Wray, W., Boulikas, T., Wray, V., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.
Yu, C., Yu, L., & King, T. (1975) *J. Biol. Chem. 250*, 1383–1392.
Yu, C., Yu, L., & King, T. (1979) in *Cytochrome Oxidase* (King, T. E., Orii, Y., Chance, B., & Okunuki, K., Eds.) pp 219–227, Elsevier/North-Holland Biomedical Press, Amsterdam.

Distribution of Proteins among Chromatin Components of Nucleoli†

Mark O. J. Olson* and Barbara A. Thompson

ABSTRACT: The nucleolus contains proteins which are believed to be involved in the organization of its subcomponents. To examine the possible locations of these proteins we have studied the release of proteins and chromatin subunits by digestion of Novikoff hepatoma nucleoli by micrococcal nuclease, deoxyribonuclease I, and ribonuclease A. Micrococcal nuclease digests of nucleoli produced typical sucrose density gradient profiles of nucleosomes showing separation of monomers, dimers, and trimers. The DNA lengths in the monomers, dimers, and trimers were generally the same as previously reported for whole nuclear chromatin of various rat tissues. Protein C23 (M_r 110 000), a putative nucleolus organizer protein which contains highly acidic phosphorylated regions, was rapidly released by micrococcal nuclease. The bulk (approximately 90%) of protein C23 released by micrococcal nuclease sedimented more slowly than mononucleosomes, at approximately 7 S. The remaining portion of protein C23 sedimented with mono-, di-, and trinucleosomes at various extents of digestion. Deoxyribonuclease I also liberated protein C23 from nucleoli. In addition, treatment of nucleoli with ribonuclease A released significant quantities of protein C23 without release of histones. These results suggest that protein C23 is associated with DNA-containing as well as RNA-containing components but that the major portion of the protein is loosely bound or associated with RNP components. However, it also appears to be present in nucleosomes to a small but significant extent. Protein B23 (M_r 37 000), which also contains highly acidic phosphorylated regions, was released by all nuclease treatments but was found to a lesser extent in nucleosome fractions. Another protein of M_r 160 000 (160K) sedimented with di- and trinucleosomes and was present in higher concentrations in nucleolar compared to whole nuclear chromatin. The 160K protein was found predominantly in the pellet after DNase I digestion of nucleoli followed by extraction with 2 M NaCl, suggesting that it is a nucleolar component of the nuclear matrix.

The nucleolus is the subnuclear organelle where preribosomal RNA is synthesized and ribosomes are assembled (Busch & Smetana, 1970). Because of these functions, the nucleolus contains both chromatin and ribonucleoprotein (RNP)[1] subcomponents. The chromatin component contains multiple copies of the genes for ribosomal RNA, although these account for less than 1% of the total DNA in rat nucleoli (Attardi & Amaldi, 1970; Attardi et al., 1965). The remainder of the DNA in nucleolar chromatin is of unknown function but includes sequences which are highly repetitive in nature (Fuke et al., 1979). The RNP components consist of preribosomal RNA at various stages of processing associated with a number of ribosomal and nonribosomal proteins (Warner, 1979; Kumar & Subramanian, 1975; Prestayko et al., 1974).

The proteins of nucleolar chromatin (Olson et al., 1975; Olson & Busch, 1978) and RNP components (Kumar & Subramanian, 1975; Prestayko et al., 1974; Olson et al., 1974) have been examined and categorized to some extent, although it is not precisely known which proteins are involved in organizing subcomponents within the nucleolus. For example, it is likely that certain proteins are responsible for producing the condensed regions of nucleolar chromatin, while other proteins may interact with chromatin and hold preribosomal RNP particles in place during RNA processing and ribosome assembly. Proteins which play these roles might be expected to have special features which allow them to engage in multiple interactions with other macromolecules.

We have isolated and partially characterized two major nucleolar proteins which have characteristics suggesting that they may serve as organizer molecules. These proteins, designated B23 and C23 by Orrick et al. (1973), have regions of high net negative charge (Mamrack et al., 1977, 1979) which may interact with histones in chromatin or ribosomal proteins in nucleolar RNP particles. The proteins are localized predominantly to nucleoli of interphase cells (Olson et al., 1981; Michalik et al., 1981), and protein C23 also resides at the nucleolus organizer regions of chromosomes (Lischwe et al., 1981). A certain portion of protein C23 which is readily extractable with low ionic strength buffers (Rothblum et al., 1977) is largely associated with preribosomal RNP particles (Prestayko et al., 1974; Olson et al., 1974), while another fraction appears to be rather tightly bound to chromatin (Olson et al., 1975; Rao et al., 1982). More recently, it was found that protein C23 binds DNA and appears to have a preference for DNA sequences upstream from the genes for ribosomal RNA.[2] These characteristics, taken together, suggest that protein C23 plays an organizational role in the nucleolus,

† From the Department of Biochemistry, The University of Mississippi Medical Center, Jackson, Mississippi 39216. *Received January 4, 1983.* This research was supported by Grant GM 28349 from the National Institutes of Health.

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; PCA, perchloric acid; RNP, ribonucleoprotein; $NaDodSO_4$, sodium dodecyl sulfate; SSC, standard saline citrate containing 0.15 M NaCl and 0.017 M sodium citrate (pH 7.0); rDNA, genes coding for ribosomal RNA; DNase I, deoxyribonuclease I; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[2] M. O. J. Olson, B. A. Thompson, and S. T. Case, unpublished results.

0006-2960/83/0422-3187$01.50/0

possibly serving as a bridge between RNP and chromatin components.

