found at the 5′ terminus of bulk yeast tRNA. Even though ψ is very rare in the acceptor stem region of tRNA, $tRNA_2^{Lys}$ has two residues in this region.

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Isolation and Characterization of the Non-Histone Chromosomal Proteins of Developing Avian Erythroid Cells†

Lee A. Sanders‡

ABSTRACT: A method originally devised for the isolation of acidic chromatin proteins from chick embryo brain [Graziano, S. L., and Huang, R. C. (1971), *Biochemistry 10*, 4770] was adapted for the isolation of non-histone chromosomal proteins from duck erythroid cells. The method involves isolation and repeated washing of cell nuclei and chromatin, dissociation of the chromatin components in 3 M NaCl, separation of soluble proteins from DNA by gel filtration on Bio-Gel A-50, and finally, separation of the histones from non-histone chromosomal proteins by chromatography on SP-Sephadex. Preliminary characterization of the non-histone proteins by both amino acid analysis and sodium dodecyl sulfate polyacrylamide gel electrophoresis indicates that they are a group of acidic proteins consisting of at least 16–22 components and ranging in size from 12,500 to 150,000 daltons. Both immature and mature erythroid cell populations manifested a major non-histone protein component of molecular weight 72,000. Non-histone proteins were also isolated from duck erythroid cells by an alternative method [Levy, S., Simpson, R. T., and Sober, H. A. (1972), *Biochemistry 11*, 1547] involving dissociation of the chromatin in 6 M urea–0.4 M Gdn·HCl, removal of DNA by ultracentrifugation, and separation of histones from non-histones by chromatography on Bio-Rex 70 resin. The latter procedure yielded at least 18–26 non-histone components with major components of apparent molecular weights 72,000 and 150,000 for both erythroid cell populations. Several significant qualitative and quantitative differences were noted in the gel electrophoretic patterns of the non-histone chromosomal proteins from immature *vs.* mature erythroid cells, suggesting that changes in the non-histone proteins may accompany the process of cellular differentiation.

In view of a number of studies, it appears that the non-histone chromosomal proteins probably play important roles in determining both the structure and function of the eukaryotic chromosome. Although many problems have been encountered in the isolation of non-histone chromosomal proteins (Graziano and Huang, 1971; Goodwin and Johns, 1972; Sanders, 1973), certain of these proteins have been shown (a) to exhibit tissue and species specificity (Teng *et al.*, 1970, 1971; Elgin and Bonner, 1970; Chytil and Spelsberg, 1971; Richter and Sekeris, 1972; MacGillivray *et al.*, 1972; Wu *et al.*, 1973), (b) to bind "selectively" to DNA (Kleinsmith *et al.*, 1970; Salas and Green, 1971; Teng *et al.*, 1971; van den Broek *et al.*, 1973), (c) to stimulate transcription *in vitro* and possibly to influence its specificity (Paul and Gilmour, 1968, 1969; Wang, 1971; Kamiyama and Wang, 1971; Spelsberg *et al.*, 1971; Stein *et al.*, 1972; Kamiyama *et al.*, 1972; Kostraba and Wang, 1972; Shea and Kleinsmith, 1973), and (d) to undergo specific changes in response to various hormones or phytomitogens (Teng and Hamilton, 1969; Shelton and Allfrey, 1970; Chung and Coffey, 1971; Levy *et al.*, 1973). In addition, several of the non-histone chromosomal proteins appear to function as enzymes, *e.g.*, histone acetyl transferases, deacetylases, kinases, and methylases; RNA polymerase, DNA polymerase, etc. (Wang, 1967; Howk and Wang, 1969; Kamiyama *et al.*, 1972; Vidali *et al.*, 1972).

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received August 17, 1973.* This work was supported in part by Grant T5-GM-01678 from the National Institutes of Health.

‡ M.D.–Ph.D. candidate, trainee of the National Institutes of Health, National Institute of General Medical Sciences (T5-GM-01678).

It has recently been indicated that changes in the non-histone proteins may occur either during the cell cycle or during the process of cellular differentiation. For example, Bhorjee and Pederson (1972) reported obtaining different non-histone protein gel electrophoretic patterns from synchronized HeLa cells at different stages of the cell cycle. Likewise, LeStourgeon and Rusch (1973) reported differences in such patterns obtained from the plasmodial slime mold *Physarum polycephalum* at different stages of cellular differentiation.

In view of the above findings in other cell systems, the apparent importance of the non-histone chromosomal proteins in nuclear function, and the recent advances in isolation procedures for the non-histone proteins, it was of interest to isolate and characterize the non-histone chromosomal proteins from developing avian erythroid cells. In an earlier comparative study (Sanders *et al.*, 1973), methods were described for obtaining populations of immature avian erythroid cells consisting predominantly of reticulocytes, which actively synthesize RNA. In contrast, mature avian erythrocytes are essentially inactive in RNA synthesis. Our preliminary evidence indicates that changes in the non-histone proteins may accompany the processes of differentiation and development in this cell system.

