Protein A24 Lyase Activity in Nucleoli of Thioacetamide-Treated Rat Liver Releases Histone 2A and Ubiquitin from Conjugated Protein A24†

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ABSTRACT: Isolated liver nucleoli from rats treated for 3 days with thioacetamide contained an enzyme activity which specifically degraded conjugate protein A24. Two-dimensional polyacrylamide gel electrophoresis indicated that the amount of protein A24 in chromatin decreased during incubation at 37 °C for 60 min with these nucleoli. Concomitantly, a marked increase was found in the content of free ubiquitin, the nonhistone component of protein A24. Incubation of ^{3}H-labeled protein A24 with the thioacetamide-treated liver nucleoli resulted in the linear release of ^{3}H-labeled histone 2A and ^{3}H-free ubiquitin in the presence of phenylmethanesulfonyl fluoride (PMSF) for 2 h. Pretreatment of the nucleoli with trypsin or by heating at 80 °C for 10 min inhibited their ability to cleave protein A24. Protein A24 lyase catalyzes the reaction: protein A24 → histone 2A + ubiquitin.

Chromosomal protein A24 was first described by its electrophoretic mobility on two-dimensional polyacrylamide gels (Orrick et al., 1973). This protein was shown to be markedly decreased in amount during nucleolar hyperfunction induced either by partial hepatectomy or thioacetamide treatment (Ballal & Busch, 1973; Ballal et al., 1974, 1975a,b). Protein A24 was identified as a conjugated chromatin protein (Goldknopf et al., 1975, 1976) composed of histone 2A (Goldknopf & Busch, 1975) and a ubiquitin moiety (Olson et al., 1976; Hunt & Dayhoff, 1977; Schlesinger et al., 1975; Goldstein et al., 1975) which was linked to histone 2A by an isopeptide bond (Goldknopf & Busch, 1977). Also, protein A24 was found to be an integral component of the nucleosome core particle (Goldknopf et al., 1977; Martinson et al., 1979; Albright et al., 1979). By use of the DNase[1] II digestion technique (Gottesfeld et al., 1974), protein A24 was shown to be depleted in the actively transcribing fraction of chromatin while ubiquitin was present, suggesting a reciprocal relationship between protein A24 and its cleavage product, ubiquitin, during chromatin activation (Goldknopf et al., 1978). Ubiquitin has been identified as a high mobility group (HMG) protein (Walker et al., 1978; Watson et al., 1978), and the HMG proteins are apparently present in active chromatin (Vidali et al., 1977; Levy et al., 1979; Weisbrod & Weintraub, 1979). This report demonstrates the presence of a specific enzyme in the hyperinduced nucleoli of livers of thioacetamide-treated rats that released histone 2A and the ubiquitin moiety from protein A24. This cleavage activity was not inhibited by the serine protease inhibitor, phenylmethanesulfonyl fluoride (PMSF), and under the conditions of incubation, the core histones were not degraded.

Materials and Methods

Rat liver nuclei and nucleoli were isolated by the sucrose–magnesium procedure as described previously (Andersen et al., 1977; Busch & Smetana, 1970). Normal liver chromatin containing protein A24 was obtained (Goldknopf et al., 1975) by two sequential extractions of nuclei with 0.075 M NaCl/0.025 M Na_2EDTA (pH 8.0)/1 mM dithiothreitol (DTT) followed by three extractions with 0.01 M Tris (pH 7.9)/1 mM DTT. The pellet was used as the substrate for the protein A24 cleaving activity.

