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A Comparison of the Loosely Bound and Tightly Bound Nonhistone Proteins from Ehrlich Ascites Tumor Chromatin[1]

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SUMMARY

The nonhistone proteins of Ehrlich ascites tumor chromatin have been separated into a loosely bound and two tightly bound protein fractions by sequential extraction of chromatin with 0.35 M NaCl and 2 M NaCl:5 M urea. The nonhistone proteins thus obtained were examined for their chemical composition and distribution of DNA polymerase, RNA polymerase, and protein kinase activities. In addition, the effect of these nonhistone proteins on transcription of DNA *in vitro* has been determined. The results indicate that these nonhistone proteins, fractionated on the basis of their extractability, exhibit varied compositional characteristics and play different functional roles in the synthesis of DNA and RNA and in the possible control of gene activity.

INTRODUCTION

When isolated chromatin is extracted with low-salt solutions, a substantial amount of NHP[2] is released (1, 2, 4, 7, 13, 20, 23, 26). Recent evidence indicates that removal of these loosely bound NHP noticeably alters the structure of the chromatin (8, 15, 16, 20) as well as its template capacity in RNA synthesis *in vitro* (1). The chromatin from human fibroblasts stimulated to proliferate, for example, usually displays an increased positive ellipticity in circular dichroism spectra and enhanced binding to ethidium bromide as compared with chromatin from quiescent cells. Extraction of chromatin with 0.25 to 0.35 M NaCl eliminates both of these increases (16, 20). A small change in circular dichroism spectra has also been observed in chick embryo brain chromatin following removal of these loosely bound NHP (8). In partially hepatectomized guinea pig, there is an increased binding of acridine orange by liver chromatin as compared with that by chromatin from normal liver. Treatment with 0.35 M NaCl causes a complete disappearance of differences in acridine orange binding between the stimulated and nonstimulated cells (15). Extraction of chromatin from stimulated WI-38 fibroblasts with 0.3 M NaCl also results in a decreased template activity of the extracted chromatin in RNA synthesis *in vitro* (1). These observations indicate that the loosely bound NHP, released by low-salt solutions, play a role in the structure and transcriptional function of chromatin.

Teng *et al.* (25), and Shea and Kleinsmith (22) first reported a nonhistone phosphoprotein fraction from rat liver that stimulates template- and enzyme-specific RNA synthesis *in vitro*. Such a stimulatory protein fraction has been demonstrated in the NHP extracted from Ehrlich ascites tumor chromatin with 0.35 M NaCl (13). Thus, the loosely bound NHR not only are involved in the conformation of chromatin but are also functional components in the control of gene activity. The question arises as to how the loosely bound NHP compare with the tightly bound NHP. In the work reported here, we prepared the loosely bound and tightly bound NHP from chromatin by extraction with 0.35 M NaCl and 2 M NaCl:5 M urea, respectively, and we show that these nonhistone chromosomal protein fractions play different roles in the function of chromatin.

MATERIALS AND METHODS

Animals. Male Albany Swiss mice, weighing 30 to 40 g, were kindly supplied by New York State Laboratory of Animal Research, Slingerland, N. Y.

Chemicals. Phenylmethylsulfonyl fluoride was purchased from Sigma Chemical Co., St. Louis, Mo. Urea, ultrapure grade, was obtained from Schwarz/Mann, Orangeburg, N. Y.; and BioRex 70, minus 400 mesh, sodium form, was obtained from Bio-Rad Laboratories, Richmond, Calif. Casein was procured from Nutritional Biochemicals Corp., Cleveland, Ohio. All other chemicals used were of highest available quality. Phenol was freshly redistilled before use.

Preparation of Chromatin. Male Albany Swiss mice, weighing 30 to 40 g were each inoculated with 10^7 Ehrlich ascites tumor cells. The mice were killed 13 days postinoculation by cervical dislocation, and the ascites fluid was harvested from the abdominal cavity. Lysis of cells and isolation of chromatin were carried out as described previously (13).