Materials and Methods

Chemicals. Urea was obtained from Baker and Adamson. Gdn·HCl[1] was obtained from Heico, Inc. Triton X-100 was purchased from Rohm and Haas Co. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Eastman Chemicals and recrystallized from chloroform and acetone, respectively, before further use. *N,N,N',N'*-Tetramethylethylenediamine was purchased from Matheson Coleman and Bell. Coomassie Brilliant Blue was obtained from Schwarz/Mann. S-100 protein prepared from porcine brain was a gift from Dr. Thomas Vanaman. Crystalline bovine serum albumin was obtained from Nutritional Biochemicals. Phosphorylase A, ovalbumin, α-chymotrypsinogen, lysozyme, and calf thymus DNA (Type I) were purchased from Sigma. Catalase was obtained from Worthington Biochemicals.

Preparation of Cells. Populations of mature and immature erythroid cells were obtained from adult white Peking ducks as described previously (Sanders and McCarty, 1972; Sanders *et al.*, 1973). All operations were performed at 0–4° unless noted otherwise. The cells were pelleted, washed once in 4 volumes of 0.14 M NaCl–0.01 M trisodium citrate, and removed from the white cell-containing buffy coats as described earlier. Typical preparations described below utilized 15–30 ml of the washed, packed erythroid cells. Cerebellar gray matter (18 g) was obtained from two adult porcine brains within 1 hr after slaughter.

Isolation of Nuclei, Chromatin, and Non-Histone Chromosomal Proteins. A method originally devised by Graziano and Huang (1971) for the isolation of acidic chromatin proteins from chick embryo brain was adapted for the isolation of nuclei, chromatin, and non-histone chromosomal proteins from duck erythroid cells. Since the washed erythroid cells lysed almost immediately when placed in 6 volumes of 0.25 M sucrose–3.3 mM $Ca(OAc)_2$–0.5% Triton X-100, they had to be pelleted by centrifugation at 7000*g* for 10 min prior to homogenization in order to avoid severe foaming problems. The crude nuclear pellets were then resuspended in 6 volumes of 0.25 M sucrose–3.3 mM $Ca(OAc)_2$–0.5% Triton X-100 and homogenized with five strokes of a Teflon–glass homogenizer (50 ml) at approximately 1200 rpm. The homogenates were filtered through four layers of cheesecloth, and centrifuged at 3500*g* in a SS-34 rotor for 20 min to pellet nuclei. The nuclear pellets were suspended in 7 volumes of 1.95 M sucrose–3.3 mM $Ca(OAc)_2$–0.5% Triton X-100 *via* seven strokes of a Dounce homogenizer A pestle, and filtered through four layers of cheesecloth. The filtrates were layered over 7-ml aliquots of 2 M sucrose in 40-ml, cellulose nitrate tubes, and centrifuged at 20,000 rpm (52,800*g* at $R_{\overline{AV}}$) for 60 min in an SW 27 rotor using a Beckman L2-65B ultracentrifuge. The resultant nuclear pellets appeared to be essentially free of cytoplasmic debris when examined by phase contrast microscopy. The nuclear preparations were lysed osmotically by successive washes in solutions of decreasing ionic strength exactly as described by Graziano and Huang (1971). This lysis procedure consists of four successive washes in 0.075 M NaCl–0.024 M EDTA (pH 7.9), followed by two washes in solutions of 50 mM Tris, 10 mM Tris, and 2 mM Tris (all at pH 7.9).

Dissociation of Chromatin Components and Separation of Chromosomal Proteins from DNA by Gel Filtration on Bio-Gel A-50. The gelatinous chromatin samples prepared as described above were solubilized by stirring for 10–12 hr in *ca.* 8 volumes of deionized water (adjusted to pH 7.1 with 0.01 volume of 2 mM Tris and several drops of 0.01 N NaOH). The chromatin solutions were adjusted to 8–15 OD ml^{-1} cm^{-1} at 260 nm and sheared in a Sorvall Omnimixer at 80 V for 90 sec. Absorbances at 230 and 280 nm were also recorded. Aliquots of the sheared chromatin preparations were withdrawn, clarified by centrifugation at 12,100*g* for 20 min (which appeared by phase contrast microscopy to pellet the remaining nuclear membranes), and frozen for later protein, DNA, and RNA determinations. The remaining chromatin samples were adjusted to 3.02 M NaCl by slow addition of solid NaCl with continuous stirring. The solutions were stirred 20–24 hr and clarified *via* centrifugation at 12,100*g* for 20 min prior to gel filtration as described below.

Salt-dissociated chromatin samples of up to 90 ml (650–1300 total OD units at 260 nm) were layered on a 4 × 125 cm column of Bio-Gel A-50 resin (extensively washed and pre-equilibrated with 3 M NaCl); 10-ml (20 min) fractions were collected, and both protein and DNA were monitored by reading eluate absorbances at 230 and 260 nm. Aliquots (50 ml) of the obtained protein peaks were dialyzed exhaustively against 0.05 M acetic acid and lyophilized prior to protein determination and sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Separation of Histones from Non-Histone Chromosomal Proteins via Ion Exchange Chromatography on SP-Sephadex. Solutions of 7 M urea–0.23 M NaCl–0.01 M NaOAc (pH 5.2) were prepared as described by Graziano and Huang (1971) except that Bio-Rad Mixed Bed Resin (AG 501-X8) was utilized for deionization of the 10 M urea stock solution. These solutions were always used within 2 days of preparation to avoid potential problems secondary to the formation of cyanate and ammonium ions. Portions (200 ml) of the protein peaks obtained from gel filtration were dialyzed against five 2-l. changes of the solution containing 7 M urea–0.23 M NaCl–0.01 M NaOAc (pH 5.2). The dialyzed protein solutions were chromatographed on a 2.0 × 39 cm column of SP-Sephadex which had been swollen and extensively washed in the above buffer at pH 5.2; 8–11-ml (20 min) fractions were collected. After elution of non-histone proteins from the

[1] Abbreviations used are: SP, sulfopropyl; Gdn·HCl, guanidine hydrochloride.

column, the histones were eluted with 7 M urea–0.8 M NaCl–0.01 M NaOAc (pH 5.2). Protein was monitored *via* absorbance at 230 nm, and various protein fractions were pooled, dialyzed exhaustively against 0.05 M acetic acid, and lyophilized prior to gel electrophoresis and amino acid analysis.

