Fractionation of Nucleoli. Enzymatic and Two-Dimensional Polyacrylamide Gel Electrophoretic Analysis†

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ABSTRACT: When nucleoli were sequentially extracted with (1) 0.075 M NaCl-0.025 M EDTA, (2) 10 mM Tris-HCl (pH 8.0), (3) 0.15 M NaCl, (4) 0.35 M NaCl, (5) 0.6 M NaCl, and (6) 3 M NaCl-7 M urea, the fractions obtained contained 19, 35, 5, 13, 14, and 14%, respectively, of the extractable nucleolar proteins. The residue contained 4% of the total nucleolar proteins. The first two buffers extracted a total of 54% of the nucleolar proteins. The major polypeptide components of these extracts were dissimilar. These differences were reflected in the distribution of RNA polymerase I and protein kinase between the two fractions. Thirteen percent of the extracted RNA polymerase I activity and 40% of the extracted protein kinase activity were in the 0.075 M NaCl-0.025 M EDTA extracts. The 10 mM Tris-HCl extract contained 87 and 60% of the solubilized RNA polymerase I and protein kinase activity, respectively. Two-dimensional polyacrylamide gel electrophoresis showed the 10 mM Tris extracts contained nucleolar preribosomal particles; the protein components of these particles were similar to those described by Prestayko et al. (Prestayko, A. W., Klomp, G. R., Schmoll, D. J., and Busch, H. (1974), *Biochemistry 13,* 1945), including a component that migrates as histone H1. The two-dimensional polyacrylamide gel electrophoresis protein patterns of the sequential 0.15, 0.35, and 0.6 M NaCl extracts were very similar; several qualitative and quantitative differences were found. The major difference was the predominant amount of histone H1 in the two-dimensional polyacrylamide gel electrophoresis of the 0.6 M NaCl extract. A distinct fraction of nucleolar nonhistone proteins was extracted with 3 M NaCl-7 M urea after the sequential saline extractions. These results show that the nucleolar proteins could be fractionated into the more soluble components which are involved in the synthesis and processing of preribosomal RNA and the less soluble components of the chromatin residue which apparently restrict the readout to rRNA (Matsui, S., Fuke, M., and Busch, H. (1977), *Biochemistry 16,* 39; Ballal, N. R., Choi, Y. C., Mouche, R., and Busch, H. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 2446).

It is well established that the nucleolus is the site of ribosomal precursor RNA synthesis (Busch and Smetana, 1970). Isolated nucleoli retain their specificity of gene expression (Ballal et al., 1977; Matsui et al., 1977). When nucleolar chromatin was prepared by sequentially washing nucleoli in 0.075 M NaCl-0.025 M EDTA[1] (pH 8.0) and 10 mM Tris-HCl (pH 8.0) the residual "chromatin" retained its transcriptional specificity (Ballal et al., 1977; Matsui et al., 1977).

Several laboratories have reported that nonhistone chromosomal proteins extracted from nuclear chromatin with solutions of low ionic strength (0.35) are responsible for structural and functional characteristics of chromatin (Augenlicht and Baserga, 1977; Hjelm and Huang, 1974; Kusch et al., 1974; Nicolini et al., 1975; Kostraba et al., 1975). On the other hand, Commings and Tack (1973) and Johns and Forrester (1969) have pointed out that many acidic nonhistone proteins may be of cytoplasmic or nucleoplasmic origin.

Montagna and Wang (1976) fractionated the proteins of Ehrlich ascites tumor chromatin into loosely bound and tightly bound fractions. They found that these fractions of nonhistone proteins contained different amounts of phosphoproteins and that there was a differential distribution of DNA-dependent RNA polymerase II and DNA-dependent DNA polymerase.

The present study was designed to evaluate the distribution of RNA polymerase I, protein kinase, preribosomal particles, and other nucleolar proteins in nucleolar fractions obtained by extraction with saline solutions of increasing ionic strength. The nucleolar chromatin was extracted sequentially with 0.15 M NaCl, 0.35 M NaCl, 0.6 M NaCl, and 3 M NaCl-7 M urea, and these subfractions of the nucleolar proteins were also compared. The present findings provide evidence that there are differences between the proteins extracted from nucleoli during the isolation of chromatin and the chromatin proteins. The more soluble components appear to be involved in synthesis and processing of preribosomal RNA and the less soluble components appear to function in regulation of the specificity of gene transcription (Ballal et al., 1977; Matsui et al., 1977).

