Ott, L. A., & Scandalios, J. G. (1978) *Genetics 89*, 137.
Reich, E., Rifkin, D. B., & Shaw, E., Eds. (1975) *Cold Spring Harbor Conf. Cell Proliferation 2*, 1.
Salmia, M. A., & Mikola, J. (1976) *Physiol. Plant. 38*, 73.
Scandalios, J. G. (1965) *J. Hered. 51*, 177.
Scandalios, J. G., & Espiritu, L. (1969) *Mol. Gen. Genet. 105*, 101.
Scandalios, J. G., & Campeau, M. A. (1972) *Mutat. Res. 14*, 397.
Schabort, J. C., Du Toit, P. J., & Kempff, P. G. (1978) *Int. J. Biochem. 9*, 161.
Shaw, E., Mares-Guia, M., & Cohen, W. (1965) *Biochemistry 4*, 2219.
Sipos, T., & Merkel, J. R. (1970) *Biochemistry 9*, 2766.
Tulley, R. E., & Beevers, H. (1978) *Plant Physiol. 62*, 746.
Vodkin, L. A., & Scandalios, J. G. (1979) *Plant Physiol. 63*, 1198.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Immunologically Specific Complexes of Chromosomal Nonhistone Proteins with Deoxyribonucleic Acid in Chicken Erythroid Nuclei†

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ABSTRACT: Erythroid cell-specific antisera capable of detecting chromosomal nonhistone protein–DNA complexes were obtained by injecting rabbits with dehistonized chicken reticulocyte chromatin. The specific antigenic nonhistone protein–DNA complexes were relatively inaccessible to the antiserum in isolated erythrocyte chromatin. However, isolation of chromatin from cells at earlier stages of erythropoiesis or treatment of isolated erythrocyte chromatin with polyanions or phenylhydrazine provided materials with significantly increased immunogolical reactivity. The altered activity was caused by changes in conformation occurring at two levels: a specific one, determined by chromosomal nonhistone proteins, and a more general one, determined by histones. Immunological examination of fractionated products obtained from limited nuclease digestion revealed the localization of the antigenic complexes in the nuclease-resistant, large fragments of erythroid chromatin. The nuclease-resistant DNA isolated from the immunologically reactive fragments migrated in gel electrophoresis as a diffuse band of between 1000 and 2000 base pairs. No preferential accumulation of globin-specifying DNA sequences could be found in this nuclease-resistant DNA. The protein fraction containing the immunologically cell-specific complexes in chicken erythrocyte chromatin was glycosylated and moderately acidic (by amino acid analysis) with an electrophoretically determined M_r of ~90 000.

Introduction of immunological methods to the studies on chromosomal proteins opened a new chapter in chromatin research. Antibodies to histones and other chromatin components are progressively applied to the investigations on chromatin structure and function (Bustin, 1978; Silver & Elgin, 1978). It was first shown by Spelsberg et al. (1971) that chromatin can be selectively dehistonized in concentrated salt–urea buffers at pH 6 or 5. When such dehistonized chromatins were used as immunogens, cell- and tissue-specific antibodies could be elicited (Chytil & Spelsberg, 1971; Wakabayashi & Hnilica, 1973). Presently, antibodies of various specificity to whole or dehistonized chromatin as well as to nuclear nonhistone protein fractions are being used to study the cell, species, and tumor specificity of the respective antigens [reviewed in Hnilica et al. (1978), Silver & Elgin (1978), and Hnilica & Briggs (1979)].

Immunization with dehistonized chicken reticulocyte chromatin produced two types of antisera. One, observed only rarely, was specific for reticulocyte chromatin and did not react significantly with dehistonized, fractionated, or sheared erythrocyte chromatin (Hardy et al., 1978). The other could be obtained more frequently and reacted extensively with chicken reticulocyte chromatin and also to a lesser, but significant, extent with isolated erythrocyte chromatin (Krajewska et al., 1979). The protein component of the nonhistone protein–DNA complex responsible for the latter reaction specificity was isolated and partially characterized. Examination of the immunological reactivity and accessibility of such complexes revealed information about their likely intrachromosomal localization as related to some general features of chromatin organization in maturing, late stage chicken erythroid cells.