Rat liver nuclear chromatin (1 mg of protein) was incubated with liver nucleoli (500 μg of protein) from 3-day thioacetamide-treated rats (50 mg/kg, ip, daily) in 0.01 M Tris (pH 7.9) and 1 mM DTT in a final volume of 1 mL at 37 °C for 60 min unless otherwise indicated. For determination of the presence of protein A24, the incubation mixture was extracted with an equal volume of 0.8 N H_2SO_4 at 0–4 °C. The acid extract was precipitated with 5 volumes of ethanol at −20 °C. The precipitated proteins were analyzed by two-dimensional gel electrophoresis according to Orrick et al. (1973). In the adaptation solutions for the second dimension, 2-mercaptoethanol (1% v/v) was included. The incubation mixture was adjusted to 0.35 M NaCl by adding 1.4 mL of 0.6 M NaCl/0.01 M Tris (pH 7.9)/1 mM DTT/1 mM PMSF at 0 °C to extract the HMG proteins. The mixture was homogenized and centrifuged at 10000*g*, and 0.2 mL of a 25% Cl_3CCOOH solution (w/v) was added to the supernatant so that the final Cl_3CCOOH concentration was 2% (Goodwin et al., 1973). The Cl_3CCOOH-soluble proteins were dialyzed against 44 mM acetic acid, lyophilized, and analyzed by two-dimensional gel electrophoresis in order to detect the presence of ubiquitin.

Protein A24 was purified according to the procedure of Goldknopf et al. (1975), labeled with ^{3}H by reductive methylation using potassium [^{3}H]borohydride (Means & Feeney, 1971), and used as substrate. ^{3}H-Labeled protein A24 (1 μg of protein) was incubated with various amounts of liver nucleoli from 3-day thioacetamide-treated rats (0–100 μg of protein) in a final volume of 30 μL containing 0.01 M Tris (pH 7.9)/1 mM PMSF/1 mM DTT (TPD) for 60 min at 37 °C. Then 30 μL of 0.1 M sodium phosphate (pH 7.1)/6 M urea/1% $NaDodSO_4$ containing 5% 2-mercaptoethanol and 10% glycerol was added to the reaction mixture, and the mixture was heated at 100 °C for 2 min and applied to one-dimensional

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[1] Abbreviations used: DNase, deoxyribonuclease; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TPD, 0.01 M Tris (pH 7.9)/1 mM PMSF/1 mM DTT; EDTA, ethylenediaminetetraacetic acid; ip, intraperitoneally; ATP, adenosine 5′-triphosphate.

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12% polyacrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate (pH 7.1), and 6 M urea gel electrophoresis (Orrick et al., 1973). The gels were fixed in 30% methanol, 10% Cl_3CCOOH, and 10% glacial acetic acid, treated with EN^{3}HANCE (New England Nuclear), and dried, and fluorography was performed to detect ^{3}H-labeled protein A24 and its cleavage products. The bands were cut out, treated with 1 mL of 30% hydrogen peroxide at 50 °C overnight, and subjected to liquid scintillation counting. For kinetic studies, the reaction mixture was mixed with 0.5 mL of 0.1 M NaCl in 0.01 M Tris (pH 7.9)/1 mM PMSF/1 mM DTT and applied to a phosphocellulose column (Whatman P11, 0.75-mL bed volume) equilibrated with the same buffer. The adsorbed products contained unreacted protein A24 and histone 2A. Liquid scintillation counting was used to quantitate the unadsorbed [^{3}H]ubiquitin.

Results

Nucleoli from livers of thioacetamide-treated rats are depleted of protein A24 (Ballal & Busch, 1973; Ballal et al., 1974, 1975a). In the incubations with normal liver chromatin, the protein A24 (Figure 1A) was derived from the chromatin substrate and not from the added nucleoli. The acid-soluble proteins from an unincubated mixture of rat liver chromatin and thioacetamide-treated rat liver nucleoli contained protein A24 seen as a dense spot by two-dimensional polyacrylamide gel electrophoresis (Figure 1A). When this mixture was incubated at 37 °C for 60 min, protein A24 (Figure 1B) was reduced to a minor spot relative to protein A24 in Figure 1A. Similarly, when chromatin was incubated alone, protein A24 did not decrease in amount (Figure 1C) indicating that the decrease in protein A24 content resulted from an activity present in the thioacetamide-treated liver nucleoli.