Preparation of Nonhistone Protein Fractions. All extractants and solutions used in the preparation of the NHP fractions were supplemented with 0.16 mM phenylmethylsulfonyl fluoride, and the operations were performed at 2°. The loosely bound NHP were isolated by extracting the purified chromatin with 0.35 M NaCl in 0.02 M Tris-HCl, pH

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[2] The abbreviation used is: NHP, nonhistone chromosomal proteins.

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7.5, as described elsewhere (13), and are referred here as 0.35 M NaCl NHP. The extracted chromatin, after removal of the 0.35 M NaCl NHP, was extracted with 2.0 M NaCl:5.0 M urea in 0.05 M Tris-HCl, pH 8.0, by stirring for 6 hr. The suspension was briefly homogenized in a loosely fitting Dounce homogenizer, adjusted to 100 μg DNA per ml with the NaCl:urea buffer, and centrifuged at 110,000 $\times$ *g* for 48 hr. The supernatant solution was collected, and the pellet was extracted once again in an identical manner. The 2 extracts were combined; dialyzed against 4 volumes of 0.02 M Tris-HCl (pH 7.0), to reduce the NaCl concentration to 0.4 M; and centrifuged at 78,000 $\times$ *g* for 1 hr. The supernatant was then dialyzed against 4 changes of 0.4 M NaCl in 0.02 M Tris-HCl, pH 7.0, and treated with Bio-Rex 70 (13). The unadsorbed proteins, referred to as 2 M NaCl:urea NHP, were dialyzed against 0.05 M NaCl in 0.01 M Tris-HCl, pH 7.0, and concentrated by lyophilization. The lyophilized powder was dissolved in and dialyzed against appropriate buffer before use.

The chromatin residue after extraction with 2 M NaCl:5 M urea as obtained from the above 48-hr centrifugation is referred to as the residual NHP.

Preparation of Phenol-soluble NHP. Phenol-soluble proteins were isolated from the 0.35 M NaCl NHP, 2 M NaCl:urea NHP, and the residual NHP fractions, obtained as described above, according to the procedure described by Teng *et al.* (25).

Amino Acid Analysis. Samples for amino acid analysis were extracted 3 times with 5% trichloroacetic acid at 90°, each time for 20 min. The samples were subsequently washed with 70, 80, 90, and 100% ethanol, followed by anhydrous ether, and air dried. The dried protein samples were hydrolyzed in constant boiling 6 N HCl at 110° in a vacuum for 24 hr. Amino acid analysis was performed with a Beckman 120C analyzer.

Preparation and Assay of RNA Polymerase. RNA polymerase II was purified from Ehrlich ascites tumor cells and assayed as described elsewhere (6).

Assay of DNA Polymerase and Protein Kinase Activities. Chromatin DNA polymerase activity was assayed according to a previously published procedure (9). Protein kinase activity was assayed by the method of Kleinsmith and Allfrey (12), using whole calf thymus histone, casein, and the phenol-soluble NHP as substrates.

Determination of Alkali-labile Phosphorus. The protein sample to be analyzed was precipitated by 10% trichloroacetic acid. The precipitate was extracted 3 times with 5% trichloroacetic acid at 90°, each time for 20 min. The sample was then washed successively with $CHCl_3$:CH_3OH (1:1), $CHCl_3$:CH_3OH (2:1):0.2 N HCl, 0.2 N HCl, and absolute ether. After drying, the sample was dissolved in 1 N NaOH and heated in a boiling water bath for 20 min. The protein was reprecipitated by addition of 4 volumes of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and the process was repeated once again. The 2 alkaline extracts were combined for phosphorus determination (3).

Other Methods. Protein was determined by the procedure of Lowry *et al.*, (17), RNA was determined by orcinol reaction (19), and DNA was determined by the method of Burton (5).