Experimental Procedures

Materials. Adult male chickens (Leghorn, Dekalb strain) were made anemic by daily injections of 1% neutralized phenylhydrazine (10 mg/kg), followed by daily bleeding until polychromatic primitive erythrocytes represented at least 95% of the total circulating red blood cells (Hardy et al., 1978). In a separate schedule, anemia (70% of polychromatic primitive erythrocytes) was obtained by extensive daily bleeding alone. Mature erythrocytes were obtained by cardiac puncture or by decapitation of the untreated animals. Blood was collected in ice-cold 0.15 M saline–15 mM sodium citrate solution containing 0.01% heparin. Erythroid nuclei were prepared either by the method of Evans & Lingrel (1969) with the addition of 0.5% Triton X-100 wash or by nitrogen cavitation (Shelton et al., 1976). Both methods gave essentially identical results. Isolated nuclei were purified by centrifugation through 1.8 M sucrose in 50 mM Tris-HCl,[1] pH 7.4, 24 mM KCl, 5

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mM $MgCl_2$, and 0.2 mM $CaCl_2$ (TKMC buffer) at 17000*g* for 30 min, and chromatin was isolated by following the procedure of Spelsberg & Hnilica (1971).

Chromatin Treatment and Digestion. Isolated chromatin was exposed in vitro to neutralized phenylhydrazine, dextran sulfate, heparin, or poly(styrenesulfonate) (all 400 μg/1000 μg of chromatin DNA), and the excess of unreacted reagents was removed by ultracentrifugation. The treated chromatins were then used in complement fixation assays. Chromatin dehistonization was accomplished by stirring chromatin (250–300 μg/mL as DNA) in 2.5 M NaCl, 5 M urea, 0.1 M sodium phosphate buffer, pH 6.0, and 0.1 mM PMSF at 0–4 °C for 6 h and by centrifugation at 100000*g* for 36 h. The pellets containing nonhistone proteins and DNA were separated from the histone-containing supernatants and used either for immunization or in reconstitution experiments. For reconstitution, chromatin components or chicken DNA purified by the modified procedure of Marmur (1961) (Spelsberg & Hnilica, 1971) were mixed with the appropriate proteins (protein/DNA = 0.4:1.0), all dissolved in 2.5 M NaCl, 5.0 M urea, 50 mM Tris-HCl buffer, and 0.1 mM PMSF, pH 8.0. The mixture was then slowly dialyzed against buffered 5.0 M urea and finally against 1.5 mM NaCl–0.15 mM sodium citrate (100 × diluted SSC).

A modified procedure of Solner-Webb & Felsenfeld (1975) was used for the digestion with micrococcal nuclease (Worthington Biochemicals Corp.). Various amounts of nuclease per milligram of DNA in 10 mM Tris-HCl, 40 mM NaCl, and 1 mM $CaCl_2$, pH 7.5, at a concentration of 0.1–1.0 mg of DNA/mL were incubated at 37 °C for specified time intervals. The reaction was terminated by making the mixtures 10 mM in respect to EDTA. After being chilled in ice, the mixtures were centrifuged at 3000*g* for 10 min to separate the digested fraction from the nuclease-resistant chromatin.

For nucleosome fractionations, 30 units of micrococcal nuclease was incubated with 1 mg of DNA (as chromatin) for 15 min, and the reaction was terminated with EDTA and centrifuged as indicated above. The nuclease digestion products were then fractionated on Bio-Gel A-50 M columns (100 × 2.5 cm) by using 10 mM Tris-HCl, 0.7 mM EDTA, and 0.1 mM PMSF, pH 7.5, for elution (Ramsay-Shaw et al., 1976).

Chromosomal Protein Fractionation. Chromatin, dehistonized according to the procedure of Spelsberg et al. (1971), was dissociated with a 2% sodium dodecyl sulfate ($NaDodSO_4$) solution in 50 mM sodium phosphate buffer, pH 7.0, and the solution was centrifuged at 110000*g* for 36 h. The upper three-fourths of the protein-containing supernatant was carefully decanted from the DNA-containing pellets, concentrated, and separated by preparative polyacrylamide gel electrophoresis using the system described by Weber & Osborn (1969). Unstained gels were cut according to stained patterns, and protein fractions were eluted from the cut gels by electrophoresis and freed of $NaDodSO_4$ by ion-exchange chromatography (Fujitani et al., 1978). The recovered protein fractions were reconstituted with purified chicken DNA and assayed by microcomplement fixation using antiserum to dehistonized chicken reticulocyte chromatin.