Examination of the Non-Histone Chromosomal Protein Preparations for Evidence of Cytoplasmic Protein Contamination. The cytoplasmic marker proteins globin (mol wt 16,500, of hemoglobin) and S-100 protein (mol wt 11,000; an acidic protein from porcine brain) were used as indicators of cytoplasmic protein contamination. Examination of the sodium dodecyl sulfate polyacrylamide gel patterns of the non-histone proteins of avian erythroid cells and porcine brain, respectively, revealed little or no protein in these molecular weight regions of the gels, suggesting cytoplasmic protein contamination to be negligible.

Estimation of Amounts of Residual Protein Remaining on DNA after Dissociation of Chromatin in 3 M NaCl. Aliquots (12 ml) of the DNA-containing peaks obtained from gel filtration on Bio-Gel A-50 were centrifuged for 46 hr at 175,000*g* (38,000 rpm) in an SW 41 rotor in a Beckman L2-65B ultracentrifuge. This procedure was sufficient to pellet *ca.* 95% of the DNA present as determined by comparisons of the absorbances at 260 nm of the respective solutions and supernatants before and after centrifugation. The DNA pellets were solubilized in 3% sodium dodecyl sulfate–4% β-mercaptoethanol–0.05 M sodium phosphate buffer (pH 7.2) by heating at 37° for 1 hr followed by incubation in a steam bath for 5–10 min. Samples containing up to 350 μg of DNA were layered on sodium dodecyl sulfate polyacrylamide gels, and DNA was removed from the gel tops after 2–3 hr of electrophoresis at reduced amperage (1.2 mA/gel, Weiner *et al.*, 1972). After removal of the DNA, electrophoresis was continued at 4 mA/gel as described below. Protein was estimated from the gels after staining, destaining, and scanning as described below.

Alternative Method for Preparation of Non-Histone Chromosomal Proteins. In addition to the method employed above, non-histone chromosomal proteins were also isolated from immature and mature avian erythroid cells essentially according to the method of Levy *et al.* (1972, 1973) with only slight modifications. In order to avoid severe foaming problems, washed, packed erythroid cells were first lysed at 4° by suspension in 9 volumes of 0.25 M sucrose–3 mM $CaCl_2$–0.01 M Tris-HCl–0.5% Triton X-100 (pH 8.0), followed by centrifugation at 5000*g* for 10 min in a Sorvall SS-34 rotor. Nuclei were isolated from the resultant pellets according to the modified procedure of Hymer and Kuff (1964) by using the pH 8.0 detergent solution noted above for repeated washes and homogenizations. As described by Huang and Huang (1969), chromatin was prepared from the isolated nuclei by extraction with solutions of decreasing ionic strength from 50 to 1 mM Tris-HCl (pH 8.0). After solubilization by stirring in 4 volumes of distilled water (adjusted to pH 8 with NH_4OH) for 4 hr, the chromatin was sheared in a Sorvall Omnimixer as described earlier.

The sheared chromatin at a DNA concentration of 0.4 mg/ml was dissociated in 6 M urea, 0.4 M Gdn·HCl, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate buffer at pH 7.0 (Levy *et al.*, 1972); 93–94% of the DNA was sedimented by centrifugation at 100,000*g* (29,500 rpm) in a no. 30 rotor for 48 hr at 4°. The combined supernatants were chromatographed on Bio-Rex 70 resin as described by Levy *et al.* (1972, 1973) except that the columns were run at 4° in an attempt to minimize potential problems due to proteolysis. Various fractions were pooled, dialyzed exhaustively against water, and lyophilized prior to analysis by sodium dodecyl sulfate gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the method suggested by Shapiro and Maizel (1969) and developed by Weber and Osborn (1969). Gels (0.5 × 12 cm) contained 6.5% acrylamide, 0.1% sodium dodecyl sulfate, 0.175% *N,N'*-methylenebisacrylamide, 0.15% *N,N,N',N'*-tetramethylethylenediamine, and 0.05 M sodium phosphate buffer at pH 7.2. Gel electrophoresis buffer consisted of 0.1% sodium dodecyl sulfate in 0.05 M sodium phosphate at pH 7.2. Protein samples (10–100 μl) were prepared at a concentration of approximately 3 mg/ml in a solution of 1% sodium dodecyl sulfate–4% β-mercaptoethanol in 0.05 M sodium phosphate buffer (pH 7.2) and incubated for 2 hr at 37°. Sucrose (3–5 μl, 40% aqueous solution) and Bromphenol Blue marker dye (5 μl, 0.1% solution in gel electrophoresis buffer) were added to each sample prior to layering on the gels. Electrophoresis was performed at 70 V (4 mA/gel) for 4.5 hr. Non-histone protein samples prepared by the method of Levy *et al.* (1972, 1973) contained significant amounts of nucleic acid. Therefore, these samples were dissolved in 5% sodium dodecyl sulfate–4% β-mercaptoethanol–0.05 M sodium phosphate and incubated as above prior to the addition of sucrose and marker dye. After 2 hr of electrophoresis of these samples at reduced amperage (1.2 mA/gel; Weiner *et al.*, 1972), nucleic acid was removed from the gel tops by gentle washing with a Pasteur pipet, and electrophoresis was continued at 70 V (4 mA/gel) for an additional 4–5 hr. All gels were stained for 5 hr at ambient temperature in 0.23% Coomassie Blue in 46% methanol–8.4% acetic acid solution and destained in 5% methanol–14% acetic acid. Gels were scanned at 562 nm with a Model 2410 Gilford linear transport scanner.