Materials and Methods

Isolation of Nucleoli. Nucleoli were isolated by sonication of 7-day-old Novikoff hepatoma ascites cells transplanted intraperitoneally in male Holtzman rats as described previously (Matsui et al., 1977). Ascites cells were freed of red blood cells by centrifugation at 1100*g* for 10 min and washed twice in Dulbecco's salt solution. These and all subsequent steps were carried out at 4 °C. The cells were then suspended in 2.0 M sucrose-12 mM $MgCl_2$-10 mM Tris-HCl (pH 7.5), and centrifuged at 7000*g* for 60 min. The cell pellet was resuspended in 0.88 M sucrose-12 mM$MgCl_2$-10 mM Tris-HCl (pH 7.5), sonicated for 15 s at 30-s intervals until no nuclei remained intact, and centrifuged at 1100*g* for 20 min. The crude nucleolar pellet was resuspended in 0.88 M sucrose-1 mM $MgCl_2$-10 mM Tris-HCl (pH 7.5), and resonicated to disperse the extranucleolar chromatin. The sonicate was centrifuged at 1100*g* for 20 min and the nucleolar pellet was washed in the

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[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; NaCl-EDTA, 0.075 M NaCl-0.025 M EDTA, pH 8.0; DEAE, diethylaminoethyl.

low magnesium–sucrose buffer. The nucleoli were stored in 0.88 M sucrose–50% glycerol–1 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5), at −76 °C.

The protease inhibitor, phenylmethanesulfonyl fluoride, was added fresh to all solutions used in this study to a final concentration of 1 mM.

Nucleolar Extractions. Nucleoli were suspended in 15–20 vol (vol/wet wt) of 0.075 M NaCl–0.025 M EDTA (pH 8.0) by gentle homogenization with three strokes of a Teflon glass homogenizer. The suspension was centrifuged for 20 min at 12 000*g*. The supernatant was decanted; the pellet was extracted twice more with NaCl–EDTA and then three times with 10 mM Tris-HCl (pH 8.0); after each extraction the suspension was centrifuged as described above. The pellet is operationally defined as nucleolar chromatin.

The nucleolar chromatin was adjusted with 10 mM Tris-HCl (pH 8.0) to a nucleic acid concentration of approximately 1 mg/mL (an absorbance of 20 at 260 nm in 0.1 N NaOH). An equal volume of a solution containing the proper molarity of NaCl and 10 mM Tris-HCl (pH 8.0) was then added dropwise with stirring to a final concentration consecutively of 0.15, 0.35, and 0.6 M NaCl. The chromatin was extracted twice with each concentration of NaCl by stirring for 1 h followed by centrifigation at 15 000*g* for 30 min. Following the second extraction with 0.6 M NaCl, the chromatin pellet was resuspended in 3 M NaCl–7 M urea–10 mM Tris-HCl (pH 8.0) and stirred overnight. The dissociated chromatin was centrifuged at 105 000*g* for 48 h and the pellet reextracted in the same buffer.

Aliquots of the respective fractions were saved for the determination of protein and enzymes and the extracts were then centrifuged at 105 000*g* for 18 h to remove sheared DNA or chromatin and the supernatant proteins were prepared for polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Protein samples were adjusted to 2 M urea by the addition of solid urea and concentrated to 4–10 mg/mL with an Amicon apparatus containing a PM-10 membrane. The samples were dialyzed against 0.9 N acetic acid–9 M urea–1% β-mercaptoethanol, two changes of 100 vol for 20 h. Two-dimensional polyacrylamide gel electrophoresis was carried out using either 10% or 6% acid–urea polyacrylamide gels in the first dimension and 12 or 8%, respectively, $NaDodSO_4$–phosphate polyacrylamide gel slabs in the second dimension as described (Orrick et al., 1973; Busch et al., 1974). For convenience, the two-dimensional electrophoretogram patterns were subdivided into either A, B, and C, or B and C, regions, and the spots numbered according to the nomenclature described previously (Olson et al., 1975; Yeoman et al., 1975; Orrick et al., 1973).

Assay of RNA Polymerase. The RNA polymerase content of isolated nucleoli and nucleolar chromatin was measured in three ways. The nucleoli or chromatin was assayed in (a) the standard reaction mixture with endogenous DNA as template, (b) the standard reaction mixture plus exogenous nucleolar DNA, and (c) the standard reaction mixture containing both exogenous DNA and endogenous DNA following solubilization and concentration (Roeder, 1974). The amount of activity measured in method c was greater than that obtained by methods a and b because the sheared endogenous nucleolar DNA was not removed. The RNA polymerase content of the nucleolar extracts was assayed either directly following dialysis against 10 mM Tris-HCl (pH 7.8)–25% v/v glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM DTT (TGMED) or following concentration by ammonium sulfate precipitation (70% of saturation). All samples were dialyzed to equilibrium with TGMED prior to assay. The standard reaction mixture contained in 200 μL: 10 μmol of Tris-HCl (pH 8.0), 1 μmol of DTT, 1 μmol of $MgCl_2$, 0.2 μmol of $MnCl_2$, 10 μmol of $(NH_4)SO_4$, 1 μmol of NaF, 0.2 μmol each of ATP, CTP, and UTP, and 0.2 μmol of [^{3}H]GTP (sp radioact. 80–100 cpm/pmol) and 25 μg of nucleolar DNA. One unit of enzyme activity incorporates 1 pmol of GMP in 10 min. After 10 min at 37 °C, reactions were terminated by pipetting aliquots onto DEAE-cellulose disks (Whatman DE-81). The filters were washed as described (Roeder, 1974) and radioactivity was determined by counting in a toluene-based fluor (Omnifluor, New England Nuclear) containing NCS solubilizer (Nuclear-Chicago).