[1] Abbreviations used: PMSF, phenylmethanesulfonyl fluoride; SSC, standard saline citrate; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate.

Polyacrylamide Gel Electrophoresis. Protein samples were dialyzed against 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 8 M urea, and 10 mM sodium phosphate, pH 7.0, and electrophoresed according to the method of Weber & Osborn (1969) with the addition of a 3% stacking gel to allow for a clear separation of proteins in the presence of DNA (Briggs et al., 1976). After electrophoresis, the gels were stained with Coomassie brilliant blue. DNA was extracted from the micrococcal nuclease digests with phenol and electrophoresed in 1.6% agarose gels using *Hae*II and *Hae*III fragments of E1 DNA as markers (Tomizawa et al., 1977; Oka & Takanami, 1976).

Preparation of Globin mRNA and cDNA. Polysomal RNA was isolated from chicken reticulocytes lysed in 1 mM $MgCl_2$ (Evans & Lingrel, 1969), and the globin mRNA was purified by repeated fractionation on oligo(dT)–cellulose columns (Bantle et al., 1976). The purified mRNA migrated in $NaDodSO_4$–polyacrylamide gel as a single band of ~10.5 S.

Globin [^{3}H]cDNA was synthesized according to Friedman & Rosbach (1977) in 0.5 mL of reaction mixture which contained 50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 6 mM $MgCl_2$, 20 mM dithiothreitol, 100 μg/mL actinomycin D (Sigma Chemical Co.), 5 μg/mL oligo(dT) 12–18 (Collaborative Research), 400 units/mL reverse transcriptase from avian myeloblastosis virus, and 15 μg/mL globin mRNA. [^{3}H]dCTP, [^{3}H]dTTP, [^{3}H]dATP, and cold dGTP were used to prepare the cDNA to a specific activity of 2.5×10^4 cpm/ng. After 30 min of incubation at 37 °C, the cDNA was extracted 3 times with 0.5% sarcosyl, 5 mM EDTA, and phenol, and the aqueous phase was treated with 0.3 M NaOH for 17 h at 37 °C and neutralized with 1 N HCl. Finally, the solution was passed through a Sephadex G-50 column (0.9 × 30 cm) preequilibrated with 1 M NaCl, 0.2 mM EDTA, and 40 mM Tris-HCl, pH 7.5. The void volume fractions were pooled and used in hybridization experiments.

The hybridization of DNA fragments obtained from nuclei digested with micrococcal nuclease with globin [^{3}H]cDNA was performed in 10 mM Tris-HCl, 0.3 M NaCl, 0.1 mM EDTA, and 0.1% $NaDodSO_4$, pH 7.4. Individual DNA preparations were mixed with cDNA at a ratio of cDNA/DNA = 1:300 000, and 20-μL aliquots were sealed in glass capillaries, denatured at 100 °C, and incubated for various time intervals at 60 °C. After incubation, the capillaries were emptied into 30 mM sodium acetate, 50 mM NaCl, and 1 mM $ZnSO_4$, pH 4.5, containing heat-denatured calf thymus DNA at 30 μg/mL, and the mixtures were incubated with and without S1 nuclease at 45 °C for 30 min. After this time the assays were terminated by adding 1 mL of 10% trichloroacetic acid and 5% sodium pyrophosphate, and the precipitated DNA was collected on Millipore filters for the determination of radioactivity.

Antisera and Immunoassays. Dehistonized chicken reticulocyte chromatin was dissolved in 2 mM Tris-HCl buffer, pH 7.5, and used to immunize white New Zealand rabbits. The immunization schedule of Chytil & Spelsberg (1971) was followed. Blood was collected by marginal ear vein bleeding 7 days after the booster injection. All sera were heat inactivated at 56 °C for 30 min.

