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A RECIPROCAL RELATIONSHIP BETWEEN CONTENTS OF FREE UBIQUITIN AND PROTEIN A24, ITS CONJUGATE WITH HISTONE 2A, IN CHROMATIN FRACTIONS OBTAINED BY THE DNase II, Mg^{++} PROCEDURE

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SUMMARY

Ubiquitin was first found in nuclei in protein A24 where its carboxyl terminal is covalently bound to histone 2A by an isopeptide linkage (Goldknopf, I. L. and Busch, H. (1977) Proc. Natl. Acad. Sci. USA 74, 864-868). Two-dimensional polyacrylamide gel electrophoresis of the 0.4 N H_2SO_4 soluble proteins from fractionated rat liver chromatin showed that protein A24 and histones H1, H2A, H2B, H3 and H4 were present in fractions P1 and P2 and markedly diminished in relative amounts in fraction S2. Conversely, a spot designated Ub was found in fraction S2 along with an increased amount and number of non-histone proteins. The Ub spot was not found in chromatin fractions P1 and P2. Ub was identified as ubiquitin by migration on two-dimensional gels and after purification by preparative polyacrylamide gel electrophoresis by its methionine NH_2-terminal amino acid and its amino acid composition.

INTRODUCTION

Structural studies of protein A24 revealed that it has a Y-branched structure (1) composed of histone 2A (2) and ubiquitin (3-5), the carboxyl terminal amino acid of which is attached through a Gly-Gly peptide to the ϵ-NH_2 of lysine 119 of histone 2A (6). Protein A24, bound to chromatin along with nucleosomal core histones H2A, H2B, H3 and H4 (7), was found in nucleosome monomers (8). It was found in approximately one-tenth of the amount of histone 2A, suggesting that it may be associated with specialized chromatin subunits (8).

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A marked decrease in nucleolar content of protein A24 during stimulation of nucleolar gene activity (9-11) indicated a possible gene inhibitory role for the conjugated protein (7) while free ubiquitin induced differentiation antigens in thymocytes and bursocytes (12), suggesting it has a role in gene activation. Accordingly, this study of the distribution of protein A24 and ubiquitin in fractionated chromatin was undertaken.

MATERIALS AND METHODS

Preparation and fractionation of chromatin - To avoid proteolysis, 1 mM phenylmethylsulfonyl fluoride was used throughout. Rat liver nuclei were isolated at 4°C by centrifugation in 2.2 M sucrose, 3 mM calcium acetate as described previously (13). To prepare chromatin, the nuclei were extracted twice with 75 mM NaCl/25 mM EDTA/pH 8.0 and twice with 10 mM Tris, pH 8.0, as described previously (7). Chromatin was fractionated into fractions S2, P1 and P2 by the method of Gottesfeld et al (14). After dialysis overnight against 200 volumes of 25 mM sodium acetate buffer, pH 6.6, the chromatin was adjusted to an A_{260} of 10, to 24°C, and digested with 100 U/ml of DNase II (Worthington HDAC) for 5 min followed by adjustment to pH 7.5 with 50 mM Tris, pH 11, and to 4°C, to stop the reaction. The chromatin fraction P1 was obtained as a pellet by centrifugation at 28,000 g for 20 min. The supernatant material was adjusted slowly to 2 mM $MgCl_2$ with 0.2 M $MgCl_2$ and stirred for 30 min followed by centrifugation to obtain pellet fraction P2 and supernatant fraction S2.

Extraction, fractionation, purification and chemical analysis of proteins - The acetone precipitated 0.4 N H_2SO_4 soluble proteins were obtained from pellets P1 and P2 as described previously (7). Supernatant fraction S2 was extracted twice with 0.4 N H_2SO_4 and the proteins were precipitated with acetone, washed and dried as previously described (8). Samples of purified ubiquitin were generously donated by Drs. Gideon Goldstein of Ortho, Allan Goldstein of George Washington University and Ed Grinnan of Eli Lilly and Co. Samples of 500 μg each of the proteins dissolved at 10 mg/ml in 0.9 N acetic acid/10 M urea/1% β-mercaptoethanol were subjected to two-dimensional polyacrylamide gel electrophoresis with a 10% acrylamide/0.9 N acetic acid/4 M urea first dimension followed by a 12% acrylamide/6 M urea/0.1% sodium dodecyl sulfate/0.1 M sodium phosphate (pH 7.1) second dimension according to the method of Orrick et al (15). The gels were stained with Coomassie Brilliant Blue R. Purified ubiquitin was prepared from the S2 fraction by preparative slab gel electrophoresis (7). Samples of 10 mg of 0.4 N H_2SO_4 soluble proteins from the S2 fraction were dissolved at 10 mg/ml in 0.9 N acetic acid/10 M urea/1% β-mercaptoethanol, diluted with 0.9 N acetic acid to 8 M urea, applied to a 14 x 28 cm slab gel (1st dimension conditions), and