The following reduced protein standards with molecular weights given in parentheses were used to calibrate the gels: phosphorylase A (94,000), catalase (60,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), and lysozyme (14,400). Standard curves of the logs of the known molecular weights *vs.* mobility were constructed as described by Weber and Osborn (1969) to calibrate the gels.

Amino acid analyses were performed after hydrolysis of samples of non-histone proteins for 24 hr at 110° *in vacuo* using a Beckman Model 120C amino acid analyzer.

Determinations of Protein and DNA. Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard (assuming $A^{1\,cm}_{280\,nm} = 0.66$ for a 1 mg/ml solution (Cohn *et al.*, 1947)). DNA was assayed by reaction with indole (Ceriotti, 1955; Short *et al.*, 1968) using calf thymus DNA (Type I) as standard.

Results

Properties of the Isolated Chromatin. Nuclei and chromatin were prepared as described above from immature and mature populations of duck erythroid cells. After solubilization in water, shearing in a Sorvall Omnimixer, and clarification (*via* centrifugation at 12,100*g* for 20 min to pellet nuclear membranes and any remaining cellular debris), the chromatin solutions manifested $A_{260/280}$ ratios of 1.73 and 1.74, respectively, and $A_{260/230}$ ratios of 1.43 and 1.28, respectively. Chemical analyses of the chromatin samples as described under Methods gave protein:DNA ratios of 1.42 ± 0.15 and 1.68 ± 0.03 for immature and mature erythroid cell

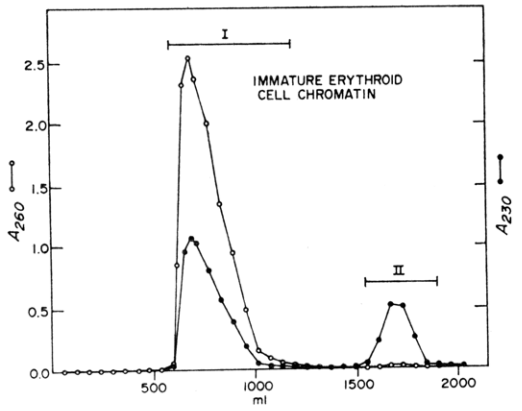

FIGURE 1: Separation of chromosomal proteins from DNA by gel filtration on Bio-Gel A-50–immature erythroid cell chromatin. A 90-ml sample of salt-dissociated immature erythroid cell chromatin containing a total of 675 OD units at 260 nm was layered on a column of Bio-Gel A-50 and chromatographed in the presence of 3 M NaCl as described under Methods. Peak I contained DNA. Peak II contained the salt-dissociated chromosomal proteins.

chromatins, respectively. [The above numbers represent the mean and standard error (SE) for 6 determinations, where $SE = ([x - \bar{x}]^2/n[n-1])^{1/2}$.]

Isolation of Non-Histone Chromosomal Proteins According to the Method of Graziano and Huang (1971). After dissociation of the immature erythroid cell chromatin samples in 3 M NaCl, chromosomal proteins were separated from DNA by gel filtration on Bio-Gel A-50 as shown in Figure 1. Gel filtration of 3 M NaCl dissociated mature erythroid cell chromatin by the same method gave a very similar chromatographic pattern, which, for the sake of brevity, is not presented here. In order to maintain reasonable flow rates and to obtain good resolution of the chromosomal proteins from DNA, it was necessary to shear the chromatin to reduce viscosity prior to gel filtration, and to utilize chromatin concentrations <15 OD/ml at 260 nm. These elution patterns are similar to those obtained by Graziano and Huang (1971) for chick embryo brain chromatin, although slightly better

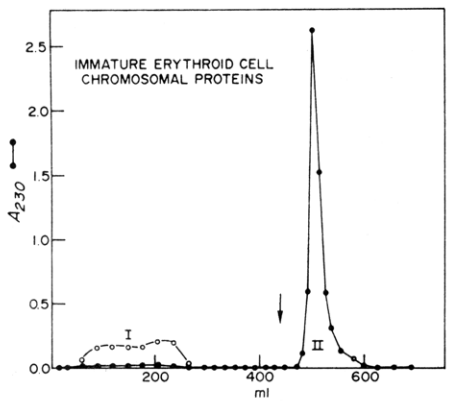

FIGURE 2: Separation of non-histone chromosomal proteins from histones *via* ion exchange chromatography on SP-Sephadex. Chromosomal proteins of immature erythroid cells were obtained from peak II of Figure 1. Chromatography was performed after dialysis of the proteins into a solution of 7 M urea–0.23 M NaCl–0.01 M NaOAc (pH 5.2) as described under Methods. Arrow denotes the start of elution of histones with 7 M urea–0.8 M NaCl–0.01 M NaOAc (pH 5.2). Note that peak I (eluting from 50–270 ml) is broad due to the large sample volume (220 ml) and the fact that the non-histone proteins did not bind appreciably to the column as did the histones. Open circles indicate peak I after magnification by a factor of 10.