Nucleases have been valuable tools to probe the structure of chromatin and to generate nucleosomes (McGhee & Felsenfeld, 1980). Although numerous nuclease digestion studies have been performed on whole nuclei, few have investigated the release of chromatin subcomponents from nucleoli. The effects of DNase I on *Tetrahymena* nucleoli have been examined (Vavra et al., 1982; Borchsenius et al., 1981), and the rDNA-containing chromatin of *Physarum* (Johnson et al., 1978) has been studied with micrococcal nuclease. However, the nucleoli of these organisms are unusual in that they contain amplified or extrachromosomal ribosomal DNA. Digestion of nucleoli of higher organisms by nucleases should provide clues regarding the extent of chromatin association and the possible locations of the putative nucleolus organizer proteins. These studies were initiated (a) to determine whether the putative nucleolus organizer proteins are released by micrococcal nuclease and other nucleases and (b) if so, to determine whether these proteins are nucleosome associated and (c) to compare the profiles of proteins released from nucleoli versus whole nuclei. It was found that the bulk of protein C23 is released as a non-nucleosomal component but that a smaller amount of it may be nucleosome associated. In addition, a higher molecular weight protein (160K) sediments with di- and trinucleosomes of nucleolar chromatin but also is found in the nucleolar component of the nuclear matrix.

Materials and Methods

Animals, Cells, and Cell Fractions. Novikoff hepatoma ascites cells, grown in male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), were harvested 6 days after transplantation. The Novikoff hepatoma cells were used for preparing nuclei and nucleoli. Nuclei were prepared by the NP-40 detergent method (James et al., 1977). Nucleoli were isolated by sonication using the magnesium–sucrose method (Rothblum et al., 1977). Nucleolar preparations were monitored by light microscopy and were essentially free of extranucleolar contamination.

Nuclease Digestion Methods. Nucleoli were suspended by gentle homogenization 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,[1] and 5 mM Pipes (pH 7.5). The concentration was determined after aliquots of the suspension were dispersed by sonication in 1% $NaDodSO_4$. Nucleoli were washed 2 times in the digestion buffer, centrifuging each for 10 min at 10000*g*. The washed nucleoli were resuspended in the digestion buffer at the above concentration and then used for digestion with various nucleases. Early in the course of these studies it was found that various proteins including C23 were released by an unidentified endogenous activity when the nucleoli were incubated at 37 °C in the absence of added enzyme. This activity was minimized when digestions were performed at 0–5 °C, which are the conditions used for most of these studies.

For micrococcal nuclease (Worthington, Freehold, NJ) the enzyme was added to a concentration of 30 units/mL, and digestion was carried out for various times. The reaction was stopped by addition of EDTA to a concentration of 5 mM, and the samples were centrifuged at 6500*g* for 10 min in an Eppendorf microcentrifuge. The supernatant from this was used for polyacrylamide gel electrophoresis or for further fractionation on sucrose density gradients. Digestions of nuclei were performed under similar conditions except that concentrations of nuclei and enzyme were doubled to obtain similar levels of protein on the sucrose density gradients. Deoxyribonuclease I (Sigma, St. Louis, MO) digestions were carried out with an enzyme concentration of 10 μg/mL. The reaction was terminated with 5 mM EDTA as with the micrococcal nuclease digests.

In the ribonuclease digestions, the pancreatic RNase A (Sigma) was heated at 95 °C in SSC for 5 min to minimize DNase activity. Digestions were done in the digestion buffer at 0–5 °C for various times with 10 μg/mL enzyme. After various times, EDTA was added to 5 mM, and the mixtures were centrifuged as above.

Preparation of Nucleolar Matrix. Nucleoli were extracted essentially according to the method used for rat liver nuclei by Berezney & Buchholtz (1981). Briefly, nucleoli were suspended in digestion buffer as above and treated with DNase I (10 μg/mL) at 0–4 °C for 30 min. This was followed by extraction with a low salt buffer containing 0.2 mM $MgCl_2$ and 10 mM Tris (pH 7.4). The pellet from this extraction was then extracted 3 times with a high salt buffer containing 2 M NaCl, 0.2 mM $MgCl_2$, and 10 mM Tris (pH 7.4) followed by one extraction with low salt buffer containing 1% Triton X-100 and two more extractions with low salt buffer. The extracts were precipitated with Cl_3CCOOH as described below, and the pellet containing the matrix was solubilized directly in $NaDodSO_4$ sample buffer.

Analytical Methods. The extent of digestion of chromatin by micrococcal nuclease was monitored by solubility in 5% perchloric acid. After various times of digestion the mixtures were made 5% in perchloric acid, allowed to stand in an ice bath for 15 min, and then centrifuged in the Eppendorf microcentrifuge for 10 min. The A_{260} of the supernatants was used to calculate the percent acid-soluble material. An identical aliquot of the undigested material was hydrolyzed in 5% PCA for 30 min at 90 °C and used as the reference for 100% acid solubility.

Chromatin material released into the supernatant by nuclease was analyzed either by ultracentrifugation in sucrose density gradients or by polyacrylamide gel electrophoresis. Isokinetic sucrose density gradients were prepared according to the method of McCarty et al. (1974) for a particle density of 1.51 by using a gradient from 5 to 27% sucrose (w/w) in a buffer containing 50 mM KCl, 1 mM EGTA, 0.1 mM PMSF, and 5 mM Pipes (pH 8.0). Centrifugation was done in a Beckman SW 41 rotor for 16 h at 35 000 rpm. The tubes were fractionated into 0.6-mL fractions in an Isco fractionator, and the profiles were monitored at 254 nm. In some cases tritiated RNAs of known *s* values (Bethesda Research Laboratories, Rockville, MD) were added to the gradients to estimate the sedimentation coefficients of gradient fractions. In other experiments ^{32}P-labeled protein C23 (Rao et al., 1982) was added to determine the sedimentation behavior of the purified protein.

