Isolation and Characterization of Protein A24, a “Histone-like” Non-Histone Chromosomal Protein*

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In earlier studies, the nucleolar levels of protein A24 were found to be markedly decreased in the nucleolar hypertrophy induced by thioacetamide or during liver regeneration (Ballal, N. R., Goldknopf, I. L., Goldberg, D. A., and Busch, H. (1974) Life Sci. 14, 1835-1845; Ballal, N. R., Kang, Y.-J., Olson, M. O.-J., and Busch, H. J. Biol. Chem. 250, 5921-5925). To determine the role of protein A24, methods were developed for its isolation in highly purified form. Milligram quantities of highly purified protein A24 were isolated from the 0.4 M H₄SO₄-soluble proteins of calf thymus chromatin by exclusion chromatography on Sephadex G-100, followed by preparative polyacrylamide gel electrophoresis. Protein A24 was highly purified as shown by its migration as a single spot on two-dimensional polyacrylamide gel electrophoresis, its single NH₂-terminal amino acid, methionine, and the production of approximately 50 peptides by tryptic digestion. Like histones 2A, 2B, 3, and 4, A24 was extractable from chromatin with 0.4 M H₄SO₄ or 3 M NaCl/7 M urea, but unlike most non-histone proteins or histone 1, protein A24 was not extracted with 0.35 M NaCl, 0.5 M HClO₄, or 0.6 M NaCl. Protein A24 was present in only 1.9% of the total amount of histones 2A, 2B, 3, and 4; its molecular weight is 27,000.

There is now considerable evidence that some of the non-histone chromosomal proteins are responsible for the specificity of gene transcription in higher organisms (1-5). Subfractions of these proteins have been purified by a variety of procedures (6-16), some of which have yielded subgroups enriched in proteins that stimulated chromatin transcription in vitro (6, 14) or exhibited specificity in binding to DNA (7, 19). In addition, several non-histone chromosomal proteins have been purified and characterized to varying extents (10, 17-21).

Protein A24, so designated because of its electrophoretic mobility on two-dimensional polyacrylamide gel electrophoresis, was detected in 0.4 M H₄SO₄-soluble proteins from nucleoli (22) and nuclei (23-26) but was not found in ribosomes or nucleolar ribonucleoprotein particles (27). The finding that rat liver nucleolar levels of protein A24 were markedly reduced during nucleolar hypertrophy induced by thioacetamide administration (25, 28) or liver regeneration (25, 29) suggested that this protein might play a role in gene control (30, 31). During liver regeneration, protein A24 was markedly decreased in nucleoli 10 hours prior to the onset of DNA synthesis (29). Since the nucleolus is the site for transcription of ribosomal cistrons in eukaryotes (30) and a protein has been implicated as a repressor of ribosomal gene activity (31), an attempt to purify protein A24 was initiated.

The present results show that A24 is a non-histone chromosomal protein with solubility properties similar to those of histones 2A, 2B, 3, and 4. In this respect, it differs from histone 1 and other non-histone chromosomal proteins. Characterization of protein A24 by amino acid composition, NH₂-terminal amino acid, molecular weight, and tryptic digestion shows that it differs from the histones and other purified non-histone chromosomal proteins (10, 17-21).

MATERIALS AND METHODS

Preparation of Rat Liver Nuclei and Chromatin—For studies on the distribution of nuclear proteins in chromatin fractions, rat liver nuclei were prepared (30, 32) and extracted at 4°. To prevent proteolysis, 1 mM phenylmethylsulfonyl fluoride was used throughout (33). Chromatin was prepared (34) by washing the nuclei twice by homogenization at 10 ml/g of nuclei with 0.075 M NaCl, 0.025 M EDTA, pH 8.0, and twice in 0.01 M Tris, pH 8.0. For each wash, centrifugation was at 18,000 x g for 10 min. For electrophoretic analysis of proteins solubilized in these washes, as well as in the subsequent chromatin washes (see "Results"), the supernatants were centrifuged at 100,000 x g for 2 hours to remove sheared deoxyribonucleoprotein, concentrated by ultrafiltration, and dialyzed against electrophoresis sample buffer (0.9 M acetic acid/10 M urea/1% β-mercaptoethanol).