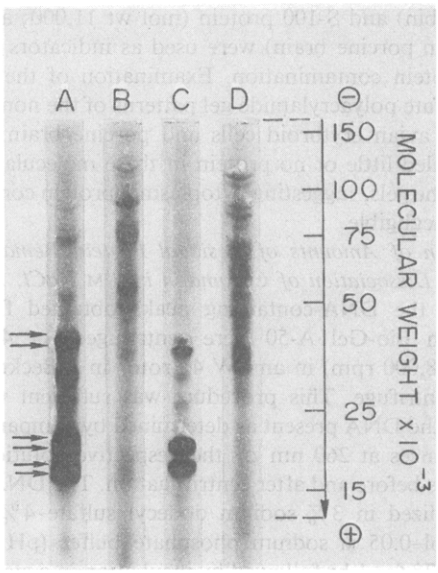

FIGURE 3: Resolution of histone and non-histone chromosomal protein preparations from mature erythroid cells by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were 0.1% in sodium dodecyl sulfate and 6.5% in acrylamide: (A) mature erythroid cell chromosomal proteins, peak II eluting from Bio-Gel A-50; (B) mature erythroid cell non-histone chromosomal proteins, peak I eluting from SP-Sephadex; (C) mature erythroid cell histones, peak II eluting from SP-Sephadex; (D) porcine brain non-histone chromosomal proteins prepared as described under Methods. Scale at right denotes molecular weight calibration of the gels as described under Methods. Dashed line near bottom of gels indicates position of marker dye at the end of electrophoresis. Arrows denote, from top to bottom, the respective positions of histones F1, F2c, F3, F2b, F2a2, and F2a1.[2]

resolution appears to have been obtained herein. The first peak in each case contained DNA almost completely free of protein, *i.e.*, <3% of the total chromosomal proteins remained bound to the DNA as determined under "Methods." The second peak contained the dissociated chromosomal proteins as evidenced by the high $A_{230/260}$ ratio. Average recoveries of DNA from the two columns were 97%, while average recoveries of protein were 89%.

As shown in Figure 2, the isolated chromsomal proteins could be further fractionated into histone and non-histone classes by ion exchange chromatography on SP-Sephadex. The relatively acidic, non-histone chromosomal proteins were readily eluted from the columns in 7 M urea–0.23 M NaCl–0.01 M NaOAc (pH 5.2). However, the basic histones which were bound to the resin under these conditions required 7 M urea–0.8 M NaCl–0.01 M NaOAc (pH 5.2) for elution from the columns. Mature erythroid cell chromosomal proteins gave an essentially identical pattern with that in Figure 2, when chromatographed on SP-Sephadex (not shown).

The various protein components of the samples obtained as described above were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were calibrated from known protein molecular weight standards by plotting the logs of the molecular weights *vs.* mobilities in the gel.

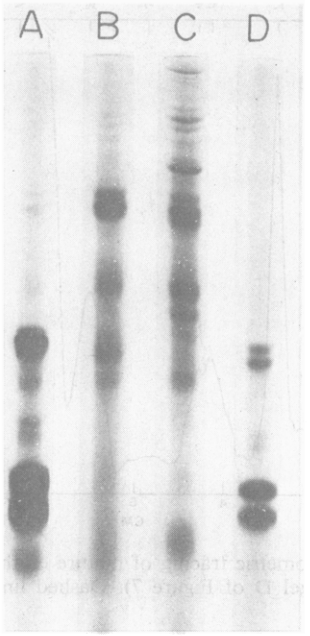

FIGURE 4: Resolution of histone and non-histone chromosomal protein preparations from immature erythroid cells by sodium dodecyl sulfate polyacrylamide gel electrophoresis: (A) immature erythroid cell chromosomal proteins, peak II of Figure 1; (B) immature erythroid cell non-histone chromosomal proteins, peak I of Figure 2; (C) mature erythroid cell non-histone chromosomal proteins, peak I eluting from SP-Sephadex; (D) immature erythroid cell histones, peak II of Figure 2.

Figure 3 presents the gel electrophoretic patterns of mature erythroid cell salt-dissociated chromosomal proteins, non-histone proteins, and histones in comparison with a preparation of the non-histone proteins obtained from porcine brain chromatin. It is important to note that although the non-histones are present in small amounts relative to the histones, these two classes of proteins have been well resolved by ion exchange chromatography. The anomalously slow migration of the histones in sodium dodecyl sulfate gels is also apparent in Figure 3. [For example, histones F1 and F2c[2] having respective molecular weights of 22,000 and 18,400 (Bustin and Cole, 1970; Greenaway, 1971) always run significantly behind the α-chymotrypsinogen standard (molecular weight 25,700). Such anomalous migration appears to be due to the high inherent positive charge of the histone molecules, which influences their electrophoretic behavior even in the presence of sodium dodecyl sulfate.] Finally, although the electrophoretic pattern of porcine brain non-histones is similar to a corresponding preparation from chick embryo brain reported by Graziano and Huang (1971), these proteins exhibit significant differences from the non-histone proteins of the mature avian erythroid cells. Such differences are consistent with the recent findings of both species and tissue specificity of the non-histone chromosomal proteins, referred to in the introduction.