Earlier, protein A24 had been shown to contain ubiquitin, the carboxyl terminus of which is covalently linked to glycylglycine in an isopeptide linkage to the ϵ-NH_2 group of lysine residue 119 of histone 2A (Goldknopf & Busch, 1975, 1977), it was of interest to determine whether free ubiquitin was produced during the decrease in protein A24 content. In the nonincubated control, the HMG fraction was extracted and fractionated by two-dimensional gel electrophoresis. It contained trace amounts of ubiquitin (Figure 2A, spot Ub) as shown by Walker et al. (1978). In the incubated sample, the concentration of ubiquitin (Figure 2B,C) increased markedly relative to the nonincubated control. Concomitant with the decrease in protein A24, there was an increase in ubiquitin when thioacetamide-treated rat liver nucleoli were incubated with chromatin. Accordingly, release of free ubiquitin from protein A24 was produced by this activity.

^{3}H-Labeled protein A24 (131 000 cpm/μg of protein) was used as substrate to determine the specificity of this activity. When ^{3}H-labeled protein A24 was incubated in the absence of nucleoli (Figure 3, lane b), no significant degradation of protein A24 occurred. With increasing amounts of nucleoli included in the incubation mixture, the amount of protein A24 decreased with a concomitant release of proteins comigrating with histone 2A and ubiquitin (Figure 3, lanes c–g). Quantitative data obtained by cutting out and counting the bands showed that the increase in histone 2A and ubiquitin accompanied the decrease in protein A24 (Figure 4). A maximum of 80% hydrolysis of ^{3}H-labeled protein A24 (1 μg of protein) was obtained at 1 h in the presence of 50 μg of nucleolar protein.

The kinetics of the reaction were studied to determine whether isolated nucleoli can hydrolyze protein A24 over an extended period of time. A linear release of ubiquitin for 2