RESULTS

Compositions of the Nonhistone Protein Fractions. Table 1A shows the relative distribution of histones and NHP in the 3 chromatin protein fractions. Extraction of chromatin with 0.35 M NaCl released 43% of the total NHP but very little (approximately 3.8%) basic proteins, presumably histones. Small amounts of basic proteins (5%) in low-salt extract of human diploid fibroblast chromatin have previously been reported by Baserga *et al.* (2). Further extraction of the Ehrlich ascites chromatin with 2 M NaCl:5 M urea completely released the histones, leaving a residue representing 9% of the total NHP. This value may be compared with the result on HeLa chromatin by Bhorjee and Pederson (4), who reported that 8% of the total NHP remain in close association with DNA when subjected to 2.5 M NaCl:5 M urea extraction.

The percentages of protein, DNA, RNA, and alkali-labile phosphorus of the NHP fractions are shown in Table 1, B and C. Most of the DNA was sedimented in the residual NHP fraction. This residual chromatin fraction also contained the highest amounts of RNA and phosphoprotein (Table 1B). Treatment of the 0.35 M NaCl NHP, the 2 M NaCl:urea NHP, and the residual NHP fractions with phenol rendered 5.9, 5.2, and 21%, of the proteins from these fractions phenol soluble, respectively, with corresponding enrichment of phosphoprotein contents 20-, 35-, and 1.6-fold (Table 1C). All the phenol-soluble proteins were free of nucleic acids.

The 3 NHP fractions also show distinctive differences in their gross amino acid compositions (Table 2). This is especially evident in the residual NHP which, when compared

Table 1

Analyses of differentially salt-extracted protein fractions prepared from Ehrlich ascites tumor chromatin

A. Distribution of histones and NHP

Fraction	% of total histone	% of total NHP
0.35 M NaCl NHP	3.8 ± 0.3	42.7 ± 1.5
2 M NaCl:urea NHP	96.2 ± 1.2	48.3 ± 1.4
Residual NHP	0	9.0 ± 0.3

B. Amounts of phenol-soluble proteins

Fraction	% of phenol-soluble NHP in each fraction
0.35 M NaCl NHP	5.9 ± 0.3
2 M NaCl:urea NHP	5.2 ± 0.1
Residual NHP	21.0 ± 0.9

C. Composition

Fraction	Wt. in each fraction (%)			
	Protein	DNA	RNA	Alkali-labile phosphorus
0.35 M NaCl NHP	97.2	0	2.8	0.03
Phenol-soluble 0.35 M NaCl NHP	100	0	0	0.90
2 M NaCl:urea NHP	98.1	1.7	0.2	0.04
Phenol-soluble 2 M NaCl:urea NHP	100	0	0	1.40
Residual NHP	28.0	57.8	14.2	3.70
Phenol-soluble residual NHP	100	0	0	5.80

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with the other 2 NHP fractions, show diminished lysine, aspartic acid, and glutamic acid and higher serine and glycine contents. Comparison of the 2 M NaCl:urea NHP with the 0.35 M NaCl NHP shows that the former contain a higher content of methionine, a lower proline content, and absence of cysteine. These data indicate that the loosely bound NHP are compositionally distinguishable from the more tightly bound NHP.

Partition of DNA Polymerase and RNA Polymerase Activity between the Loosely Bound and Tightly Bound NHP Fractions. Chromatin DNA polymerase activity has been commonly extracted from chromatin with 1 to 2 M NaCl (9, 18). Since the high-salt extract of chromatin contains both the 0.35 M NaCl NHP as well as most of the 2 M NaCl:urea NHP, it was of interest to determine whether DNA polymerase activity is localized in 1 of the NHP fractions. Also, since nRNA polymerase activity is tightly bound to DNA and released only after sonic extraction of isolated nuclei in high salt, this enzyme is probably present in the 2 M NaCl:urea NHP. Initial experiments showed that by preparing the NHP using 2 M NaCl:5 M urea extraction of chromatin and subsequently removing the salt and urea by dialysis, only insignificant DNA polymerase and RNA polymerase activities were recovered from the 2 M NaCl:urea NHP. Two M NaCl without urea was therefore used for the extraction of the tightly bound NHP for examining the distribution of DNA polymerase and RNA polymerase activities. The results are given in Table 3. It can be seen that approximately 96% of the chromatin DNA polymerase activity is concentrated in the 0.35 M NaCl NHP, while the high-salt-extracted NHP contain 93% of the RNA polymerase activity.