TABLE I: Distribution of Nucleolar Proteins.[a]

Fraction	% total nucleolar protein	% total dissociated nucleolar chromatin protein
NaCl–EDTA	19 ± 3	
10 mM Tris	35 ± 5	
Chromatin	46 ± 5	100
0.15 M NaCl	5 ±1	14 ± 1.4
0.35 M NaCl	13 ± 4	27 ± 4.0
0.6 M NaCl	14 ± 4	30 ± 4.8
3 M NaCl–7 M urea	14 ± 6	28 ± 7.6

[a] Nucleoli were extracted as described in Materials and Methods, and the amount of protein extracted was determined and is represented here as the percent of the total amount extracted. Data presented are the mean ± the standard error of the mean.

Assay of Protein Kinase. Protein kinase was assayed by modifying the filter disk method of Corbin and Reimann (1974) using casein as the substrate. The final concentration of $MgCl_2$ was 10 mM. Reactions were run for 30 min, after which aliquots were spotted on cellulose filter disks. The disks were first washed twice in 15% trichloroacetic acid, 2 mM ATP, 1 mM NaH_2PO_4, and 1.5% $Na_4P_2O_7$, then once in 15% trichloroacetic acid, 1 mM NaH_2PO_4, and 1.5% $Na_4P_2O_7$, followed by one wash with 95% ethanol and a final wash in ether.

Other Methods. Protein was determined by the method of Lowry et al. (1951). DNA was determined by a modification of the diphenylamine reaction as described by Richards (1974), and RNA by the orcinol reaction (Hurlbert et al., 1954).

Results

Distribution of Nucleolar Proteins. When nucleoli were extracted with 0.075 M NaCl–0.024 M EDTA, 19% of the nucleolar proteins were removed (Table I). A subsequent wash with 10 mM Tris extracted another 35% of the nucleolar proteins. These two buffers extracted 54% of the nucleolar proteins.

The remainder of the protein was removed by salt extractions, and may be more tightly bound to the DNA or chromatin. The chromatin was only extracted two times at each stage as it was determined that a third extraction would remove less than 5% of the total protein extractable at a particular molarity of NaCl. The 0.15 M NaCl extraction contained 5% of the nucleolar proteins and the subsequent extracts with 0.35 M NaCl, 0.6 M NaCl, and 3 M NaCl–7 M urea contained 13, 14, and 14% of the nucleolar proteins (Table I).

Distribution of RNA Polymerase and Protein Kinase. Both RNA polymerase I and protein kinase (Kang et al., 1974) activities were assayed in whole nucleoli and various fractions. The NaCl–EDTA and 10 mM Tris washes contained 40 and 60% of the extracted nucleolar protein kinase activity, respectively. However, only 13% of the extracted RNA poly-