The proteins soluble in 0.4 M H₄SO₄ were ethanol-precipitated and dried as previously described (17).
Fig. 1. Two-dimensional polyacrylamide gel electrophoretic analyses (22) of the distribution of rat liver nuclear proteins during chromatin preparation. Electrophoresis was from right to left in the first dimension and top to bottom in the second. The gels were stained in Coomassie brilliant blue. The histones (31) are spots GAR (histone 4), A1 (histone 2A), A2 (histone 2B), A3-5, 7 (histone 3) and A11, 17-19 (histone 1). a, 0.4 N H₄SO₄-soluble nuclear proteins; b, proteins solubilized in two washes of 0.075 M NaCl/0.025 M EDTA, pH 8.0/1 mM PhCH₂SO₄F; c, proteins solubilized in two washes of 0.01 M Tris, pH 8.0/1 mM PhCH₂SO₄F; d, 0.4 N H₄SO₄-soluble chromatin proteins.

Using the stained sections as a guide, protein A24 was cut out of the unstained slabs and subjected to electrophoresis at 120 volts for 24 hours into dialysis bags in 0.9 N acetic acid (17, 22).

Two-Dimensional Polyacrylamide Gel Electrophoresis—Electrophoresis was carried out as described previously (22) on 10% acrylamide/4.5 M urea/0.9 N acetic acid first dimension and 12% acrylamide/6 M urea/0.1% Na dodecyl-SO₄, 0.1 M sodium phosphate, pH 7.1, second dimension gels. For each gel, 0.5 A₄₆₀ unit of the dialyzed supernatant proteins or 500 µg of dried proteins dissolved at 10 mg/ml in the same buffer were applied per gel. Aliquots of purified protein A24 were dialyzed against electrophoresis sample buffer, and approximately 30 µg were used for analytical electrophoresis.

Amino Acid Analysis and NH₂-terminal Analysis—For amino acid and NH₂-terminal amino acid analysis, aliquots of 100 µg of purified A24 were dialyzed against 0.01 N HCl and lyophilized. Amino acid compositions were determined with the aid of a Beckman model 121 amino acid analyzer by the method of Spackman et al. (36) after hydrolysis of the protein with 5.7 N HCl at 110°C for 22 hours. Tryptophan was determined by hydrolysis with mercaptoethane sulfonic acid (37). The NH₂-terminal amino acid was determined by the dansyl technique (38) using polyamide thin layer sheets (7.5 x 7.5 cm) for chromatography and visualization by fluorescence (39), with the modification that dansyl amino acids were solubilized in acetone/acetic acid (3/2).

Determination of Molecular Weight—The molecular weight of A24 was determined essentially by the method of Shapiro et al. (40) using the mobility of proteins of known molecular weight in the second dimension (Na dodecyl-SO₄) of the two-dimensional gel system (22).

Mapping of Tryptic Peptides—Protein A24 (500 µg) was digested.
Fig. 2. Distribution of rat liver chromatin proteins during extraction with various solutions. Electrophoresis conditions were the same as in Fig. 1 (22). a, proteins solubilized from chromatin by 3 extractions of 0.35 M NaCl/0.01 M Tris, pH 8.0, 1 mM PhCH$_2$SO$_4$F; b, 0.4 N H$_2$SO$_4$-soluble proteins of the residual deoxyribonucleoprotein after 0.35 M NaCl extraction. Note the presence of histone 1 (A11, 17-19). (spots A11, 17-19) compared to b.

c, proteins dissociated from chromatin in 3 M NaCl/7 M urea/0.05 M sodium acetate, pH 6.0/1 mM PhCH$_2$SO$_4$F after the DNA was pelleted by centrifugation at 214,000 x g for 24 hours. d, 0.4 N H$_2$SO$_4$-soluble proteins of the residual DNP after three (10 ml/g of nuclei) extractions of chromatin with 0.6 M NaCl/0.01 M Tris, pH 8.0/1 mM PhCH$_2$SO$_4$F. Note the markedly reduced amounts of histone 1 (spots A11, 17-19) compared to b.

twice for 2 hours each with 5 µg of trypsin in 0.1 M N-ethylmorpholine acetate, pH 8.0, at 37°. The peptides were separated by chromatography in butanol/acetic acid/H$_2$O (4/1/5) followed by electrophoresis in acetic acid, pyridine, and H$_2$O, pH 3.6, as described previously (41).