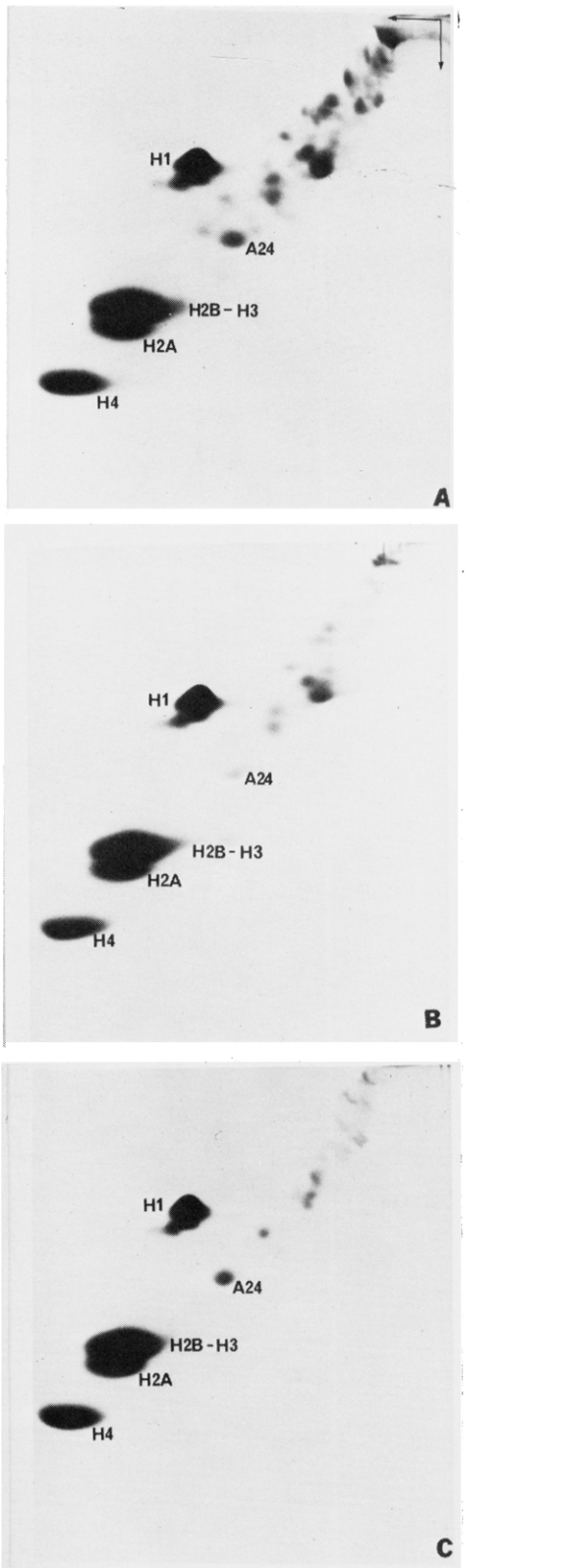

FIGURE 1: Two-dimensional polyacrylamide gel electrophoresis of 0.4 N H_2SO_4 soluble proteins (150 μg). The first dimension is 10% polyacrylamide, 4.5 M urea, and 0.9 N acetic acid (right to left). The second dimension is a sodium dodecyl sulfate system (top to bottom) in 12% polyacrylamide gels as described under Materials and Methods. (A) Normal liver chromatin mixed with thioacetamide-treated rat liver nucleoli at 0 °C. (B) Normal liver chromatin mixed with thioacetamide-treated rat liver nucleoli and incubated at 37 °C for 60 min. (C) Normal liver chromatin incubated at 37 °C for 60 min in the absence of thioacetamide-treated liver nucleoli.

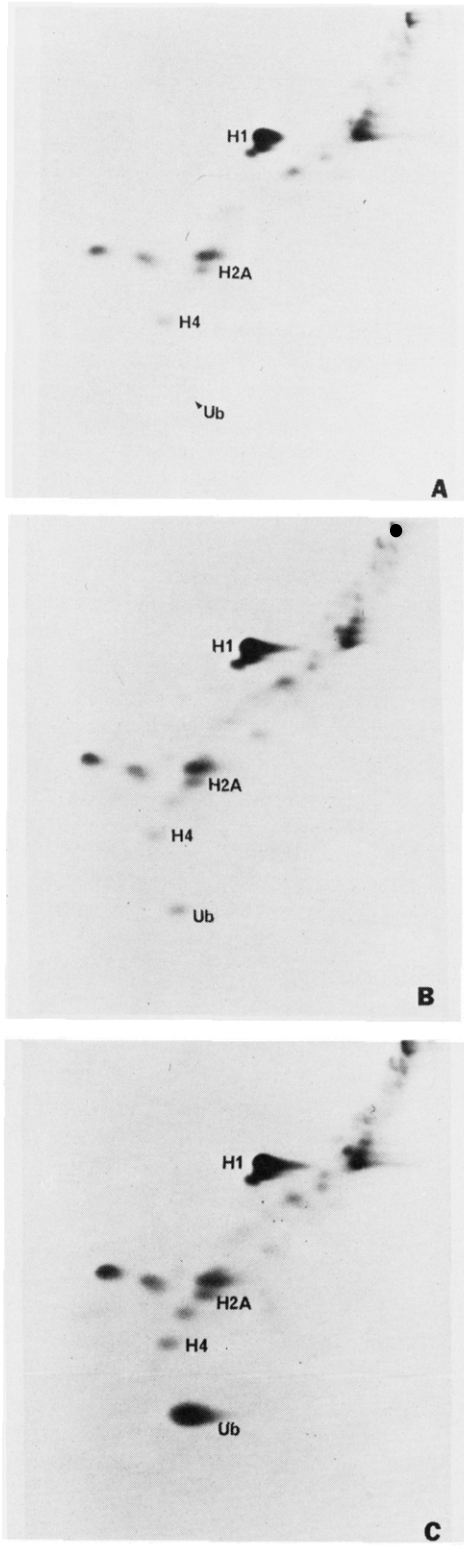

FIGURE 2: Two-dimensional polyacrylamide gel electrophoresis as described in Figure 1. Incubated samples were extracted with 1.4 volumes of 0.6 M NaCl, 0.01 M Tris (pH 7.9), 1 mM DTT, and 1 mM PMSF, and the HMG fraction from this extract was isolated as described under Materials and Methods. (A) The HMG fraction of normal liver chromatin mixed with thioacetamide-treated nucleoli at 0 °C and immediately extracted. (B) The HMG fraction from the identical sample incubated at 37 °C for 60 min in the presence of 1 mM PMSF prior to extraction. (C) The same fraction as in Figure 2B plus 10 μg of purified ubiquitin.

h was obtained by using 7 μg of nucleolar protein/30-μL assay, as determined by isotope not absorbed by phosphocellulose (Figure 5). The linear kinetics imply that a stable catalytic activity cleaved protein A24.