For polyacrylamide gel electrophoresis, supernatants of nuclease digests or gradient fractions were precipitated with 15% Cl_3CCOOH after the addition of 20 μg of poly(adenylic acid) as carrier, and the precipitates were washed with ethanol and ethanol–ether as described by Valenzuela et al. (1976). The precipitates were dissolved in sample buffer and applied to Laemmli type $NaDodSO_4$ gels containing 12 or 15% polyacrylamide (Laemmli, 1970). Gels were stained with Coomassie blue, destained in 10% acetic acid and 10% 2-propanol, and in some cases scanned at 540 nm with a Transidyne densitometer.

For analysis of DNA, gradient fractions were made 1% in $NaDodSO_4$ and digested directly with proteinase K (Beckman Instruments, Palo Alto, CA) at a concentration of 100 μg/mL

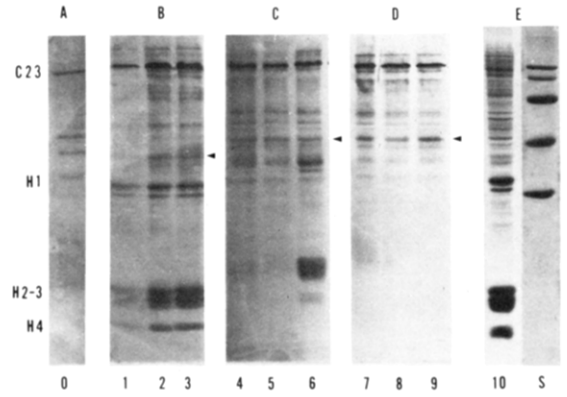

FIGURE 1: Proteins released from nucleoli by various enzymic treatments. Nucleoli were suspended in digestion buffer at a concentration of 20 A_{260} units/mL and digested as described under Materials and Methods. Each lane contains material released from 0.3 mL of nucleoli after digestion and treatment with 5 mM EDTA. (A) (lane 0) Undigested control kept at 0 = 4 °C for 40 min; (B) digestion with 30 units/mL micrococcal nuclease for 3 (lane 2), 10 (lane 3), and 40 min (lane 3); (C) digestion of nucleoli with 10 μg/mL DNase I for 5 (land 4), 10 (lane 5), and 40 min (lane 6); (D) digestion with 10 μg/mL RNase A for 3 (lane 7), 10 (lane 8), and 40 (lane 9); (E) pellet after digestion with micrococcal nuclease for 10 min (lane 10). The load in lane 10 is one-third that of lanes 0–9. S, molecular weight standard consisting of (from top to bottom) myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase B (M_r 97 400), bovine albumin (M_r 66 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 29 000). Protein C23 is designated by its number. Protein B23 is indicated by the pointer. Histones H1 and H4 run as separate bands as indicated. Histones H2A, H2B, and H3 run as a cluster of bands as designated by H2-3.

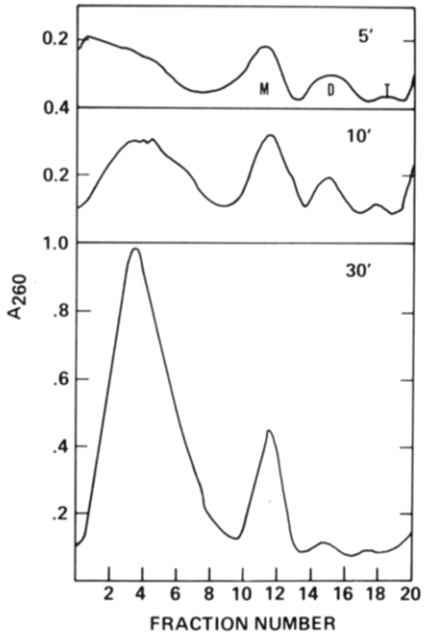

FIGURE 2: Separation of micrococcal nuclease digests of nucleoli by sucrose density gradient centrifugation. Nucleoli were digested as described under Materials and Methods with micrococcal nuclease for the time indicated in the panels and applied to isokinetic sucrose gradients containing 5–27% sucrose. Each gradient contained the EDTA-solubilized material after digestion of 0.5 mL of 20 A_{260} units/mL nucleoli. M, D, and T indicate mono-, di-, and trinucleosome peaks, respectively.

for 1 h at room temperature. The samples were made 0.3 M in ammonium acetate, and the DNA was precipitated with 2.5 volumes of ethanol at −20 °C overnight. The precipitated DNA was then taken up in sample buffer and run on 5% polyacrylamide gels (Maniatis et al., 1975). Markers run in parallel consisted of *Eco*RI restriction endonuclease digested rat whole nuclear DNA which produced DNA lengths in multiples of 93 base pairs visible up to octamers (Fuke & Busch, 1979). The gels were stained with ethidium bromide and viewed and photographed under ultraviolet light.