Figure 4 shows the gel electrophoretic patterns of immature

[2] The histone nomenclature used is that of Johns and Butler (1962) as modified by Hnilica (1964). The corresponding designations of Rasmussen *et al.* (1962) as modified by Neelin *et al.* (1964) and Fambrough *et al.* (1968) are given in parentheses as follows: F1 (I), F2a1 (IV), F2a2 (IIb1), F2b (IIb2), F2c (V), and F3 (III).

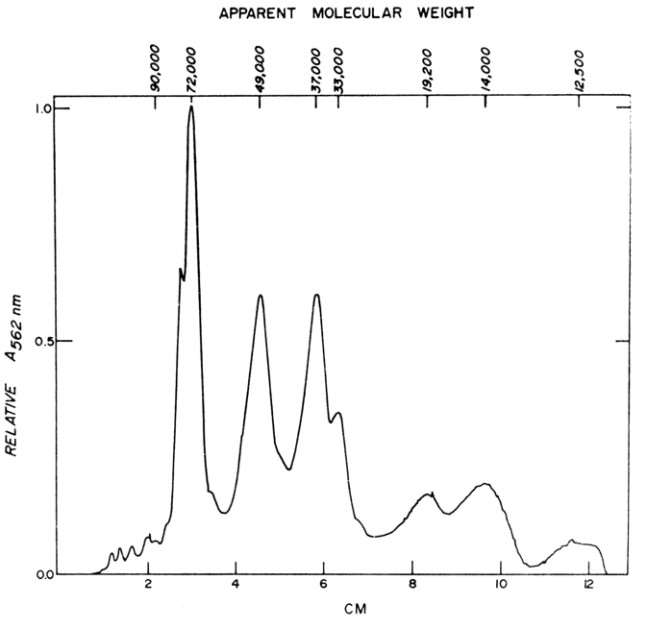

FIGURE 5: Densitometric tracing of non-histone proteins after sodium dodecyl sulfate gel electrophoresis–immature erythroid cells (gel B of Figure 4).

erythroid cell chromosomal proteins, non-histone proteins, and histones. Again, note that good resolution of non-histones from histones was obtained by ion exchange chromatography on SP-Sephadex. However, there exist qualitative and quantitative differences in the non-histone protein patterns of immature *vs.* mature erythroid cells as shown in gels B and C, respectively. These differences are also apparent in the respective densitometric tracings presented in Figures 5 and 6. As shown, the most notable similarity between the immature and mature erythroid cell patterns is the major non-histone component of apparent molecular weight 72,000 in each preparation.

The non-histone proteins isolated as described above appear to be relatively acidic as a group. This characteristic is

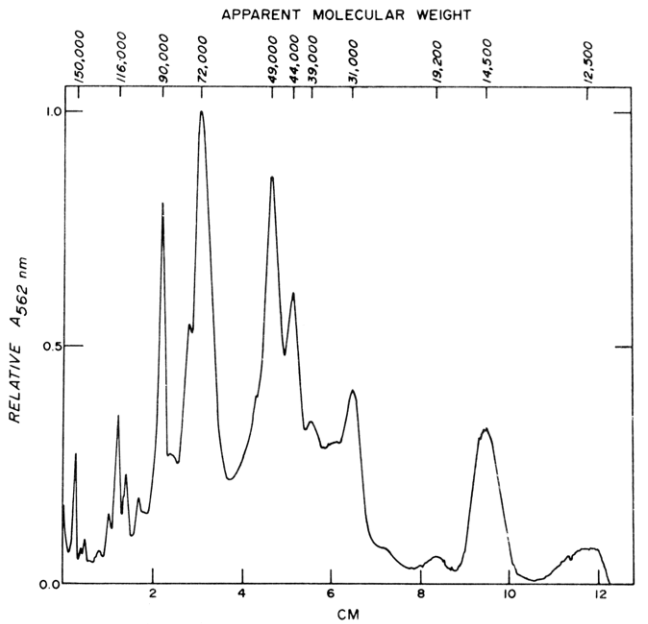

FIGURE 6: Densitometric tracing of non-histone proteins after sodium dodecyl sulfate gel electrophoresis–mature erythroid cells (gel C of Figure 4)

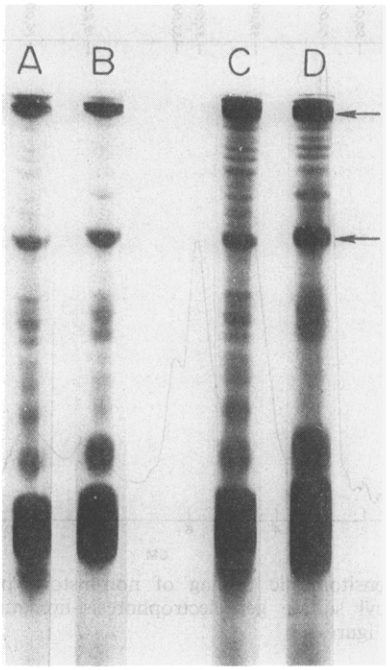

FIGURE 7: Sodium dodecyl sulfate gel electrophoresis of avian erythroid cell chromosomal proteins prepared by the method of Levy *et al.* (1972, 1973). Samples were obtained from the following sources: (A) immature erythroid cells; (B) mature erythroid cells; (C) immature erythroid cells; (D) mature erythroid cells. Samples A and B were derived from *ca.* 200 μg of chromatin DNA; samples C and D were derived from *ca.* 300 μg of chromatin DNA. Arrows indicate apparent molecular weights of 150,000 and 72,000 from top to bottom, respectively.