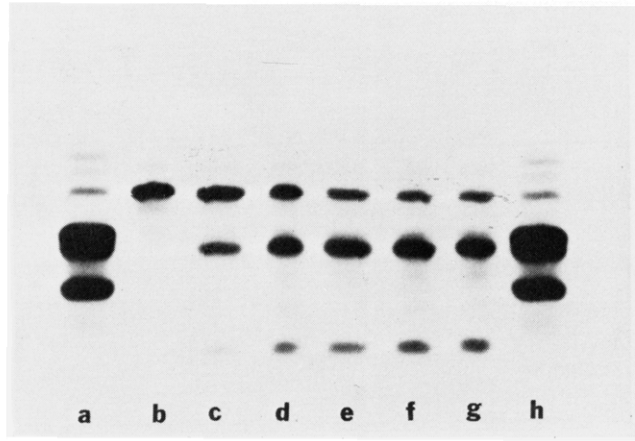

FIGURE 3: One-dimensional $NaDodSO_4$–12% polyacrylamide gel electrophoresis of ^3H-labeled protein A24 (131 000 cpm/μg of protein) and its hydrolysis products. Electrophoresis is identical with the second dimension of the two-dimensional polyacrylamide gel electrophoresis described by Orrick et al. (1973). After electrophoresis, samples were fixed in 30% methanol, 10% Cl_3CCOOH, and 10% glacial acetic acid for 1 h, treated with EN^{3}HANCE (New England Nuclear) for 1 h, washed with deionized water while shaking for 1 h, and dried under vacuum on Whatman 3MM paper. Fluorography was performed on the dried down gel. (Lanes a and h) ^3H-Labeled core histones (80 000 cpm/μg of protein). (Lane b) ^3H-Labeled protein A24 was incubated at 37 °C for 60 min in the absence of the nucleoli. ^3H-Labeled protein A24 (1 μg of protein) was incubated with various amounts of thioacetamide-treated liver nucleoli: (lane c) 6.25 μg, (lane d) 12.5 μg, (lane e) 25 μg, (lane f) 50 μg, and (lane g) 100 μg of protein.

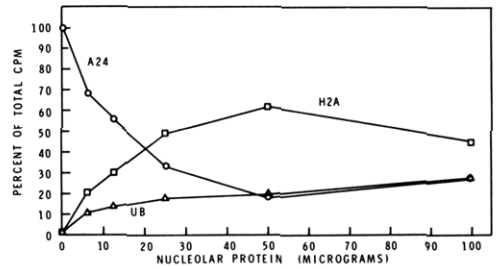

FIGURE 4: Bands from the dried down gel as described in Figure 3 were cut out and treated overnight with 1 mL of 30% hydrogen peroxide at 50 °C. 10 mL of Aquasol-2 (New England Nuclear) was added and counted in a liquid scintillation counter. Total counts equal the sum of the counts for the protein A24 band, the histone 2A band, and the ubiquitin band. Depending upon the sample on the gel, the total counts ranged from 46 141 cpm for lane b of Figure 3 to a maximum of 86 408 cpm for lane d. Therefore, the ordinate was plotted as the percent of total counts.

For further characterization of the protein A24 hydrolytic activity, thioacetamide-treated liver nucleoli were pretreated with trypsin (Figure 6, lane d) and then incubated with ^3H-labeled protein A24. The protein A24 lyase activity of nucleoli was almost completely inhibited (Figure 6, lanes d and e). Incubation of ^3H-labeled protein A24 with nucleoli in the presence of trypsin inhibitor did not inhibit the protein A24 lyase activity (Figure 6, lane f), which indicates that the trypsin inactivated the nucleolar activity but the trypsin inhibitor did not. When thioacetamide-treated liver nucleoli were heated at 80 °C for 10 min prior to incubation with ^3H-labeled protein A24 (Figure 6, lane g), the A24 lyase activity was essentially inactivated. The trypsin sensitivity and heat lability of the protein A24 lyase indicate this activity resides in a heat labile enzyme.