electrophoresed at 120 volts, 4°C, until a cytochrome C marker in a companion gel migrated 16 cm. Side strips and a center strip were then stained to locate the proteins. The corresponding portions of the unstained gels were cut out and protein eluted by electrophoresis into dialysis tubing (Spectrapore 3) as previously described (7).

Amino acid analyses were performed after hydrolysis in 5.7 N HCl as previously described (7). Amino terminal amino acid analysis was performed by a modification of the dansyl method as previously described (7).

RESULTS

Two-dimensional polyacrylamide gel electrophoretograms of 0.4 N H_2SO_4 soluble proteins obtained from the chromatin fractions produced by this procedure are shown in Figure 1. The electrophoretic patterns obtained with proteins from chromatin fractions P1 and P2 contained almost identical amounts and relative proportions of proteins A24, B7 and histones H1, H2A, H2B, H3 and H4. These patterns were very similar to those obtained previously with 0.4 N H_2SO_4 soluble proteins from unfractionated chromatin (7). However, variations were noted in amounts of minor protein species including B13, B18, BU and C2.

The two-dimensional gel pattern obtained with proteins from the chromatin fraction S2 was considerably different from those of the pellet fractions. There were marked reductions in the relative content of protein A24 and histones H1, H2A, H2B, H3 and H4. There were increases in content of many nonhistone proteins including B18, BU, B33, C2, C14 and C18 and a larger number of nonhistone proteins were present than found in fractions P1 and P2. In addition, the low molecular weight protein spot designated Ub was found in this fraction; it migrated in the position of free ubiquitin. The corresponding Ub spot was not detected among the proteins of fractions P1 and P2.

To confirm the identity of spot Ub, a sufficient quantity of this protein was prepared (Fig. 2) from samples of 0.4 N