Results

Proteins Released by Nuclease Digestion of Nucleoli. To establish conditions for release of proteins and nucleosomes from nucleoli, Novikoff hepatoma nucleoli were washed twice in digestion buffer and then treated with various nuclease digestion conditions. Proteins released into the supernatant after digestion and EDTA treatment were displayed on 15% polyacrylamide Laemmli type NaDodSO$_4$ gels. This method allowed the visualization of histones as well as high molecular weight polypeptides. Figure 1 indicates that (other than histones) the predominant polypeptide released by all nucleases was the 110 000-dalton protein C23. Under standard digestion conditions, protein C23, as well as histone H1, the nucleosomal histones, B23, and numerous other proteins, was rapidly released by micrococcal nuclease (lanes 1–3, Figure 1). DNase I digestions also released protein C23 and histones, although less effectively than micrococcal nuclease under the same conditions (Figure 1, lanes 4–6). Ribonuclease A, which had been heat treated to remove DNase activity, was approximately as effective in releasing proteins C23 and B23 as was DNase I, but histones were not released (Figure 1, lanes 7–9). Only trace amounts of protein C23 and other proteins were liberated when the washed nucleoli were allowed to stand at 0–5 °C for up to 40 min and then treated with 5 mM EDTA (lane 0, Figure 1). Thus, the release of protein C23 was not simply due to EDTA treatment, but the protein could be liberated from nucleoli by either ribonuclease or deoxyribonuclease activity.

Protein C23 was also released more rapidly than the histones by micrococcal nuclease. Lane 10 of Figure 1 shows the proteins remaining in the pellet after 10 min of micrococcal nuclease digestion. A small amount of C23 remained in the pellet, although most of protein C23 was removed (75–85% as estimated by gel scans). On the other hand, 70–80% of the nucleosomal histones remained in the pellet. Visual examination of the ratio of protein C23 to histones indicates that the ratio is much greater in supernatant fractions than in pellet fractions (Figure 1, lanes 2 and 10), indicating that protein C23 is released more rapidly by micrococcal nuclease than general nucleosomal components.

Proteins Associated with Nucleosomes of Nucleoli. Of the digestion methods tested micrococcal nuclease provided the most effective method of simultaneously releasing histones (presumably in nucleosomes), protein C23, and other proteins from nucleoli. The kinetics of release of 5% perchloric acid soluble material from nucleoli were similar to nuclei; i.e., the release of PCA-soluble material proceeded rapidly over a 20-min period and then approached a plateau of approximately 10% acid solubility after 60 min of digestion. In order to determine which proteins were associated with nucleosomal fractions, washed nucleoli were digested for various times and then fractionated in isokinetic sucrose gradients. Figure 2 indicates that typical nucleosome patterns were obtained at various times of digestion with several sedimentation classes: First, slowly sedimenting material appeared near the top of the gradient. Second, there was a broad peak which presumably consisted of mononucleosomes (designated M) with a peak *s* value near 11 S at all time points. Third, the lower part of the gradient consisted of apparent dimers, trimers, and sometimes traces of oligomers. After 30 min of digestion the components larger than monomers were proportionately lower,

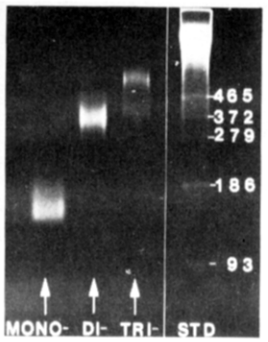

FIGURE 3: Electrophoretic analysis of DNA in mono-, di-, and trinucleosomes. Nucleoli were digested for 10 min with micrococcal nuclease as described under Materials and Methods and applied to sucrose gradients as in Figure 2. DNA was extracted from pooled monomer, dimer, and trimer peaks and run on 5% polyacrylamide gels. The mono-, di-, and trinucleosome peaks from the 10-min digests are indicated. The standard (STD) consisted of an *Eco*RI restriction endonuclease digest of rat whole nuclear DNA with the major bands represented by the repeating sequences with lengths in base pairs as indicated on the right hand side of the figure.

and the slowly sedimenting material and monomers were the major components.

To positively identify the peaks on the gradients the lengths of DNA from the monomers, dimers, and trimers of the gradients of the 10-min digest (Figure 2) were measured on 5% polyacrylamide gels (Figure 3). The DNA near the top of the gradient was of a size too small to measure by using this electrophoretic system. The mononucleosome peak contained DNA in the range 140–200 base pairs. The DNA in the dimer was approximately 330–400 base pairs, and the trimer contained DNA lengths of 520–600 base pairs, although the trimer peak was slightly contaminated with dimers. Thus, the nucleosomes from nucleoli fit into the generally established nucleosome structure from various rat tissues, i.e., 192–198 base pair repeat lengths (Compton et al., 1976).

To determine the protein composition of the nucleolar nucleosomal fractions, each fraction was precipitated with Cl_3CCOOH and then run on $NaDodSO_4$ Laemmli type gels. Figure 4a shows that in a 10-min digest the nucleosomal histones were first seen in trace amounts at fraction 10 and then reached a peak at fraction 12. This is graphically illustrated by plotting quantities of various proteins estimated by densitometry of the gel patterns (Figure 4b). The relative amounts of nucleosomal histones generally followed the optical density profile of the gradient; that is, the peaks of the histones roughly coincided with the monomer, dimer, and trimer peaks of the gradient. However, histone H1, represented by two bands, began at the heavy side of the monomer peak (near tube 13) and was present in all subsequent fractions down to the bottom of the gradient. This is consistent with H1 being located at the internucleosomal spacer region; that is, histone H1 is only found in those chromatin fragments which contain the core particle plus all or a portion of the spacer. Thus, the histones were distributed in the same general pattern in nucleolar nucleosomes as normally found in nuclear chromatin.