Discussion

This study showed that purified protein A24 was specifically cleaved to release intact histone 2A and ubiquitin by isolated

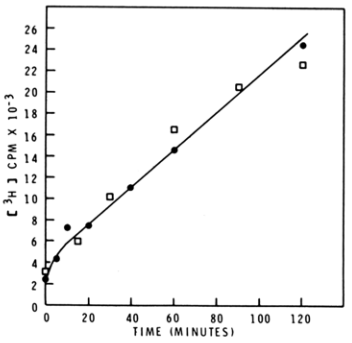

FIGURE 5: ^{3}H-Labeled protein A24 (1 μg/30-μL assay mix) was mixed with thioacetamide-treated rat liver nucleoli (7 μg of protein/30-μL assay mix) in a final volume of 225 μL containing 0.01 M Tris (pH 7.9), 1 mM DTT, and 1 mM PMSF. The mixture was incubated at 37 °C, and 30-*mL* aliquots were mixed at various times of incubation with 0.5 mL of 0.1 M NaCl in TPD. The mixture was applied to a phosphocellulose column, and the unadsorbed counts ([^{3}H]ubiquitin) were determined by liquid scintillation counting.

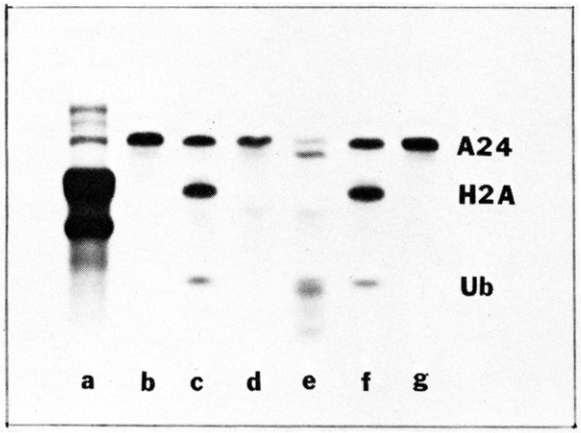

FIGURE 6: One-dimensional $NaDodSO_4$–12% polyacrylamide gel electrophoresis of ^{3}H-labeled protein A24 and its hydrolysis products as described in Figure 3. (Lane a) ^{3}H-Labeled core histones (80 000 cpm/μg of protein). (Lane b) ^{3}H-Labeled protein A24 (1 μg of protein) incubated at 37 °C for 60 min in the absence of nucleoli. (Lane c) ^{3}H-Labeled protein A24 incubated in the presence of nucleoli (25 μg of protein) from livers of rats treated for 3 days with thioacetamide. (Lane d) Same as Lane c except that the nucleoli were pretreated with 1 μg of trypsin at 37 °C for 30 min which was then inactivated with 2 μg of lima bean trypsin incubator. (Lane e) ^{3}H-Labeled protein A24 (1 μg of protein) incubated with 0.1 μg of trypsin at 37 °C for 60 min. (Lane f) ^{3}H-Labeled protein A24 incubated with nucleoli (25 μg) in the presence of 2 μg of lima bean trypsin inhibitor. (Lane g) Nucleoli were heated at 80 °C for 10 min prior to incubation with ^{3}H-labeled protein A24.

nucleoli in vitro, a result which implies a site-specific cleavage at or near the isopeptide linkage between histone 2A and the ubiquitin moiety of protein A24. Recently, Ciechanover et al. (1978) described a heat-stable polypeptide component (APF-1) of an ATP-dependent proteolytic system from rabbit reticulocytes which has been proposed to conjugate with endogenous proteins (Ciechanover et al., 1980a; Hershko et al., 1980) to act as an intermediate under conditions of ATP-dependent protein degradation (Ciechanover et al., 1980b). Also, Wilkinson et al. (1980) identified ubiquitin as the ATP-dependent proteolysis factor I (APF-I) of rabbit reticulocytes. Since protein A24 is a conjugated chromatin protein consisting of ubiquitin and histone 2A (Goldknopf & Busch, 1975; Olson et al., 1976; Hunt & Dayhoff, 1977; Schlesinger et al., 1975; Goldstein et al., 1975), its cleavage might involve a mechanism similar to the reticulocyte proteolytic system (Ciechanover et al., 1978). However, protein A24 is cleaved specifically to release apparently intact histone 2A. Thus, in this system the ubiquitin may have a role other than catabolism of histone 2A; the cleavage may be related to gene activation (Ballal & Busch, 1973; Ballal et al., 1974, 1975a,b; Goldknopf et al., 1978).