The DNA polymerase activity was ascertained by its absolute requirements for all 4 deoxyribonucleotides and a DNA template, preferentially a native DNA partially digested with pancreatic DNase. The RNA polymerase activity was verified by chromatography of the tightly bound NHP on DEAE-Sephadex A-25 (21). The chromatographed RNA polymerase activity peak, II, shown in Chart 1, was inhibited by α-amanitin. RNA polymerase I (or A) was present in trace activity, probably as a result of inactivation during the fractionation procedure. The results indicate that the 0.35 M NaCl NHP are distinguished from the tightly bound NHP by their compartmentalization of DNA polymerase and RNA polymerase activities.

Protein Kinase Activities in the Loosely Bound and Tightly Bound NHP. Multiple nuclear protein kinases have been shown to phosphorylate NHP, histones, and other substrates (10, 11). Since the enzymes appear to be localized in different intranuclear sites, protein kinase activity was examined in the differentially salt-extracted NHP fractions. As shown in Table 4, protein kinase activity is mainly present in the 0.35 M NaCl NHP. However, the kinase activity of the 0.35 M NaCl NHP catalyzes more active phosphorylation of casein and phenol-soluble 0.35 M NaCl NHP, but relatively less active phosphorylation with histones. In contrast, the high-salt-extracted NHP contain only significant histone kinase activity. Acidic protein kinase activity, therefore, appears to be mostly concentrated in the loosely bound NHP fraction, whereas histone kinase activity is distributed more in the loosely bound NHP and less in the tightly bound NHP.

Localization of Protein Factor(s) That Stimulates Tran-

Table 2

Amino acid compositions of the loosely bound and the tightly bound nonhistone protein fractions from Ehrlich ascites tumor chromatin

	Loosely bound NHP		Tightly bound NHP		Residual NHP	
Amino acid	Total	Phenol-soluble	Total	Phenol-soluble	Total	Phenol-soluble
Lysine	8.5	6.2	7.3	6.4	6.6	5.0
Histidine	2.4	2.4	2.2	2.8	2.4	2.1
Arginine	6.0	5.2	6.6	5.6	5.2	3.1
Aspartic acid	10.6	10.0	9.8	8.9	8.4	10.0
Threonine	5.3	5.8	5.4	5.9	5.9	6.7
Serine	6.2	6.5	5.9	4.5	12.0	17.5
Glutamic acid	15.3	14.0	16.4	11.3	9.7	11.8
Proline	4.2	4.5	1.8	3.8	2.3	1.6
Glycine	6.7	6.5	5.7	6.4	10.5	10.8
Alanine	7.6	7.5	8.3	7.7	9.3	9.5
Half cystine	0.6	0.8	0.0	0.0	0.0	0.0
Valine	6.6	6.7	7.0	7.9	6.3	5.4
Methionine	0.4	1.2	2.1	1.6	1.3	0.1
Isoleucine	4.9	5.1	5.9	6.4	5.0	3.7
Leucine	10.3	11.4	11.1	11.8	9.6	9.3
Tyrosine	1.4	2.4	1.3	3.9	2.2	1.8
Phenylalanine	3.6	3.9	3.2	5.1	3.5	1.6
Glu + Asp	25.9	24.0	26.2	20.0	18.1	21.9
Lys + His + Arg	17.0	13.8	16.1	14.8	14.2	10.2
$\frac{\text{Glu + Asp}}{\text{Lys + His + Arg}}$	1.5	1.7	1.6	1.4	1.3	2.1