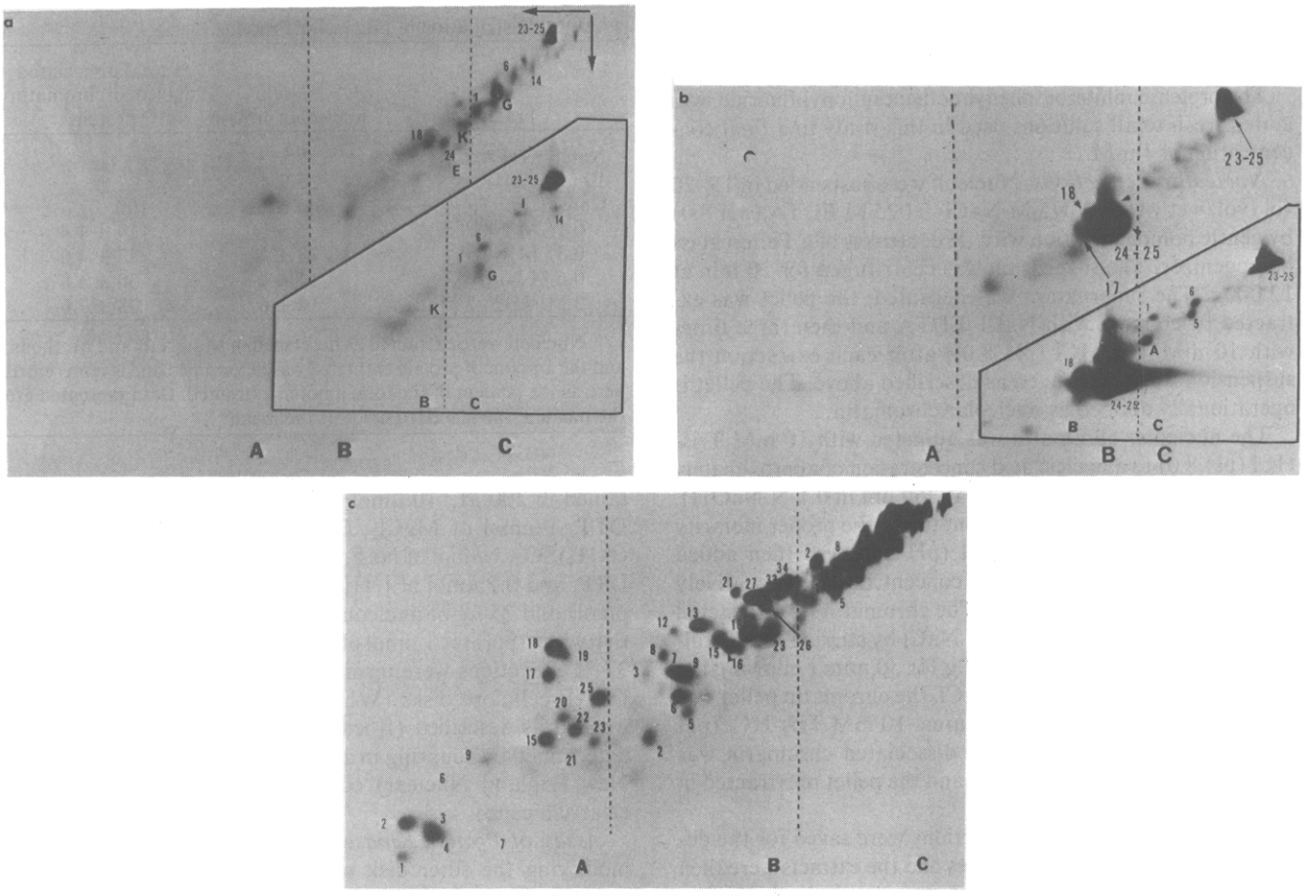

FIGURE 1: (a) Two-dimensional polyacrylamide gel electrophoresis of 200 μg of Novikoff hepatoma nucleolar proteins extracted with NaCl–EDTA. Samples were run in the first dimension (horizontal arrow) on disc gels of 10% acrylamide–6 M urea–0.9 N acetic acid. For the second dimension electrophoresis (vertical arrow), a 12% acrylamide–0.1% $NaDodSO_4$ slab gel was run. Such runs are referred to as 10/12 two-dimensional polyacrylamide gel electrophoresis. Inset (enclosed by solid lines): Two-dimensional polyacrylamide gel electrophoresis of Novikoff hepatoma nucleolar proteins extracted with NaCl–EDTA. Samples were run in the first dimension on the disc gels of 6% acrylamide–6 M urea–0.9 N acetic acid. For the second dimension an 8% acrylamide–0.1% $NaDodSO_4$ slab gel was run; such runs are referred to as 6/8 two-dimensional gel electrophoresis. The electrophoretograms were divided, as indicated by the dashed lines, into three regions A, B and C, or B and C; A is the region of most rapid migration of the proteins in the first dimension; the protein spots designated by numbers were originally found in acid extracts of whole nucleoli (Orrick et al., 1973), those designated by upper case letters were originally found in nucleolar chromatin fraction II (Olson et al., 1975), and those designated by lower case letters were originally found in acid extracts of nuclei (Yeoman et al., 1973). (b) 10/12 two-dimensional gel electrophoresis of 110 μg of Novikoff hepatoma nucleolar proteins extracted with 10 mM Tris-HCl (pH 8.0). Inset: 6/8 two-dimensional gel electrophoresis of Novikoff hepatoma nucleolar proteins extracted with 10 mM Tris-HCl (pH 8.0). (c) 10/12 two-dimensional gel electrophoresis of 300 μg of Novikoff hepatoma nucleolar preribosomal particle proteins extracted with 10 mM Tris-HCl (pH 8.0). Proteins were extracted from the particles with 3 M NaCl–7 M urea–20 mM Tris-HCl (pH 8.0).

TABLE II: Distribution of RNA Polymerase I and Protein Kinase in the NaCl–EDTA and Tris Extracts of Nucleoli.[a]

	Protein kinase[b] (cpm $\times 10^{-5}$)	RNA polymerase[b] (units $\times 10^{-3}$)
NaCl–EDTA	11.2 (40)	10.4 (12.5)
10 mM Tris	18.4 (60)	67.9 (87.5)

[a] Enzyme activities are expressed as counts per minute of ^{32}P incorporated into casein for protein kinase and as units for RNA polymerase. Numbers in parentheses are the percent of the total extracted. For further details see Materials and Methods. [b] Protein kinase represents 77% of the total nucleolar activity; RNA polymerase represents 73% of the total nucleolar activity.

TABLE III: Distribution of RNA Polymerase I and Protein Kinase in Subnucleolar Fractions.[a]

	RNA polymerase I			Protein kinase
	I	II	III	
Nucleoli	20.8	106.6	170.6	38.4
Chromatin	4.8 (23)	22.5 (21)	38.6 (23)	8.8 (23)

[a] RNA polymerase activity determined as described in Materials and Methods, using: I, endogenous template; II, endogenous template and added nucleolar DNA; and III, nucleoli or chromatin extracted as described by Roeder (1974). Protein kinase activity is expressed as counts per minute of ^{32}P incorporated into casein. Numbers in parentheses are the percentages of the total nucleolar activity. For further details refer to Materials and Methods.

merase I was in the NaCl–EDTA washes; 87% of the RNA polymerase I was in the 10 mM Tris washes (Table II).