Most of protein C23 did not cosediment with nucleosome fractions. The bulk of protein C23 recovered from the gradient (approximately 90% by gel scans; Figure 4b) sedimented slower than mononucleosomes at approximately 7 S. The remaining 10% of the protein was seen in the monomer, dimer, and trimer regions, all the way to the bottom of the gradient, suggesting that it was partially associated with nucleosomes. This was further shown by analyzing monomer, dimer, and trimer regions of gradent runs of material released from nu-

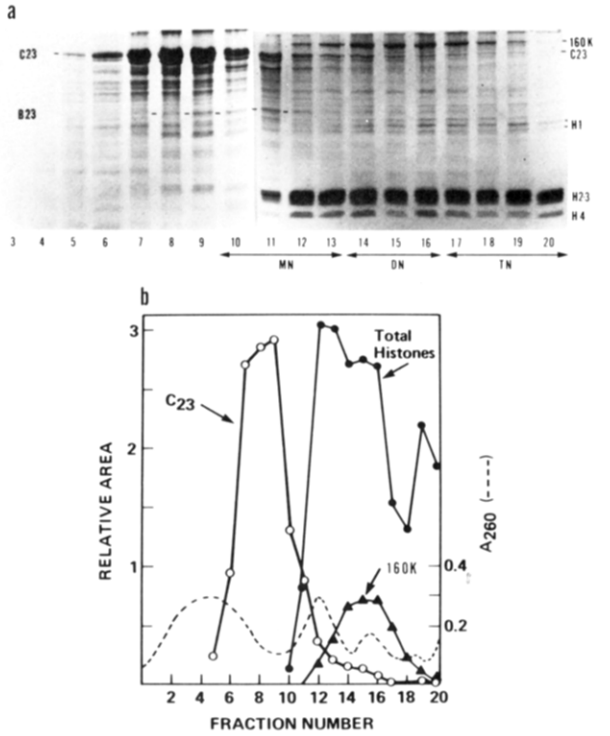

FIGURE 4: Proteins associated with nucleolar nucleosomal fractions separated on sucrose gradients. (a) Individual tubes from a gradient separation of a 10-min micrococcal digest were Cl_3CCOOH precipitated and applied to a 12% polyacrylamide Laemmli type gel. The numbers at the bottom indicate tube numbers. The tubes in the monomer, dimer, and trimer regions are designated by Mn, Dn, and Tn, respectively. The numbers at the sides indicate the positions of various proteins. Histones H2A, H2B, and H3 are indicated by H2-3. (b) The relative areas of protein C23, total histones, and the 160K protein were obtained by densitometry of the gel lanes in (a) and plotted according to the tube number. The dashed line indicates the profile of the original sucrose density gradient.

cleoli at various times of micrococcal nuclease digestion (Figure 5a). A fraction of protein C23 was found associated with monomers, dimers, and trimers at all times of digestion from 2 to 12 min. The protein was present in greater quantities in the dimers and trimers obtained at the shorter times of digestion than at longer times of digestion. However, the histone content of the mono-, di-, and trinucleosome peaks increased with increasing times of digestion, indicating that the total amount of nucleosomal material released increased with time. Thus, the ratio of protein C23 to histone in the dimers and trimers decreased with time of digestion. This suggests that protein C23 is partially removed by micrococcal nuclease from already released dimers and trimers as the digestion proceeds.

In order to show that the presence of small quantities of protein C23 in oligonucleosomes was not due to tailing of the main peak of C23 or aggregation of the protein, the dinucleosome peak was rerun on a sucrose gradient under identical conditions. Electrophoretic analysis (Figure 5b) showed that protein C23 still sedimented with the dimers and little, if any, protein C23 was seen in the 7S region of the gradient. Furthermore, when ^{32}P-labeled purified protein C23 was run on an identical gradient, essentially all of the radioactivity sedimented in the 7S region prior to the mononucleosome peak, suggesting that the cosedimentation of C23 with nucleosomes was not simply due to aggregation of the protein. Thus, it seems likely that a small portion of the total C23 is associated with nucleosomes.

Protein B23, another nucleolar phosphoprotein with characteristics similar to those of C23, was also released by mi-

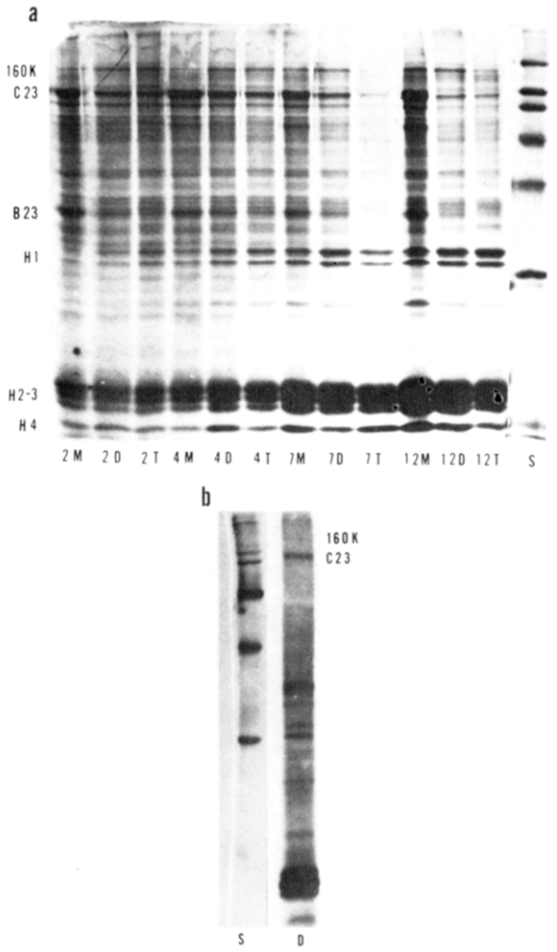

FIGURE 5: Proteins associated with mono-, di-, and trinucleosome regions of sucrose density gradients of micrococcal nuclease digests of nucleoli. (a) The digests are fractionated as in Figure 2, and the peaks were pooled and run on 12% polyacrylamide gels as in Figure 4. The numbers at the bottom indicate the times of digestion in minutes, and the letters refer to the region of the gradient; i.e., M, D, and T refer to monomer, dimer, and trimer, respectively. The numbers at the left indicate the positions of various proteins. The molecular weight standard (S) contains, from top to bottom, myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase B (M_r 97 400), bovine albumin (M_r 66 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 29 000). (b) The dinucleosome peak of the 7-min digest was pooled and run on an identical sucrose gradient. The resulting dinucleosome peak was precipitated with Cl_3CCOOH and run on a 12% polyacrylamide gel as in (a). Note the presence of protein C23 in the recentrifuged dimer.