This study showed that the specific enzyme in isolated nucleoli cleaved protein A24 in chromatin in vitro to release free ubiquitin (Figures 1 and 2). A protease described by Eickbush et al. (1976) cleaved histone 2A at valine-114 which would be five amino acid residues from the isopeptide linkage of protein A24 (Goldknopf & Busch, 1977). However, the products of such a cleavage of protein A24 would not comigrate with free ubiquitin or histone 2A. The ubiquitin would have a higher molecular weight and would be more positively charged due to the presence of the basic carboxyl terminus of histone 2A. Also, the H2A portion would be cH2A, a polypeptide which does not comigrate with H2A on $NaDodSO_4$ gels (Eickbush et al., 1976). In addition, that enzyme required 1 M NaCl for maximum activity (Eickbush et al., 1976), but the nucleolar activity reported here did not.

Since the cleavage reaction also occurred when intact chromatin was used as substrate, it is probable that the protein A24 which was cleaved was part of the nucleosomal core complex (Goldknopf et al., 1977; Martinson et al., 1979; Bonner & Stedman, 1979). This result implies that protein A24 was cleaved only at a site which was not protected by the interactions between core histones within the nucleosomal particle (Kornberg, 1974; Olins & Olins, 1974; Hewish & Burgoyne, 1973; Sahasrabuddhe & Van Holde, 1974; Oudet et al., 1975). The H2A portion of protein A24 is involved in nucleosome core formation (Goldknopf et al., 1977; Martinson et al., 1979; Albright et al., 1979), but the ubiquitin portion does not interfere with histone–histone interactions (Martinson et al., 1979; Bonner & Stedman, 1979; Nelson et al., 1979). Further, that portion of histone 2A which is linked to the ubiquitin moiety is available in the chromatin structure for release by the lyase described here (Bohm et al., 1980).

The presence of this activity agrees with the earlier observations of a decrease of protein A24 in isolated liver nucleoli during thioacetamide treatment and regeneration after partial hepatectomy (Ballal & Busch, 1973; Ballal et al., 1974, 1975a,b), both of which are accompanied by an increased transcription of preribosomal RNA (Andersen et al, 1977; Ballal et al., 1975a,b). There is also a decrease in protein A24 in the transcriptionally active chromatin fraction (Gottesfeld et al., 1974) which contains free ubiquitin (Goldknopf et al., 1978; Watson et al., 1978). Altered chromatin conformation due to the cleavage of protein A24 and release of free ubiquitin may be involved in nucleolar transcriptional activation. Further studies on the isolation and characterization of the protease described here should aid in the analysis of the structural and functional alterations of chromatin produced by the cleavage of the protein A24.

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Differences in Rearrangements of H1 and H5 in Chicken Erythrocyte Chromatin†

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ABSTRACT: H1 can rearrange in chicken erythrocyte polynucleosomes in 80 mM NaCl buffers. These rearrangements have been studied by sedimentation analysis. H1 redistributes between polynucleosomes as well as between polynucleosomes and monosomes. In these rearrangements H1 molecules move to free DNA sites. In contrast to H1, the chicken erythrocyte specific lysine-rich histone H5 does not show any of these dynamic properties. This difference in mobility of H1 and H5 also manifests itself in the selective extraction of H1 from H1, H5 containing polynucleosomes by the cation-exchange resin AG 50W-X2 at 80 mM NaCl.

Histone H1 is involved in the packing of nucleosomes into a 200-300-Å fiber. Different models—a solenoidal architecture (Finch & Klug 1976; Thoma et al. 1979), a higher order unit (superbead) (Hozier et al., 1977), and other packing models (Bradbury, 1977)—have been put forward. From the experimental work leading to various models it has become clear that the observed chromatin superstructure is very dependent on the ionic strength conditions. The recent electron microscopy work of Thoma et al. (1979) shows that in the

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