The chromatin residue after the NaCl–EDTA and 10 mM Tris extractions contained only 22% of the nucleolar RNA polymerase I activity, with three different assay methods, and 25% of the nucleolar protein kinase (Table III). NaCl (0.15 M) extracted another 17% of polymerase I and 15% of the protein kinase from the chromatin residue. After two 0.35 M NaCl extractions, less than 3% of the polymerase activity remained associated with the chromatin.

Analysis of Nucleolar Proteins. The first two nucleolar extractions, i.e. NaCl–EDTA and 10 mM Tris, extracted more than 50% of the nucleolar proteins. The proteins of these

TABLE IV: Protein Components of the Sequential Nucleolar Extracts.[a]

Fraction	Gel region A	B	C
0.075 M NaCl–0.025 M EDTA		*18, 24, K*	*6, G,* 14, I, *23–25*
10 mM Tris		17, *18, 24, 25*	A, 1, 5, 6, *23–25*
10 mM Tris pellet	1, 2, 3, 4, 6, 7, 9, 15, 17, 18, 19, 20, 21, 22, 23, 25	2, 3, 5, 6, 7, 8, 9, 13, 15, 16, 18, 21, 23, 26, 27, 33, 34	1, 2, 5, 8
0.15 M NaCl		*17, 18,* 28, 35	6, 8, M″, 1′, 16, 18, g′, Q, 20, 24
0.35 M NaCl	*17, 19,* 24, 25	1′, 5, 7, *18,* 24, 25, I, 28, 33	5, 6, 8, 13, 14, I′, g′, 15, 16, 17, 18, Q, 22, *24,* 24′, z, z′
0.6 M NaCl	*17, 18, 19*	*18,* 33′, 25	5, 6, 8, 11′, 13, 14, 15′, g′, 17, 18, *24,* 24′, 27, 27A, 26A, z, z′
3 M NaCl–7 M urea	*GAR, 1, 2, 3, 4, 7,* 15, 24, 25	2, 7, 9, 13, 16, 26, 27, J, 33, 34	A, B, C, G, 6, 8′, M, M′, I, g′, P, 18, Q, 21, S, R, T, U, M

[a] The nomenclature used pertains to the region, either A, B, or C, of the two-dimensional polyacrylamide gel electrophoresis. Protein spots GAR, 1, 2, 3, 4, 7 and 17, 18, 19 correspond to histones H4, H2a, H2b, H3, and H1, respectively. Italic peptide numbers indicate a dominant component.

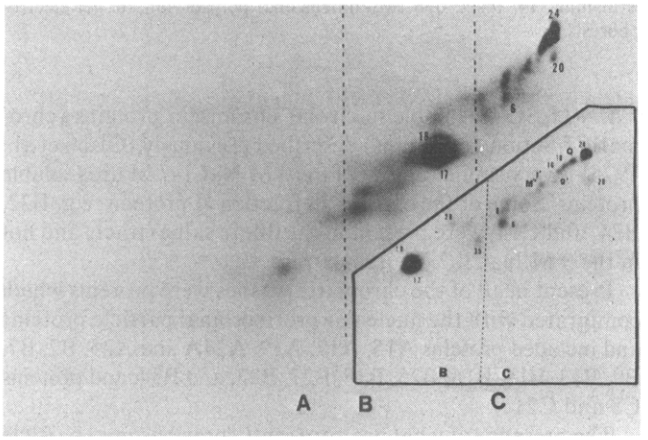

FIGURE 2: Two-dimensional polyacrylamide gel electrophoresis of 200 μg of the proteins extracted with 0.15 M NaCl from nucleolar chromatin. 10/12 two-dimensional polyacrylamide gel electrophoresis. Inset: 6/8 two-dimensional polyacrylamide gel electrophoresis.

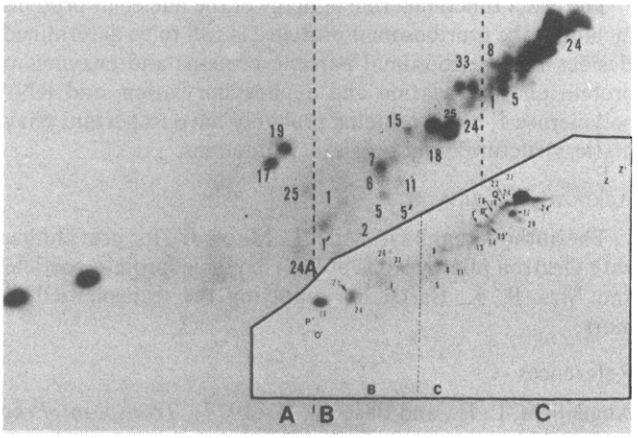

FIGURE 3: Two-dimensional polyacrylamide gel electrophoresis of 550 μg of the proteins extracted with 0.35 M NaCl from nucleolar chromatin. 10/12 two-dimensional polyacrylamide gel electrophoresis. Inset: 6/8 two-dimensional polyacrylamide gel electrophoresis.