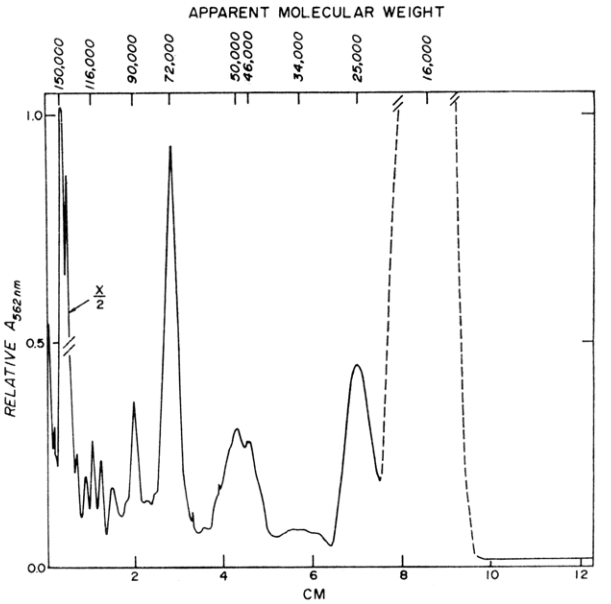

FIGURE 9: Densitometric tracing of mature erythroid cell chromosomal proteins (gel D of Figure 7). Dashed lines denote histone contaminants..

suggested not only by their inability to bind to the SP-Sephadex resin during chromatography, but also by their amino acid composition. For example, the ratios of acidic: basic amino acids for the non-histone proteins of immature erythroid cells, mature erythroid cells, and porcine cerebellar gray matter were 1.46, 1.53, and 1.55, respectively. [In contrast, purified avian erythrocyte histone F2c (Sanders and McCarty, 1972) has an acidic:basic ratio of 0.18.]

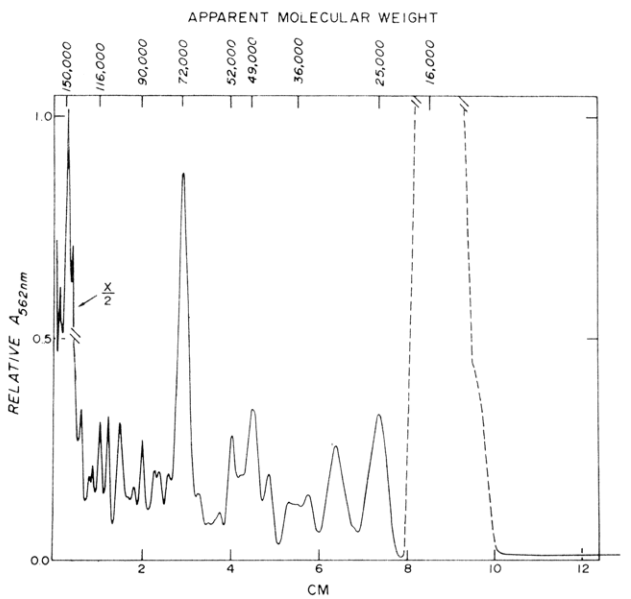

FIGURE 8: Densitometric tracing of immature avian erythroid cell chromosomal proteins (gel C of Figure 7). Dashed lines denote presence of histone contaminants remaining even after chromatography on Bio-Rex 70 resin at 4°.

Isolation of Non-Histone Chromosomal Proteins According to an Alternative Method. For the sake of comparison and in order to examine the non-histone chromatin proteins in further detail, these proteins were also prepared from avian erythroid cells according to the method developed by Levy *et al.* (1972, 1973). This method utilizes 6 M urea, 0.4 M Gdn·HCl, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate buffer at pH 7.0 for dissociation of the chromatin components, followed by ultracentrifugation to remove nucleic acid, and chromatography on Bio-Rex 70 resin to separate histones from non-histone chromosomal proteins. Although this method of isolation proved to be less time consuming than that of Graziano and Huang (1971), two potential problems were encountered: first, only 93–94% of the nucleic acid was removed from the dissociated chromatin solutions *via* ultracentrifugation; and second, with the exception of histone F2c, the histones were only partially removed from the non-histones by chromatography on Bio-Rex 70 resin. [It should be noted that the latter problem may have arisen because the columns were run at 4° (in our attempt to avoid potential problems due to proteolysis) rather than at ambient temperature as recommended by Levy *et al.* (1972).]

In spite of the above problems, examination of the non-histone chromosomal proteins was still possible *via* sodium dodecyl sulfate gel electrophoresis. As shown in Figures 7–9, the non-histone proteins prepared by this method manifested major components of apparent molecular weights 72,000 and 150,000 for both immature and mature avian erythroid cell populations. As in the earlier preparations, qualitative and quantitative differences may be noted in the non-histone protein electrophoretic patterns from immature *vs.* mature erythroid cells (Figures 7–9).