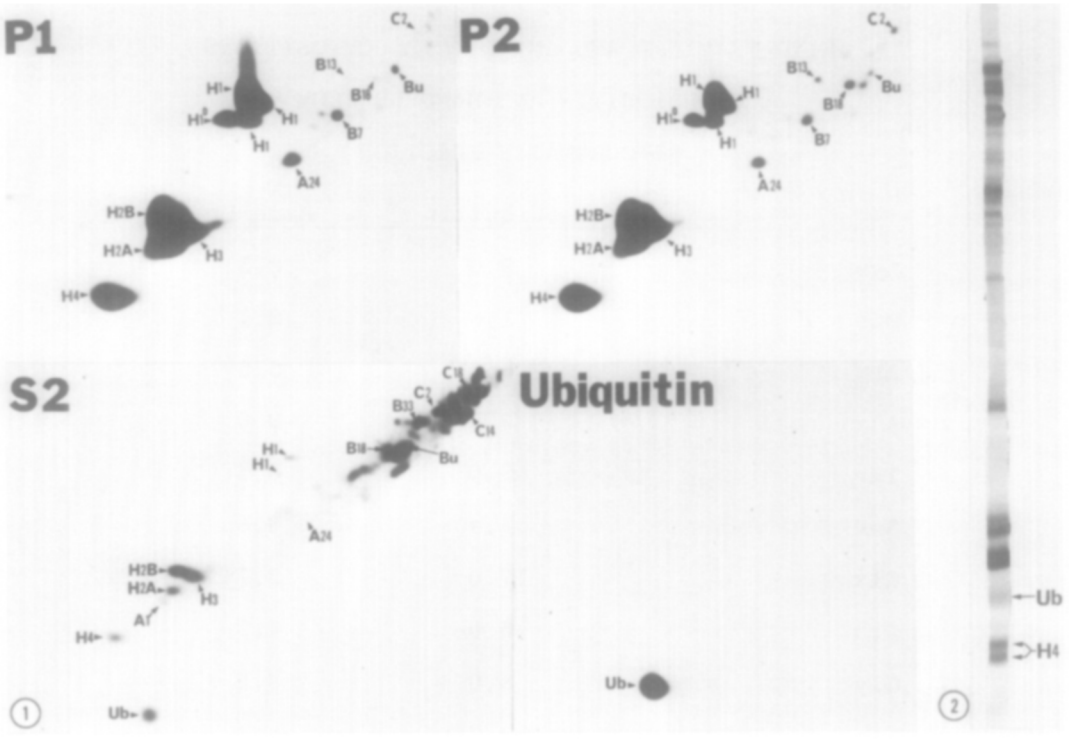

Figure 1 Two-dimensional polyacrylamide gel electrophoretic fractionation of 500 μg each of 0.4 N H_2SO_4 soluble proteins and 25 μg of ubiquitin. The gels were obtained using proteins from pellet (P1 and P2) and supernatant (S2) chromatin fractions as well as known samples of purified ubiquitin (lower right). Electrophoresis as in Methods was from right to left in the first dimension and top to bottom in the second.

Figure 2 Preparative slab gel electrophoresis with the first dimension acid urea system (10% acrylamide/0.9 N acetic acid/4 M urea) of 0.4 N H_2SO_4 soluble proteins from supernatant chromatin fraction S2. The conditions are given in Materials and Methods. The positions of ubiquitin and histone 4 in the amido black stained vertical center strip, as indicated by arrows, were used to locate the proteins in the unstained slab for preparative elution by electrophoresis as in Materials and Methods.

H_2SO_4 soluble proteins of fraction S2 by preparative slab gel electrophoresis (7). The amino acid composition of Ub purified from the gel was very similar to that of purified ubiquitin and the amino terminal amino acid of both was methionine (Table I).

Table I

AMINO TERMINAL AND AMINO ACID COMPOSITIONS OF UBIQUITIN AND BAND UB FROM S1 CHROMATIN FRACTION

	Ubiquitin	Band Ub
Lys	9.0	8.5
His	1.6	2.1
Arg	5.3	5.5
Asx	9.5	9.0
Thr	8.0	8.5
Ser	4.4	6.6
Glx	17.0	13.7
Pro	5.0	4.9
Gly	8.0	8.3
Ala	3.7	5.1
Val	5.4	5.6
Met	1.2	1.2
Ile	8.4	6.2
Leu	11.1	9.2
Tyr	1.4	1.6
Phe	2.4	2.8
NH_2-Terminal	Met	Met

DISCUSSION

The organization of the genome of higher organisms into subunits or nucleosomes is now well established (16-25). Although subunits are present in both active and inactive chromatin (26,27), the DNA of transcribed sequences has an altered conformation (28) which renders it more susceptible to DNase I, DNase II and brief micrococcal nuclease digestion (29-41).

TABLE II

CHROMATIN FRACTIONATION

Reference (No)	Tissue Studied	Hybridization Probe Used	Enrichment of Actively Transcribed Genes in the S2 Fraction
Gottesfeld et al, 1974 (14)	Rat Liver	Total cell RNA	4-fold
Gottesfeld and Partington, 1977 (32)	Friend Cell	Globin cDNA	2.4-4.4-fold
Gottesfeld and Partington, 1977 (32)	Friend Cell	cDNA to Poly A+ mRNA	6-fold
Bonner et al, 1978 (40) Wallace et al, 1977 (33)	Friend Cell	Globin cDNA	7-fold
Matsui and Busch, 1977 (41)	Novikoff Hepatoma Nucleoli	18 and 28S rRNA	8.5-fold

The DNase II, Mg^{++} precipitation procedure used here (14) produces a soluble fraction S2 that is relatively enriched in DNA sequences from active genes (Table II). It also differs from inactive chromatin in the higher sedimentation coefficients of its subunits (38). Preliminary electron microscopic data indicated that the S2 fraction contains spherical particles larger than nucleosomes. On the other hand, the two pellet fractions (P1 and P2) also produced by this procedure are not enriched in transcribed DNA sequences (14,32,33).

The reciprocal distribution of ubiquitin and protein A24 among these fractions suggests that conjugation of ubiquitin with histone 2A may alter the structure of chromatin to decrease its template activity. Apparently, cleavage of protein

A24 may increase availability of the chromatin for transcription either indirectly or by increasing the concentration of ubiquitin.

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*Recently, Walker et al (42) have confirmed the presence of ubiquitin in chromatin by locating it in free form among the high mobility group (HMG) nonhistone chromosomal proteins of calf thymus.