fractions were dissimilar by two-dimensional polyacrylamide gel electrophoresis. The NaCl–EDTA extract of nucleoli was heterogeneous containing a minimum of 25 polypeptides (Figure 1a). In the C region, the most prevalent proteins were C23–25, C6, C14, CI, and CG. In contrast, the 10 mM Tris extract consisted predominantly of proteins B18, B24–25, and C23–25 (Figure 1b).

The initial RNA:DNA ratio of nucleoli was 1:1; following the NaCl–EDTA and 10 mM Tris extracts, the RNA:DNA ratio of the residue was 0.2:1. Accordingly, a significant amount of the nucleolar ribonucleoproteins had been extracted. Tris extracts (10 mM) were sedimented at 105 000*g* for 18 h over a 1 M sucrose cushion. The proteins of the pelleted particles, which composed 10% of the extracted proteins, were extracted from the pellet with 3 M NaCl–7 M urea and 20 mM Tris-HCl (pH 8.0). The two-dimensional electrophoretogram (Figure 1c) of these proteins was essentially identical with those of the nucleolar ribonucleoprotein particles (Prestayko et al., 1974; Daskal et al., 1974). Furthermore, these pellets were essentially free of DNA.[2]

The 0.15 M NaCl extract of chromatin (Figure 2) consisted mainly of proteins with molecular weights greater than 30 000.[3] The sample was of limited heterogeneity; proteins B17, B18, and C24 were the densest spots present. The 0.35 M NaCl (Figure 3) extract of chromatin contained many proteins not extracted by 0.15 M NaCl (Table IV). For example, proteins B24, B25, C17, and C27 which were dense spots in the 0.35 M NaCl extract (Figure 3 and inset) were not found in the 0.15 M NaCl extract (Figure 2 and inset). On the other hand, proteins B35 and B17 were not present in the 0.35 M NaCl extract; they were in the 0.15 M NaCl extracted group of proteins.

The H1 histone spots (A17, A18, and A19) were dense and large in the 0.6 M NaCl extract (Figure 4); proteins C17 and C24 also were dense spots. When the post 0.6 M NaCl chromatin residue was extracted with 3 M NaCl–7 M urea, the histones (GAR, A1, A2, A3, and A4), other than the H1 histones, were the major polypeptides present (Figure 5).

In addition, several low molecular weight proteins including A7, A15, A24, and A25 were also found in the 3 M NaCl–7 M urea extract. Additional dense spots in this fraction were B7, B9, B13, BJ, and CA, C6, CM, CM′, CI, CP, and CQ which were previously found by Yeoman et al. (1975) in

[2] Scanning electron micrographs of these pellets showed that they were a homogeneous preparation of particles. Their diameters were similar to that reported by Koshiba et al. (1971) for ribonucleoprotein particles isolated from the nucleoli of thioacetamide-treated rat livers.

[3] As determined by comparison with electrophoretograms of molecular weight standards: bovine serum albumin, 67 000; ovalbumin, 45 000; and cytochrome *c*, 12 000.

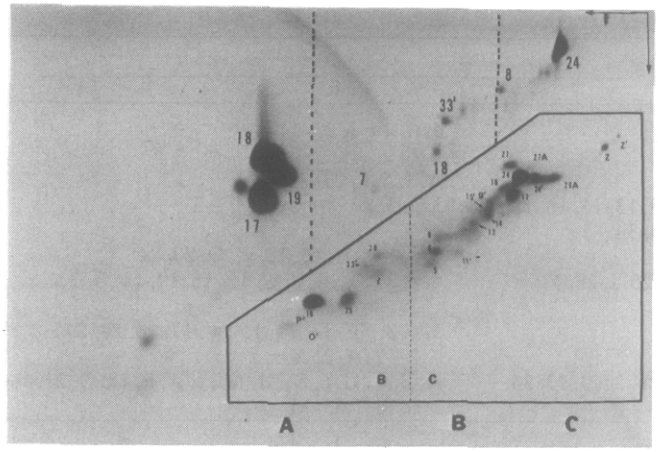

FIGURE 4: Two-dimensional polyacrylamide gel electrophoresis of 575 μg of the proteins extracted with 0.6 M NaCl from nucleolar chromatin. 10/12 two-dimensional polyacrylamide gel electrophoresis. Inset: 6/8 two-dimensional electrophoresis run. Spots 17–19 are histone H1.