The quantitative complement fixation assay of Wasserman & Levine (1961) was used to test the immunological activity of the chromatin preparations. Washed sheep red blood cells (GIBCO diagnostic) were activated with antisheep red blood cell serum (Capell Laboratories). Guinea pig serum complement was titrated to give 100% red blood cell lysis after 30 min of incubation at 37 °C. Various chromatin dilutions

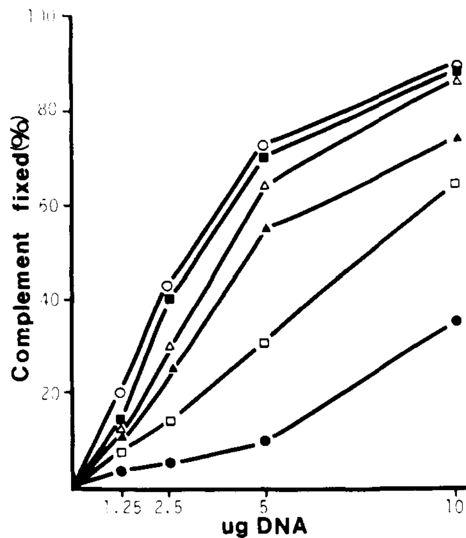

FIGURE 1: Effects of chromatin treatment with various polyanions and phenylhydrazine. The complement fixation assays were performed in the presence of 1:100 diluted antiserum to dehistonized chicken reticulocyte chromatin. Isolated erythrocyte chromatins were treated as indicated under Experimental Procedures. Reticulocyte (■) and erythrocyte (●) untreated control chromatins. Chromatins treated with dextran sulfate (○), heparin (△), poly(styrenesulfonate) (□), and phenylhydrazine (▲).

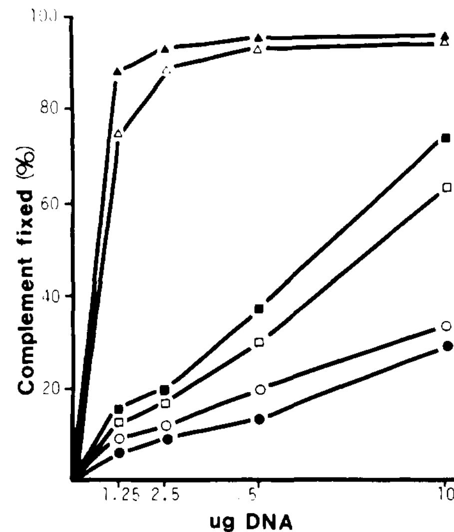

FIGURE 2: Effects of chromatin dehistonization and histone reconstitution on complement fixation activity in the presence of antiserum (dilution 1:100) to dehistonized chicken reticulocyte chromatin. Dehistonized reticulocyte (▲) and erythrocyte (△) chromatins. Dehistonized reticulocyte chromatin reconstituted with reticulocyte (■) and erythrocyte (□) histones. Dehistonized erythrocyte chromatin reconstituted with erythrocyte (●) and reticulocyte (○) histones.

were incubated for 18 h at 4 °C in the presence of titrated complement with 0.1 mL of antiserum diluted 1:100. Activated sheep red blood cells were added, and, after 30 min of incubation at 37 °C, the extent of red blood cell lysis was determined spectrophotometrically at 413 nm. For each new chromatin preparation, a standard amount of chromatin was incubated with subsequent dilutions of antiserum to determine the optimal titers. All assays were tested for anticomplementarity.

Analytical Procedures. Protein, DNA, and RNA concentrations were respectively determined by the methods of Lowry et al. (1951), Burton (1956), and Mejbaum (1939). The DNA concentration in normal, dehistonized, and reconstituted chromatins was determined spectrophotometrically at 260 nm. Protein samples for amino acid analysis were dialyzed against deionized water, lyophilized, suspended in 5.5 N HCl, sealed under vacuum, and hydrolyzed at 110 °C for 24 h. The hydrolyzed samples were evaporated in vacuo and analyzed with a JEOL JLC-6 AH amino acid analyzer.

Results

The specificity of our antisera was assayed by complement fixation in the presence of intact or dehistonized chromatins. In accord with our previous experience (Krajewska et al., 1979), antibodies to dehistonized chicken reticulocyte chromatin did not react immunologically with intact or dehistonized chromatins isolated from various nonerythroid tissues of domestic fowl or other vertebrate species. Complement fixation observed with intact chicken erythrocyte chromatin was only marginal, indicating that considerable qualitative or quantitative changes might have occurred in erythroid chromatin during its final maturation.