crococcal nuclease, but it was seen in quantities much smaller than C23 (Figures 4 and 5). Its distribution in the gradient roughly followed that of C23, but in the upper part of the gradient it appeared to sediment faster than C23, with maximal concentration slightly ahead of the monomer peak. Traces of B23 were also found in dimers and trimers at various times of digestion (Figure 5).

Also seen in Figure 4a is a prominent band (160K) having a molecular weight of approximately 160 000. This polypeptide was found in fractions at the heavy side of the monomer down to the bottom of the tube, as is histone H1. The peak of concentration of this polypeptide was reached in the dimer region (Figure 4b), suggesting that it may also be located in the internucleosomal spacer region or that the protein itself sediments in that position.

Proteins Associated with Nucleosomes of Whole Nuclei. To compare the proteins of chromatin subcomponents of whole nuclear vs. nucleolar chromatin, Novikoff hepatoma nuclei prepared by the NP-40 detergent method were digested with

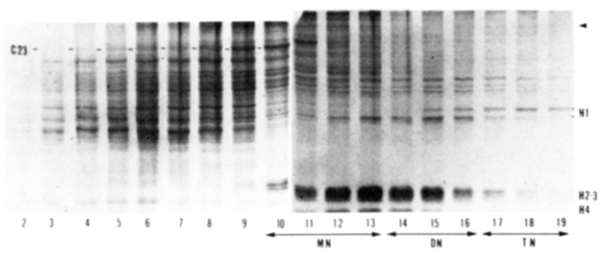

FIGURE 6: Proteins associated with nuclear nucleosomal fractions separated by sucrose gradient centrifugation. Micrococcal nuclease digested nuclei were centrifuged as in Figure 2. Proteins from each fraction were run on 12% polyacrylamide gels as in Figure 4. Positions of C23 and the histones are indicated. Pointer designates position of 160K protein, when present.

micrococcal nuclease. Concentrations of nuclei and enzyme were adjusted to obtain a level of released nucleosomes similar to that obtained from nucleoli. Typical gradient patterns were obtained as with the nucleolar digests, and the fractions were analyzed by polyacrylamide gel electrophoresis (Figure 6). As with the nucleolar pattern (Figure 4a), histones, which were seen beginning at fraction 10, were found all the way to the bottom of the gradient, and histone H1 increased in concentration at the heavy side of the monomer region. Interestingly, the slower moving H1 component was seen only in the dimers or larger fragments.

Proteins C23 and 160K were present in markedly different amounts in the nuclear gradient compared to the nucleolar gradient profile. Protein C23, although visible in fractions 5–10 in Figure 6, was considerably reduced in concentration compared to the predominant band of C23 in Figure 4a. Furthermore, the 160K protein was barely visible in the nuclear profile. These results confirm that protein C23 and 160K protein are predominantly localized to the nucleolus.

Protein Associated with the Nucleolar Matrix. Franke et al. (1981) have reported the presence of a predominant high molecular weight protein in the skeleton or matrix of *Xenopus* oocyte nucleoli. In order to determine whether the 160K protein is also matrix associated, the matrix fraction of these nucleoli was analyzed. The procedure of Berezney & Buchholtz (1981), which employs DNase digestion followed by extraction by high molarity salt, was found to be the most practical for this purpose. Figure 7 shows the proteins extracted by the various treatments en route to the matrix fraction. Small quantities of protein C23 and histone were released by DNase I treatment (Figure 7, lane 1). The bulk of protein C23 and histones were extracted by 2 M NaCl after DNase treatment (Figure 7, lane 2). Only traces of the 160K protein were seen in this extract, even though this lane of the gel was overloaded. Virtually none of the 160K protein was seen in the Triton X-100 wash (lane 3) or in the subsequent low molarity salt wash (not shown). In the final pellet or nucleolar matrix preparation (Figure 7, lane 4), the predominant polypeptides were the 160K protein, protein C23, and the polypeptides that migrated in the vicinity of the bovine serum albumin standard. The latter group of proteins is commonly associated with nuclear matrix (Berezney & Buchholtz, 1981). Therefore, the 160K protein appears to be predominantly localized to nucleolar component of the nuclear matrix.

Discussion

These studies indicate that the chromatin component of nucleoli contains typical nucleosomal structures. This conclusion is based on results of experiments in which the sucrose density gradient profiles of micrococcal nuclease digested nucleoli contained mono-, di-, and trinucleosomes, and the

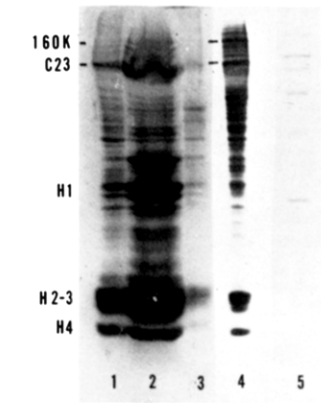

FIGURE 7: Proteins associated with the nucleolar portion of the nuclear matrix. Nucleoli were extracted after DNase I treatment as described under Materials and Methods. Equivalent amounts of each of the following washes were applied to a 12% polyacrylamide Laemmli type $NaDodSO_4$ gel: (lane 1) proteins liberated by DNase I into low salt buffer; (lane 2) proteins extracted by high salt buffer; (lane 3) proteins extracted by Triton X-100 wash; (lane 4) proteins remaining after extractions (nucleolar matrix); (lane 5) molecular weight standard consisting of (from top to bottom) myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase B (M_r 97 400), bovine albumin (M_r 66 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 29 000). Numbers at left designate the 160K protein, protein C23, and the histones.