RESULTS

Similarities in Solubility of Protein A24 and Histones 2A, 2B, 3, and 4—The position of protein A24 in the two-dimensional polyacrylamide gel electrophoresis map of the 0.4 N H$_2$SO$_4$-soluble rat liver nuclear proteins is shown in Fig. 1a. During the preparation of chromatin, protein A24 and the histones were not detected in either the saline/EDTA (0.075 M NaCl/0.025 M EDTA, pH 8.0) (Fig. 1b) or the Tris (Fig. 1c) washes but were found in the 0.4 N H$_2$SO$_4$-soluble proteins of chromatin (Fig. 1d). On the other hand, most of the other non-histone proteins were present in reduced amounts in the 0.4 N H$_2$SO$_4$ extract of chromatin (compare Fig. 1, a and d). Protein A24 and the histones were not solubilized when the chromatin was treated with 0.35 M NaCl (Fig. 2a), which extracts many non-histone chromosomal proteins (12, 13).

Similar results were obtained when chromatin was prepared from the nucleolar and extranucleolar fractions.

When the histones and most of the non-histone chromosomal proteins were extracted from chromatin with 3 M NaCl/7 M urea, protein A24 was also extracted (Fig. 2e). Protein A24 and the histones reassociated with the DNA upon one-step or gradient dialysis to low ionic strength. Thus, protein A24 had tight chromatin binding characteristics similar to those of histones 2A, 2B, 3, and 4, but it was present in much lower amounts than the histones.

Isolation of Electrophoretically Homogeneous Protein A24 from Calf Thymus—Studies on the isolation of histones (42) demonstrated that calf thymus tissue was a good source of large amounts of nuclear protein. As a result of the solu-
b. Sephadex G-100 column chromatography of the protein A24-enriched acid-soluble proteins from calf thymus tissues (prepared as described under “Materials and Methods”). The fractions containing protein A24 and histones 2B and 3 (indicated by the shaded area of the graph) were pooled, treated as described under “Materials and Methods,” and used for preparative electrophoresis (Fig. 4).

c. Two-dimensional gel electrophoresis of purified calf thymus protein A24 prepared in Figs. 3b and 4. d. Estimation of the molecular weight of protein A24. The molecular weight was determined as described under “Materials and Methods,” using Na dodecyl-SO₄ gels and known molecular weight markers (37).

Protein A24 was isolated from chromatin after pretreatment with 0.35 M NaCl and 0.5 M HClO₄ to selectively extract most non-histone proteins and histone 1; protein A24 and histones 2A, 2B, 3, and 4 were then extracted from the chromatin residue in 0.4 N H₂SO₄. Protein A24 was further purified on Sephadex G-100 and by preparative gel electrophoresis. Its purity was established by one- and two-dimensional polyacrylamide gel electrophoresis, NH₂-terminal and amino acid analysis and tryptic digestion. The results obtained show that protein A24 has the unique feature that as a non-histone protein its solubility and its tight binding to chromatin are like those of histones 2A, 2B, 3, and 4. However, unlike these histones which are present in 1:1:1:1 molar ratios (44), protein A24 is present in much lower amounts.

Analysis of A24—Based on relative electrophoretic mobility in the Na dodecyl-SO₄, second dimension of the two-dimensional gels to that of standard proteins (Fig. 3d), the molecular weight of protein A24 is approximately 27,000. Amino acid analyses (Table I) showed that A24 has an acidic/basic amino acid ratio of 0.93; the most plentiful amino acids of A24 are glutamic acid, lysine, and leucine; its content of tyrosine, phenylalanine, and methionine is low (Table I). Protein A24 does not contain tryptophan. The molecular weight figure was supported by minimum molecular weight calculations (43) based on the finding that lysine and arginine constituted 11.3 and 7.4 mol %, respectively, of the amino acids in protein A24. Thus, there should be 47 residues of these amino acids in one Mₐ = 27,000 molecule of protein A24. Tryptic digestion of protein A24 and two-dimensional separation of the digest (41) resolved approximately 50 peptides (Fig. 5) in agreement with these calculations. Determination of the NH₂-terminal amino acid by the dansyl reaction (38, 39) showed that it is methionine (Table I). No other Nα-dansyl amino acids were detected using 100 μg of protein.