Chromatin conformation may play a considerable role in its reactivity to antisera directed to relatively bulky complexes of DNA and specific chromosomal nonhistone proteins. Isolated erythrocyte chromatin was exposed in vitro to selected polyanions as well as phenylhydrazine and Isoniazid (isonicotinic acid hydrazide) to test the effects of chromatin condensation during erythroid maturation on its immunological activity with antisera to dehistonized reticulocyte chromatin. These compounds are known to effect a marked decondensation of the treated chromatin which was accompanied by increased immunological activity. As illustrated in Figure 1, poly(styrenesulfonate) was least active, followed by phenylhydrazine, Isoniazid (data not shown), dextran sulfate, and heparin. These last two polyanions produced erythrocyte chromatins similar in their immunological reactivity to that of the reticulocytes. Immunoabsorbtion of the diluted reticulocyte antiserum with untreated erythrocyte chromatin removed all its complement fixing activity, indicating that the low reactivity of erythrocyte chromatin with the reticulocyte antiserum was principally caused by limited accessibility and not by qualitative changes of the antigenic protein–DNA complexes.

Both the Histones and Chromosomal Nonhistone Proteins Influence the Extent of Chromatin Condensation. Although the exact mode of action of polyanions on chromatin is not clear, it appears that histone displacement or even dissociation from DNA may be involved (Ansevin et al., 1975; Taylor & Cook, 1977; Adolph et al., 1977a). The possibility of histone displacement by polyanions employed in our experiments is supported by dehistonization experiments. As can be seen in Figure 2, removal of histones by extraction with concentrated NaCl and urea at pH 6.0 made the residual chromatins of both the chicken reticulocytes and erythrocytes much more reactive and abolished any differences in complement fixation between these two cell types. However, since the polyanions at a ratio of 400 μg/1000 μg of DNA (as chromatin) increased the immunological activity of erythrocyte chromatin only to the level of reticulocyte chromatin, we conclude that little, if any, histones became actually dissociated. This conclusion is supported by our findings that only small amounts of histones could be detected in the high-speed centrifugation supernatants of chromatins exposed to these relatively high concentrations of polyanions.

Furthermore, when the erythrocyte histones were added back to either erythrocyte or reticulocyte dehistonized chromatin (and vice versa), the reconstitution products exhibited immunological reactivity determined by the origin of the de-

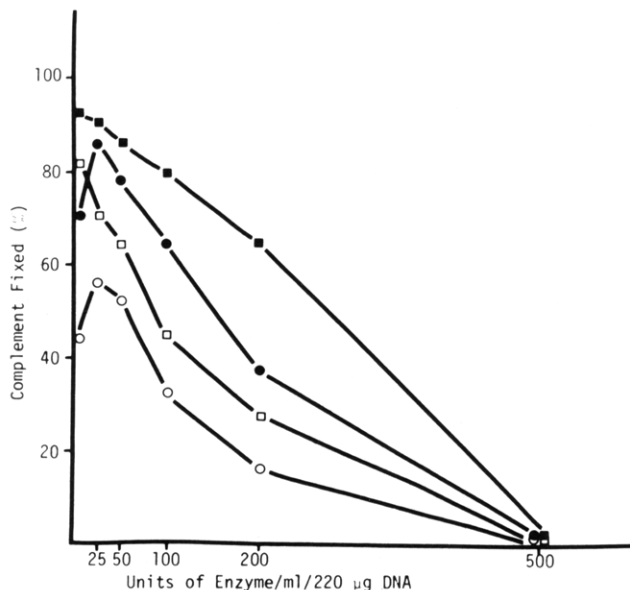

FIGURE 3: Digestion of chicken erythroid chromatin with micrococcal nuclease. The experimental conditions are described in the text. All complement fixation assays were performed in the presence of antiserum (dilution 1:100) to dehistonized chicken reticulocyte chromatin and 2.5 μg (○) or 5 μg (●) of erythrocyte chromatin DNA as well as 2.5 μg (□) or 5 μg (■) of reticulocyte chromatin DNA.