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Table 3

Relative distribution of DNA polymerase and RNA polymerase activities in the loosely bound and tightly bound nonhistone proteins from Ehrlich ascites tumor chromatin

The 0.35 M NaCl NHP were prepared by extraction of chromatin with 0.35 M NaCl as described in "Materials and Methods" and dialyzed against TGMED[a] before use. To prepare the 2 M NaCl-soluble NHP, the extracted chromatin was suspended in 2.0 M NaCl:TGMED and stirred for 2 hr. The mixture was centrifuged at 105,000 × g for 1 hr. The supernatant fluid was collected, dialyzed against 4 volumes of TGMED, and centrifuged to remove some insoluble material. The supernatant was treated with BioRex 70 and dialyzed against TGMED. Assays for DNA polymerase and RNA polymerase activities were performed as described in "Materials and Methods." The percentage of distribution of these 2 enzymic activities is calculated on the basis of the sum total of the assayed enzymic activities of the 2 NHP fractions.

	DNA polymerase activity		RNA polymerase activity	
	nmoles [^{3}H]dTMP incorporation	%	pmoles [^{3}H]UMP incorporation	%
Experiment 1				
0.35 M NaCl NHP (3.36 mg)	1.58	94.7	0.88	6.8
2 M NaCl NHP (1.59 mg)	0.09	5.3	12.0	93.2
Experiment 2				
0.35 M NaCl NHP (3.13 mg)	1.69	96.8	0.80	5.5
2 M NaCl NHP (1.34 mg)	0.05	3.2	13.8	94.5

[a] TGMED, 50 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 25% glycerol.

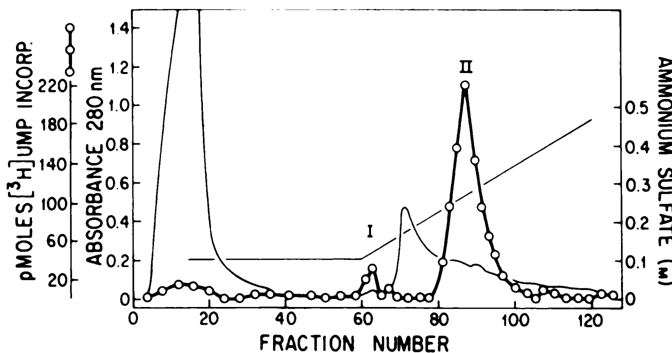

Chart 1. The tightly bound NHP prepared from 2 M NaCl chromatin extract was chromatographed on DEAE-Sephadex A-25 with 0.1 to 0.6 M ammonium sulfate according to the method of Roeder and Rutter (21) and assayed as described in "Materials and Methods." Incubation was at 30° for 10 min. RNA polymerase activity is expressed as [^{3}H]UMP incorporated per eluted fraction, collected in 2 ml each.

scription of DNA. We have previously reported (13) that the 0.35 M NaCl NHP contain an active fraction, obtained either by phenol extraction or by selective DNA-binding, which stimulates template- and enzyme-specific transcription of DNA. Such a stimulatory factor appears to be absent in the tightly bound NHP. As shown in Chart 2, the phenol-solubilized 2 M NaCl:urea NHP had no effect on the DNA-dependent RNA polymerase II reaction. Further, the phenol-soluble fraction of the residual NHP inhibited RNA synthesis. This is harmonious with our previous finding of a tightly bound NHP that inhibits transcription of DNA *in vitro* (14).