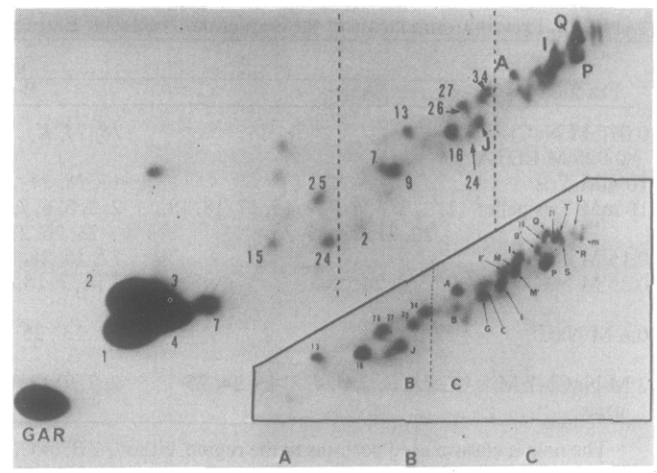

FIGURE 5: Two-dimensional polyacrylamide gel electrophoresis of 600 μg of the residual chromatin proteins extracted by 3 M NaCl–7 M urea. 10/12 two-dimensional polyacrylamide gel electrophoresis. Protein GAR is histone H4. Inset: 6/8 two-dimensional polyacrylamide gel electrophoresis.

chromatin fraction II, and by Olson et al. (1975) in nucleolar chromatin fraction II.

Discussion

In the procedure for isolating nucleolar chromatin a number of soluble fractions were obtained that contain nucleolar proteins responsible for transcription and processing, as well as the nucleolar preribosomal particles which are the products of synthesis and assembly.

The extraction of nucleoli with 0.075 M NaCl–0.025 M EDTA removes 19% of the nucleolar proteins; the polypeptide composition of the extract is heterogeneous. This fraction contains 40% of the extractable nucleolar protein kinase and 13% of the extractable RNA polymerase I.

The 10 mM Tris extracts of nucleoli contained 87% of the extracted RNA polymerase I and 60% of the protein kinase. These extracts removed 35% of the nucleolar proteins. The amount of protein extracted by the NaCl–EDTA and Tris washes, 54% of the nucleolar protein, was similar to that reported by dePomerai et al. (1974) for nuclear chromatin. Two-dimensional polyacrylamide gel electrophoresis showed that 8 polypeptides were the predominant protein species of the 100 000*g* supernatant of the Tris extract. In addition, 10 mM Tris also extracts a 100 000*g* pellet which contains nucleolar preribosomal particles. The protein complement of these particles is identical with that reported (Prestayko et al., 1974) for nucleolar preribosomal particles prepared by the EDTA method.

These results confirm that nucleolar preribosomal particles contain proteins A17, A18, and A19, which have been shown to comigrate with Novikoff hepatoma nuclear histone H1 (Goldknopf and Busch, 1973). The role of this histone is uncertain; it is not known whether it may have a specific structural role or is incorporated into the particles during their formation. It might also become associated with the preribosomal particles during the extraction procedure.

The nucleolar chromatin was sequentially extracted with increasing molarities of NaCl, followed by a final extraction with 3 M NaCl–7 M urea. These buffers extract approximately 90% of the nucleolar chromatin proteins. The proteins of the three dilute NaCl extracts of chromatin were qualitatively similar, and only a few differences were observed (Table IV). The more tightly bound proteins, those which were extracted with 3 M NaCl–7 M urea, were significantly different from the loosely bound proteins. The electrophoretic pattern of the 0.4 N H_2SO_4 insoluble nucleolar chromatin proteins (chromatin fraction II proteins) described previously (Olson et al., 1975) is very similar to that of the 3 M NaCl–7 M urea soluble proteins. Some of the chromatin fraction II proteins, e.g. B22, B24, and C14, were present in the dilute salt extracts and not in the 3 M NaCl–7 M urea extract.

Present in all of the chromatin washes were proteins which comigrated with the nucleolar preribosomal particle proteins and included proteins A15, A17, A19, A24A and A25, B2, B7, B9, B13, B15, B16, B25, B26, B27, B33, and B34, and proteins C8 and C21.

The presence of what are apparently preribosomal particle proteins in the chromatin protein fractions may be due to the presence of such proteins in association with transcriptionally active chromatin. Recently, Bolla et al. (1977) reported a stimulatory effect of ribosomal proteins on ribosomal RNA synthesis by isolated nuclei in vitro.

The exact functional role of many of the nucleolar proteins, including the preribosomal proteins, is still to be determined. Besides the preribosomal particle proteins and enzymes of protein phosphorylation and dephosphorylation, and RNA polymerase I, other proteins probably have important enzymatic, structural, and regulatory functions.

Acknowledgments

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References

Augenlicht, L. H., and Baserga, R. (1977), *Transplant. Proc. 5,* 1177.

Ballal, N. R., Choi, Y. C., Mouche, R., and Busch, H. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 2446.

Bolla, R., Roth, H., Weissbach, H., and Brot, N. (1977), *J. Biol. Chem. 252,* 721.

Busch, G. I., Yeoman, L. C., Taylor, C. W., and Busch, H. (1974), *Physiol. Chem. Phys. 6,* 1.