DNA of these nucleosomes falls within the repeat lengths previously reported for chromatin of various rat tissues (Compton et al., 1976). Electrophoretic analyses of proteins sedimenting with various nucleolar nucleosomal fractions showed that histones followed the nucleosomal gradient profile as normally seen in whole nuclear chromatin. In addition, the putative nucleolus organizer protein C23 was released concomitantly with nucleosomes, but the bulk of it was not nucleosome associated. Protein B23, a nucleolar phosphoprotein with characteristics in common with C23, also sedimented in the prenucleosomal region of the gradient. Another prominent protein band (160K) was also released by micrococcal nuclease and sedimented along with dimers and trimers of the nucleosome gradient. The latter protein appears to be part of another structural component of the nucleolus, the nuclear matrix. Thus, one can envisage various components of the nucleolus being organized by several different proteins.

If protein C23 serves as an organizer molecule in the nucleolus as has been proposed (Olson et al., 1981; Lischwe et al., 1981; Olson, 1982), it would be expected to interact with chromatin as well as with RNP components, possibly serving as a bridge between the two structures. Recent studies suggest that protein C23 may be capable of binding DNA with preference for a region upstream from genes coding for ribosomal RNA.[2] The protein has also been found in the nucleolus organizer regions of chromosomes (Lischwe et al., 1981) as well as in the fibrillar elements of the nucleolus (Daskal et al., 1980). Association with DNA is supported by the partial release of protein C23 from nucleoli by DNase I. In addition, a small but significant fraction of protein C23 was found associated with nucleosomes in these studies. Although the peak of slowly sedimenting C23 (near 7 S) overlapped with the mononucleosome peak, and it cannot be conclusively stated that protein C23 is in monosomes, small amounts of the protein were always found in dimers and trimers at various times of digestion. Furthermore, protein C23 remained associated with dimers after the pooled dinucleosome fractions were recentrifuged under the same sucrose density gradient conditions. Thus, it is likely that a small portion of protein C23 is nucleosome associated, although the bulk of it is either loosely associated with nucleolar components of RNP bound. Consequently, if protein C23 is involved in organization of the nucleolus, the majority of the protein's interactions are probably not at the DNA level.

Earlier studies showed that protein C23 is also associated to some extent with nucleolar RNP particles (Prestayko et al., 1974; Olson et al., 1974). This portion of the protein would be expected to be released by ribonuclease activity. These studies show that ribonuclease A digestion releases protein C23, suggesting that the protein is associated to a significant extent with RNP elements. Micrococcal nuclease, which acts on RNA as well as DNA, also releases protein C23, and this may be partially due to action on RNA. Approximately 90% of protein C23 recovered from the gradient sediments more slowly than mononucleosomes (approximately 7 S). Thus, it is unlikely that this portion of the protein is associated with intact preribosomal RNP particles since the isolatable preribosomal RNP particles have much higher *s* values (30–80 S) (Olson & busch, 1978).

Studies on whole nuclear chromatin have shown that transcriptionally active genes are preferentially digested by DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1977) and micrococcal nuclease (Bellard et al., 1978; Bloom & Anderson, 1978; Levy-Wilson et al., 1979). Protein C23 appears to be present to a limited extent in nucleosomal structures which were released at short times of digestion (2–4 min), rendering 2–4% of the chromatin acid soluble. However, at this time we have no positive evidence to indicate that the sequences released are derived from actively transcribing ribosomal genes or that protein C23 is associated with transcribed rDNA in vivo.

The studies reported here are consistent with previous studies showing that protein C23 is distributed among various fractions of the nucleolus, e.g., loosely bound or RNP associated and tightly bound to chromatin (Prestayko et al., 1974; Olson et al., 1975; Rothblum et al., 1977). It is conceivable that protein C23 plays a dynamic role, possibly shuttling between chromatin and RNP components. Recent ultrastructural studies (Hernandez-Verdun et al., 1982) have shown that silver-staining nucleolus organizer proteins are in close association with DNA as well as with RNP fibrillar components of the nucleolus. The presence of such proteins is an indication of the activity of the nucleolus in transcription of the ribosomal gene (Miller, D. A., et al., 1976; Miller, O. J., et al., 1976). Thus, protein C23, which is a major silver-staining protein of the nucleolus (Lischwe et al., 1979), may be present at the start of the transcriptional and ribosome assembly processes and may then move with nascent preribosomal RNA as the RNP components are formed.

These studies revealed the presence of a 160K protein that is sequestered in the nucleolus. Since this polypeptide cosedimented predominantly with di- and trinucleosomes, it may be present in the internucleosomal spacer region of nucleolar chromatin. Alternatively, it may sediment as an aggregate or as part of some other macromolecular complex which cosediments with di- or trinucleosomes. The relationship of this protein to other nucleolar proteins is unknown; however, its molecular weight is similar to that of nucleolar skeletal protein from amplified *Xenopus laevis* nucleoli. Furthermore, in the studies reported here the 160K protein was found in the residual nucleolar matrix fraction prepared by DNase I digestion and high salt extraction (Berezney & Buchholtz, 1981). Work in progress suggests that this protein is a component of the nuclear matrix which is uniquely nucleolar.[3] Thus, the

[3] M. O. J. Olson and B. A. Thompson, unpublished results.