DISCUSSION

In the Ehrlich ascites tumor chromatin, some compositional features of the 0.35 M NaCl NHP and the 2 M NaCl:urea NHP appear to be similar. This is seen by their approximately equal amount of NHP yield (43 and 48%), phenol-soluble proteins (5.9 and 5.2%), and alkali-labile phosphorus content (0.03 and 0.04%). However, the gross amino acid compositions of these NHP fractions are different. More importantly, the differentially salt-extracted NHP fractions are functionally different in the 3 studies described in this report. The chromatin DNA polymerase activity and the acidic protein kinase activity are confined to the 0.35 M NaCl NHP, whereas RNA polymerase activity, as expected, is concentrated in the high-salt-extracted NHP. Moreover, the phosphoprotein fraction that stimulates DNA- and enzyme-specific RNA synthesis *in vitro* is shown to reside exclusively in the 0.35 M NaCl NHP. These results indicate that the NHP, bound in the chromatin with various affinities

Table 4

Distribution of protein kinase activity in the loosely bound and tightly bound nonhistone protein fractions

Each assay tube contained 25 moles of Tris-HCl, pH 7.5; 2.5 moles of $MgCl_2$; 100 pmoles of [γ-^{32}P]ATP (specific activity, 1.5 mCi/mmole); 150 μg of each substrate as noted; and, where indicated, either 4.3 mg of the 0.35 M NaCl NHP or 4.6 mg of the 2 M NaCl-soluble NHP. The reaction mixtures were incubated at 37° for 15 min. Controls using each of the 3 substrates without either of the nonhistone protein fractions incorporated $<0.1 \times 10^{-2}$ pmole of [γ-^{32}P]ATP.

	pmoles [γ-^{32}P]ATP incorporated (× 10^2)	
Substrate	0.35 M NaCl NHP	2 M NaCl-soluble NHP
Histones	16.7	8.5
Casein	98.5	0.9
Phenol-soluble 0.35 M NaCl NHP	81.3	2.9

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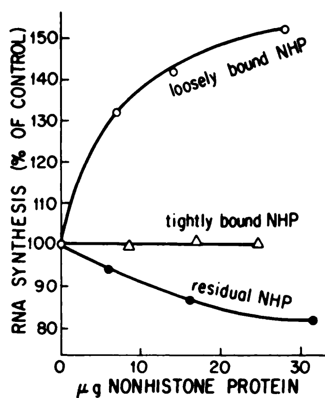

Chart 2. Effect of phenol-solubilized 0.35 M NaCl NHP (○), 2 M NaCl:urea NHP (△), and residual NHP (●) on transcription of DNA *in vitro*. The assay was conducted in Ehrlich ascites tumor RNA polymerase reaction, using 10 µg DNA template, as described in "Materials and Methods." *Abscissa*, amounts of the various phenol-soluble NHP fractions added. The control assay, without the addition of any of the NHP fractions, gave an incorporation of 2120 cpm from [^{3}H]UTP into acid-insoluble material and is taken as 100% template activity (*ordinate*).

and presumably related to the structure of chromatin, are specifically involved in replication and transcription of DNA and in the control of gene activity. The ease with which the 0.35 M NaCl NHP are prepared and the absence of DNA in this NHP preparation may help to simplify the procedures for isolation of chromatin DNA polymerase and acidic protein kinase activities.

One interesting result in the present study concerns the tightly bound NHP that are not extractable by 2 M NaCl:5 M urea. This residual NHP fraction contains a high phosphoprotein content (3.7%), which is in good agreement with its high phenol-soluble protein (21%) and high serine (12 mole % of the total amino acids analyzed) contents. Its chemical composition is quite different from that displayed by the 0.35 M NaCl NHP and the 2 M NaCl:urea NHP. When this residual NHP fraction was solubilized by phenol treatment and added to a DNA-dependent RNA polymerase II reaction, it inhibited RNA synthesis *in vitro*. This finding was not surprising, since Spelsberg *et al.* (24) have reported that calf thymus and rat liver chromatin, after extraction with 2 M NaCl:5 M urea at pH 6, transcribes only at 67 to 76% efficiency of that for pure DNA. In a previous work (6), we have also shown that the template activity of chromatin, which had been extracted with 2 M NaCl:5 M urea thus devoid of histones and most of the NHP (Table 1A), for RNA synthesis was not elevated to the level of that by naked DNA. The residual NHP solubilized by phenol treatment must therefore contain the factor(s) responsible for the restricted template activity of the extracted chromatin in transcription.

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