Busch, H., and Smetana, K. (1970), The Nucleolus, New York, N.Y., Academic Press.

Comings, D. E., and Tack, L. O. (1973), *Exp. Cell Res. 82,* 175.

Corbin, J. D., and Reimann, E. M. (1974), *Methods Enzymol. 38,* 287.
Daskal, Y., Prestayko, A. W., and Busch, H. (1974), *Exp. Cell Res. 88,* 1.
dePomerai, D. I., Chesterton, C. R., and Butterworth, P. H. W. (1974), *Eur. J. Biochem. 46,* 461.
Goldknopf, I. L., and Busch, H. (1973), *Physiol. Chem. Phys. 5,* 131.
Hjelm, R. P., and Huang, R. C. (1974), *Biochemistry 13,* 5275.
Hurlbert, R. B., Schmitz, H., Brumn, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209,* 23.
Johns, E. W., and Forrester, S. (1969), *Eur. J. Biochem. 8,* 547.
Kang, Y.-J., Olson, M. O. J., and Busch, H. (1974), *J. Biol. Chem. 249,* 5580.
Koshiba, K., Thirumalachary, C., Daskal, Y., and Busch, H. (1971), *Exp. Cell Res. 68,* 235.
Kostraba, N. C., Montagna, R. A., and Wang, T. Y. (1975), *J. Biol. Chem. 250,* 1548.
Kusch, A. A., Kolesnikov, V. A., and Zelenin, A. V. (1974), *Exp. Cell Res. 86,* 419.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Matsui, S., Fuke, M., and Busch, H. (1977), *Biochemistry 16,* 39.
Montagna, R. A., and Wang, T. Y. (1976), *Cancer Res. 36,* 3138.
Nicolini, C., Ng, S., and Baserga, R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2361.
Olson, M. O. J., Ezrailson, E. G., Guetzow, K., and Busch, H. (1975), *J. Mol. Biol. 97,* 611.
Orrick, L. I., Olson, M. O. J., and Busch H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1316.
Prestayko, A. W., Klomp, G. R., Schmoll, D. J., and Busch, H. (1974), *Biochemistry 13,* 1945.
Richards, G. M. (1974), *Anal. Biochem. 57,* 369.
Roeder, R. G. (1974), *J. Biol. Chem. 249,* 241.
Yeoman, L. C., Taylor, C. W., and Busch, H. (1973), *Biochem. Biophys. Res. Commun. 51,* 956.
Yeoman, L. C., Taylor, C. W., Jordan, J. J., and Busch, H. (1975), *Exp. Cell Res. 91,* 207.

Yeast Mutants Blocked in Removing the Methyl Group of Lanosterol at C-14. Separation of Sterols by High-Pressure Liquid Chromatography†

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ABSTRACT: Sterols of a nystatin resistant mutant and of the wild type parent of *Saccharomyces cerevisiae* were separated by a newly developed procedure involving high-pressure liquid chromatography and were identified. The mutant contained larger amounts of squalene and lanosterol (I) than the wild type, as well as 4,14-dimethylcholesta-8,24-dien-3β-ol (II), 4,14-dimethylergosta-8,24(28)-dien-3β-ol (III), and 14-methylergosta-8,24(28)-dien-3β-ol (IV), which were not hitherto found in yeast. These results indicated a block in removal of the methyl group at C-14 of lanosterol. An ergosterol requiring derivative of the mutant which carried in addition a mutation in heme biosynthesis had the same sterols as the parent, but at one-third the concentration. The low level of sterols may be due to a requirement for a heme or cytochrome in oxygenation reactions between lanosterol and ergosterol.

Yeast mutants deficient in ergosterol formation have received attention recently in several laboratories (Parks et al., 1972; Gollub et al., 1974; Fryberg et al., 1974; Barton et al., 1974). In most cases the mutants were isolated by selection for resistance to nystatin or other polyene antibiotics and were blocked in a step beyond lanosterol formation. In this report we describe a mutant deficient in removal of C-32 of lanosterol, i.e., the methyl group attached at C-14 of the sterol nucleus. This step is generally accepted as the first step in the transformation of lanosterol to ergosterol in yeast (Goad, 1970; Fryberg et al., 1973; Barton et al., 1973). We also describe a procedure for separating sterols by high-pressure liquid chromatography.[1] A preliminary communication of the results has appeared (Trocha et al., 1974).

Experimental Section

Strains of Yeast. The wild type strain D587-4B (*αhis1-1*) of *Saccharomyces cerevisiae* was from Dr. F. Sherman's collection and was a gift from Dr. L. Skogerson. Strain SG1 (*erg11-1*), a nystatin resistant derivative of strain D587, and strain SG100 (*erg11-1hem3-1*), a sterol requiring derivative of SG1, were isolated in this laboratory (Gollub et al., 1974). These strains were previously named *Nys1* and *Erg3*, respec-

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[1] Abbreviations used: LC, high-pressure liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; Eu(dpm)$_3$, tris(dipivalomethanato)europium; UV, ultraviolet.