Discussion

The goals of the studies presented here were essentially threefold: first, to provide a preliminary characterization of

the non-histone chromosomal proteins of avian erythroid cells; second, to compare these non-histone proteins after isolation by at least two independent methods in order to determine the extent to which the preparative methods themselves may influence the relative amounts of the various non-histones obtained; and finally, to compare the non-histone proteins obtained from immature *vs.* mature avian erythroid cells in order to ascertain whether changes in these proteins may accompany the process of cellular differentiation.

From both the SP-Sephadex columns and the sodium dodecyl sulfate polyacrylamide gels of the various protein preparations, it is apparent that in avian erythroid cells, the non-histone chromosomal proteins account for a relatively small proportion (<15%) of the total chromosomal proteins. As noted earlier, these proteins as a group are relatively acidic.

The sodium dodecyl sulfate gel patterns presented in Figures 3–9 indicate that avian erythroid cell non-histone chromosomal proteins consist of at least 16–26 polypeptide components and range in size from 12,500 to 150,000 daltons. When prepared according to the 3 M NaCl dissociation method (Graziano and Huang, 1971), both immature and mature erythroid cell non-histone proteins manifested a major component of molecular weight 72,000. When prepared according to the 6 M urea–0.4 M Gdn·HCl method (Levy *et al.*, 1972, 1973), the non-histone proteins of both cell populations manifested major components of 72,000 and 150,000 daltons. The above findings appear to be in disagreement with those of MacGillivray *et al.* (1972), who reported that after preparation by chromatography on hydroxylapatite, "most of the nonhistone proteins obtained from this tissue [mature duck erythrocytes] were of low molecular weight . . .," *i.e.*, in the range of 13,000–20,000 daltons. (It may be noted that the sodium dodecyl sulfate gel patterns of "duck erythrocyte non-histone proteins" presented by these workers look similar to our gel patterns for histones rather than non-histones. It is also interesting to note that our gel patterns for duck erythroid cell non-histones more closely resemble these workers' patterns for mouse kidney, liver, or brain non-histones than their own preparations for duck erythrocyte non-histones.)

While this manuscript was in preparation, Vidali *et al.* (1973) reported that changes in the phenol-soluble nuclear acidic proteins occur during the embryonic development of duck erythroid cells. Although these workers observed numerous high molecular weight nuclear acidic proteins (40,000–150,000 daltons), they obtained markedly different gel electrophoretic patterns from those presented herein. Such differences are not surprising, however, in view of the use of saline-washed nuclei in their preparations *vs.* solubilized chromatin in ours. [For example, Bhorjee and Pederson (1973) have demonstrated that HeLa nuclei contain some acidic proteins which are not found in isolated chromatin.] Furthermore, different preparative procedures were employed in each case.

Although a variety of methods are now available for the isolation of "non-histone chromosomal proteins" (Sanders, 1973), very little data are currently available on the extent to which the different preparative methods themselves might influence the relative amounts of various non-histones obtained from a given tissue. Our data suggest that the preparative methods employed have a significant influence on the final non-histone patterns obtained. As noted above, when avian erythroid cells are used as the source of non-histone chromosomal proteins, one method of isolation (Graziano and Huang, 1971) yields a major component of molecular weight 72,000, while the other method (Levy *et al.*, 1972, 1973) yields major components of molecular weights 72,000 and 150,000. Other differences between the final non-histone preparations are also apparent from inspection of Figures 3–9, and these appear to be readily reproducible. The attainment of such differences may be due (1) to the different methods used for the isolation of nuclei (see "Methods") and/or (2) to differential proteolytic activity occurring *in vitro* in the different isolation solutions employed in each case.[3]

Within each preparative procedure employed herein, the observed differences in the gel electrophoretic patterns of non-histone proteins from immature *vs.* mature erythroid cells could be due to several factors. First, to a certain extent these differences may reflect actual *in vivo* differences of the proteins present within the chromatin matrix. Second, the differences may reflect differential losses of particular non-histones occurring during the various chromatin isolation and washing procedures. Perhaps different degrees of chemical modification, *e.g.*, phosphorylation of serine and threonine residues (Gershey and Kleinsmith, 1969; Shelton and Neelin, 1971; Shelton *et al.*, 1972), and/or differences in the inherent chromatin structures (Kernell *et al.*, 1971) could account for such losses, if they indeed exist. Finally, the observed differences could be due to specific losses secondary to proteolysis occurring *in vivo* and/or *in vitro*. One could speculate that in the developing avian erythroid cell system, certain proteases might actively degrade particular non-histone proteins. Such proteases might themselves be subject to control by degradation or inactivation during the course of erythroid cell development, perhaps playing some role in the progressive turnoff of RNA synthesis which accompanies terminal differentiation. Clearly, further work is necessary to distinguish among the above possibilities, and studies along these lines will be performed in our laboratory in the future.

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[3] The absence of the 150,000-dalton component in the non-histones prepared by the method of Graziano and Huang (1971) does *not* appear to be due to the inability of 3 M NaCl to extract such a high molecular weight component, since examination of the 3 M NaCl-extracted DNA by sodium dodecyl sulfate gel electrophoresis (see "Methods") revealed negligible residual protein of molecular weight 150,000.

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