DISCUSSION

Protein A24 was isolated from chromatin after pretreatment with 0.35 M NaCl and 0.5 M HClO₄ to selectively extract most non-histone proteins and histone 1; protein A24 and histones 2A, 2B, 3, and 4 were then extracted from the chromatin residue in 0.4 N H₂SO₄. Protein A24 was further purified on Sephadex G-100 and by preparative gel electrophoresis. Its purity was established by one- and two-dimensional polyacrylamide gel electrophoresis, NH₂-terminal and amino acid analysis and tryptic digestion. The results obtained show that protein A24 has the unique feature that as a non-histone protein its solubility and its tight binding to chromatin are like those of histones 2A, 2B, 3, and 4. However, unlike these histones which are present in 1:1:1:1 molar ratios (44), protein A24 is present in much lower amounts.
FIG. 4. Purification of protein A24 by preparative electrophoresis. a, an Amido black-stained vertical side strip cut from a preparative 10% polyacrylamide slab gel after electrophoresis of the pooled fractions obtained from column chromatography on Sephadex G-100 (Fig. 3a). The position of protein A24 and histones 2B (A2) and 3 (A3-5, 7) are indicated. Such vertical strips from both sides and the center were used as a guide to cut out horizontal sections of the unstained remainder of the slabs which contained the protein A24 band. The protein A24 was then obtained by electrophoresis out of the gel sections into dialysis tubing. b, re-electrophoresis of purified protein A24 obtained in (a) on 10% polyacrylamide gels.

TABLE I

| Amino acid composition and NH₂-terminal amino acid of A24 |
|----------------------------------------------------------|
| Amino acid     | Mole percent |
|----------------|--------------|
| Ala            | 9.6          |
| Arg            | 7.4          |
| Asx            | 7.3          |
| Gly            | 9.2          |
| Glx            | 12.3         |
| His            | 2.4          |
| Ile            | 5.8          |
| Lys            | 11.3         |
| Met            | 0.3          |
| Phe            | 0.9          |
| Pro            | 5.6          |
| Ser            | 4.5          |
| Thr            | 6.5          |
| Trp            | 0.0          |
| Tyr            | 1.3          |
| Val            | 4.9          |
| Lys + His + Arg| 21.1         |
| Glx + Asx      | 19.6         |
| Glx + Asx/Lys + His + Arg | 0.93 |
| NH₂-terminal   | Methionine   |

The combination of metabolic, solubility, and analytical features of protein A24 are unlike those of any histones or other non-histone proteins. The amino acid composition of protein A24 differs from those reported previously for the histones (45) as well as those of other purified non-histone proteins (17, 18, 20, 21). In addition, although the 0.35 M NaCl-soluble chromatin proteins and the non-histone chromosomal proteins extractable with 3 M NaCl/7 M urea are strikingly similar (13, 14) and promoted specific DNA transcription (14), A24 was not among the 0.35 M NaCl extracts but was more tightly bound to chromatin. Non-histone proteins tightly bound to chromatin have been described (16, 33) but they were not acid-soluble.

A number of analytical features differentiate protein A24 from other non-histone proteins. The acidic/basic amino acid molar ratio of protein A24 is 0.93. For several other proteins the values are: nucleolar band 15, 1.75 (17); lac repressor, 1.72 (46); pancreatic ribonuclease, 1.5 (47); lysozyme, 1.35 (47); cytochrome c, 0.74 (47); and histones, 0.17 to 0.58 (45). Protein A24 differs in glutamic acid and lysine content from non-histone chromosomal proteins which contained 21.8% lysine, 18.3% glutamic acid (20); 23.9% lysine, 17.8% glutamic acid (18); and 6% lysine, 16% glutamic acid (21). The NH₂-terminal amino acid of protein A24 differs from those of the histones (45).

Garrard and Bonner (48) reported a "minor protein band" designated X which was found among the rat liver histones obtained from Bio-Rex 70 columns. They also mentioned that it was present in all their histone preparations and was observed by Panyim and Chalkley (49) as well. They reported a high turnover rate for this band during rat liver regeneration. Band X had an electrophoretic mobility similar to protein
A24\(^8\), and in a preliminary report (26) protein A24 was shown to bind and elute from Bio-Rex 70 along with the histones.

Since protein A24 has properties of both histones and nonhistone proteins, it may have unique biological properties. Its localization in nuclear and nucleolar chromatin, and the marked decrease in nucleolar levels of protein A24 during nucleolar hypertrophy (25, 28) suggests it may have a possible role as a rDNA repressor (31). Crippa (31) has reported a protein that inhibits transcription of ribosomal cistrons in amphibian oocytes. The decreased nucleolar content of protein A24 during thioacetamide administration (25, 28) and in liver regeneration (25, 29) suggests a possible relationship of protein A24 to the amphibian protein since decreases in Mr. Mark Mamrack and Dr. Young C. Choi for many helpful discussions.

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