160K protein may be important in organizing the nucleolus at another level, possibly by promoting an interaction of nucleolar chromatin with the nuclear matrix.

Acknowledgments

We thank Ruth Andersen for technical assistance in the early stages of this work. We also thank Drs. Brad Chaires, Steven Case, and Donald Sittman for their critical review of this work and Romie Brown for typing the manuscript.

References

Attardi, G., & Amaldi, F. (1970) *Annu. Rev. Biochem. 39*, 183–226.

Attardi, G., Huany, P. C., & Kabat, S. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 185–192.

Bellard, M., Cannon, F., & Chambon, P. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 779–787.

Berezney, R., & Buchholtz, L. A. (1981) *Biochemistry 20*, 4995–5001.

Bloom, K. S., & Anderson, J. N. (1978) *Cell (Cambridge, Mass.) 15*, 141–149.

Borchsenius, S., Bonven, B., Leer, J. C., & Westergaard, O. (1981) *Eur. J. Biochem. 117*, 245–250.

Busch, H., & Smetana, K. (1970) *The Nucleolus*, Academic Press, New York.

Compton, J. L., Bellard, M., & Chambon, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4382–4386.

Daskal, Y., Smetana, K., & Busch, H. (1980) *Exp. Cell Res. 127*, 285–291.

Franke, W. W., Kleinschmidt, J. A., Spring, H., Krohne, G., Grund, C., Trendelenburg, M. F., Stoehr, M., & Scheer, U. (1981) *J. Cell Biol. 90*, 289–299.

Fuke, M., & Busch, H. (1979) *FEBS Lett. 99*, 136–140.

Fuke, M., Davis, F. M., & Busch, H. (1979) *FEBS Lett. 102*, 46–50.

Garel, A., & Axel, R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3966–3970.

Hernandez-Verdun, D., Derenzini, M., & Bouteille, M. (1982) *Chromosoma 85*, 461–473.

James, G. T., Yeoman, L. C., Matsui, S., Goldberg, A. H., & Busch, H. (1977) *Biochemistry 16*, 2384–2389.

Johnson, E. M., Allfrey, V. G., Bradbury, E. M., & Matthews, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3966–3970.

Kumar, H., & Subramanian, A. R. (1975) *J. Mol. Biol. 94*, 409–420.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–681.

Levy-Wilson, B., Connor, N., & Dixon, G. H. (1979) *J. Biol. Chem. 254*, 609–620.

Lischwe, M. A., Smetana, K., Olson, M. O. J., & Busch, H. (1979) *Life Sci. 25*, 701–708.

Lischwe, M. A., Richards, R. L., Busch, R. K., & Busch, H. (1981) *Exp. Cell Res. 136*, 101–109.

Mamrack, M. D., Olson, M. O. J., & Busch, H. (1977) *Biochem. Biophys. Res. Commun. 76*, 150–157.

Mamrack, M. D., Olson, M. O. J., & Busch, H. (1979) *Biochemistry 18*, 3381–3386.

Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787–3794.

McCarty, K. S., Vollmer, R. T., & McCarty, K. S. (1974) *Anal. Biochem. 61*, 165–183.

McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 115–1156.

Michalik, J., Yeoman, L. C., & Busch, H. (1981) *Life Sci. 28*, 1371–1379.

Miller, D. A., Dev, V. G., Tantravani, R., & Miller, O. J. (1976) *Exp. Cell Res. 101*, 235–243.

Miller, O. J., Miller, D. A., Dao, V. G., Tantravani, R., & Croce, C. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4531–4535.

Olson, M. O. J. (1982) in *Biochemistry and Biology of the Cell Nucleus* (Hnilica, L. S., Ed.) Vol. II, CRC Press, Boca Raton, FL (in press).

Olson, M. O. J., & Busch, H. (1978) in *Methods in Chromosomal Protein Research* (Stein, G., Stein, J., & Kleinsmith, L., Eds.) pp 163–210, Academic Press, New York.

Olson, M. O. J., Prestayko, A. W., Jones, C. E., & Busch, H. (1974) *J. Mol. Biol. 90*, 161–168.

Olson, M. O. J., Ezrailson, E. G., Guetzow, K., & Busch, H. (1975) *J. Mol. Biol. 97*, 611–619.

Olson, M. O. J., Guetzow, K., & Busch, H. (1981) *Exp. Cell Res. 135*, 259–265.

Orrick, L. R., Olson, M. O. J., & Busch, H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1316–1320.

Prestayko, A. W., Klomp, G. T., Schmoll, D. J., & Busch, H. (1974) *Biochemistry 13*, 1945–1952.

Rao, S. V. V., Mamrack, M. D., & Olson, M. O. J. (1982) *J. Biol. Chem.* (in press).

Rothblum, L. I., Mamrack, P. M., Kunkle, H. M., Olson, M. O. J., & Busch, H. (1977) *Biochemistry 16*, 4716–4721.

Valenzuela, P., Weinberg, F., Bell, G., & Rutter, W. J. (1976) *J. Biol. Chem. 251*, 1464–1470.

Vavra, K. J., Colavito-Shepanski, M., & Gorovsky, M. A. (1982) *Biochemistry 21*, 1772–1781.

Warner, J. R. (1979) *J. Cell Biol. 80*, 767.

Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 93*, 848–858.