fractionated by sucrose density centrifugation. Many of the lower molecular weight polypeptides of the nucleolar matrix fraction comigrate electrophoretically with polypeptides in the soluble RNP fraction and are presumed to be ribosomal proteins as has been shown previously (Kumar & Subramanian, 1975; Prestayko et al., 1974; Warner, 1974, 1979). The remaining insoluble polypeptides are limited in number and are generally in the molecular mass range of 30–50 kDa. These are similar in molecular weight to the hnRNP-associated proteins found in the nuclear protein matrix by van Eekelen & van Venrooij (1981). Although it is possible that these polypeptides constitute the actual nucleolar matrix to which the RNP particles are in some way attached, they may simply be contaminants or denatured insoluble polypeptides that remain after the extraction procedure. Except for traces of filamentous material, this final residue appears amorphous by electron microscopy. Thus, if this is the true nucleolar matrix, it must have lost most resemblances to cytoskeletal structures during preparation.

Although these results do not disprove the existence of a true nucleolar matrix an alternative interpretation is that proteins of the RNP particles themselves, in association with chromatin proteins, may organize the various components into a three-dimensional array in the absence of an underlying nucleolar matrix. This concept has been proposed by Labhart et al. (1984) and is supported in recent work by Scheer et al. (1984). In the latter study it was shown that when production of large ribosomal subunits was inhibited by the drug 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), the genes for ribosomal RNA dispersed throughout the nucleus and nucleoli essentially disappeared. Furthermore, the tendency of nucleoli to fuse is increased in cells having increased metabolic activity (Wachtler et al., 1984). In other words, nucleolar material, presumably of an RNP nature, tends to aggregate with itself or other nucleolar macromolecules to organize the chromatin-containing genes for preribosomal RNA to form nucleoli. This is consistent with the proposed nucleolus organizer role for protein C23 (Lischwe et al., 1979; Long & Ochs, 1982; Olson, 1983; Olson et al., 1981). These and earlier studies (Olson et al., 1984; Olson & Thompson, 1983; Prestayko et al., 1974) as well as work in progress (A. H. Herrera and M. O. J. Olson, unpublished results) show that protein C23 is at least partially RNP associated. Recent studies also show that protein C23 may preferentially bind to spacer regions between the genes for preribosomal RNA (Olson et al., 1983). Thus, it is tempting to speculate that protein C23 serves as a bridge between chromatin and RNP components in its role as a nucleolus organizer.

ACKNOWLEDGMENTS

We thank Dr. Motohiro Fuke for his gift of cloned fragments of rDNA and Barbara Alexander for technical assistance in the early stages of this work. We also appreciate the helpful discussions with Drs. Donald Sittman and H. W. Detrich, III, and the typing done by Romie Brown.

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In Nondividing Cells, Histone $H1^0$ Is Synthesized and Deposited onto Chromatin without Accompanying Phosphorylation†

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Received May 20, 1985

ABSTRACT: The phosphorylation of H1 histone subfractions was measured in mouse neuroblastoma cells stopped from dividing by three treatments that block cell division: 5 mM butyrate, 2% dimethyl sulfoxide, and serum withdrawal. H1 histone phosphorylation decreased in response to all three treatments, but the response differed in its timing and its extent for the different H1 subfractions. The different decreases in phosphorylation correlated well with the differential decreases in biosynthesis of the individual H1 subfractions; however, an exception to this parallel decrease in synthesis and phosphorylation was observed in the case of histone $H1^0$. Phosphorylation of $H1^0$ was absent in each of the three treatments after 2 days, despite the continued synthesis and deposit of $H1^0$ on the chromatin. Thus, despite the fact that $H1^0$ was being synthesized and that the other newly synthesized H1 subfractions were phosphorylated at this time, the phosphorylation of $H1^0$ became uncoupled from its synthesis after prolonged treatments blocking cell division.

Phosphorylation of H1 histone was first observed in 1966 (Kleinsmith et al., 1966; Ord & Stocken, 1966) and is known to occur at multiple sites on the H1 molecule (Langan, 1982). Although there is some minor hormonally dependent phosphorylation that is independent of the cell cycle (Langan, 1971), almost all histone H1 phosphorylation is correlated with the cell cycle. Major H1 histone phosphorylation has been observed during S phase when DNA replication and most H1 synthesis occurs and during late G_2, immediately prior to the beginning of metaphase (Bradbury et al., 1974). In dividing cells the G_2 phosphorylation accounts for the majority of phosphorylation on H1 molecules, but S-phase phosphorylation is a subtantial fraction. A modest amount of phosphorylation also occurs late in G1 (Gurley et al., 1974). Although the exact role of phosphorylation is not understood, some suggestions have been put forward that distinguish between possible roles of G_2- and S-phase phosphorylation.

The notion that phosphorylation of H1 histone in late G_2 phase of the cell cycle is related to chromosome condensation and the initiation of mitosis has been proposed because of coincidence of the two events (Gurley et al., 1974) and because of the advance of mitosis by exogenous kinase (Bradbury et al, 1974). Moreover, a temperature-sensitive mutant hamster cell line characterized by premature chromosome condensation was found to exhibit extensive phosphorylation of both histones H1 and H3 at the nonpermissive temperature when the premature chromosome condensation occurred (Inglis et al., 1976).

In addition to its occurrence in the late G_2 phase of the cell cycle, phosphorylation of H1 has been observed in S phase, coincident with histone synthesis (Balhorn et al., 1972; Gurley et al., 1974). In this regard, Sung et al (1977) observed that deposition of histone H5 onto the chromatin of chicken erythrocytes is linked to its prior phosphorylation (Sung et al., 1977; Wagner et al., 1977). These workers suggested that phosphorylation may modulate the interactions of histone and DNA while newly synthesized chromatin is annealed to its nature structure [see also Louie & Dixon (1972) and Ruiz-

† This research was supported by grants from the NIH (GMS20338, NIEHS ITES07075, and ES01896) and by the Agricultural Research Station.

0006-2960/86/0425-0491$01.50/0