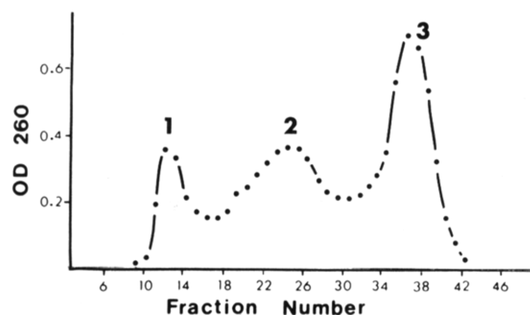

FIGURE 4: Gel filtration on Bio-Gel A-50m of chicken erythrocyte chromatin digested with micrococcal nuclease (30 units/mg of DNA for 15 min).

histonized chromatin. In other words, the erythrocyte histones did not suppress the immunological activity of the reconstituted chromatins to the level found for erythrocytes. These findings indicate some specific involvement of chromosomal nonhistone proteins in chromatin condensation.

Associations of Cell-Specific Complexes of Chromosomal Nonhistone Proteins and DNA with Structural Elements of Chromatin. Since the immunological specificity could be observed only when nonhistone proteins were associated with DNA and not when pure DNA or nonhistone proteins were assayed alone (Krajewska et al., 1979), it was decided to study the antigenic complexes in context with chromatin structure as revealed by digestion with nucleases. Extensive digestion with nucleases completely abolished the complement fixation activity of reticulocyte chromatins (Figure 3). Interestingly, exposure of erythrocyte chromatin to small amounts of nuclease (25 or 50 units/220 μg of DNA) resulted in its initial decondensation as manifested by an increased immunological activity (Figure 3). The relationship between the extent of complement fixation and chromatin digestion as measured by the amount of nuclease used per unit of DNA was nearly linear for both the erythrocytes and reticulocytes.

Digestion of erythroid cell nuclei with micrococcal nuclease (30 units/mg of DNA for 15 min) released ~50% of the nuclear DNA into a form which did not sediment at 3000g

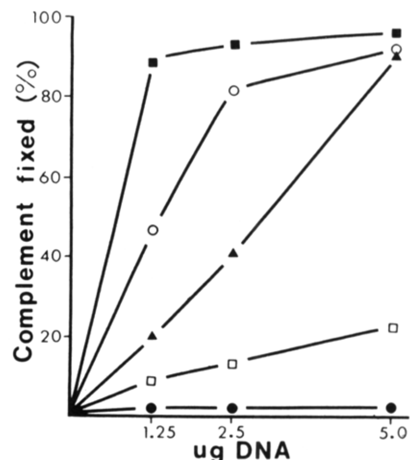

FIGURE 5: Complement fixation of materials recovered from peaks 1, 2, and 3 (Figure 4) of chicken erythrocyte nuclei digested with micrococcal nuclease for 15 min. The complement fixation assays were performed in the presence of antiserum to dehistonized chicken reticulocyte chromatin (1:100 dilution). Intact nuclei (○), unfractionated nuclease released material (▲), and peaks 1 (■), 2 (□), and 3 (●) from Figure 4.

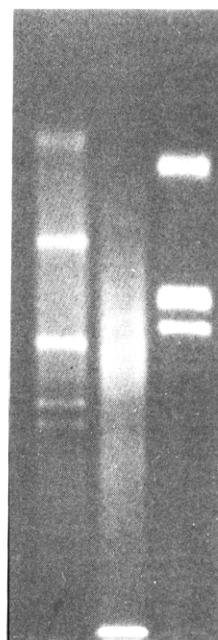

FIGURE 6: Polyacrylamide gel electrophoresis of DNA recovered from peak 1 (15-min digestion with micrococcal nuclease fractionated as shown in Figure 4). *Hae*III and *Hae*II fragments of *Escherichia coli* E1 DNA are used as markers. Lane 1, *E. coli* DNA digested with *Hae*III; lane 2, Chicken DNA fragments recovered from peak 1 in Figure 4; lane 3, *E. coli* digested with *Hae*II.

for 10 min. Separation of the released fragments on Bio-Gel A-50 columns resulted in three well-resolved peaks (Figure 4). Over 90% of the immunological activity was associated with the high molecular weight material (peak 1 in Figure 4) as documented by the complement fixation assays (Figure 5). This suggests either that the antigenic complexes require for their immunological activity DNA segments longer than those associated with the oligonucleosomes present in the second Bio-Gel A-50m peak or, perhaps more likely, that they are protected from the nuclease action. Polyacrylamide gel electrophoresis showed these DNA fragments to be represented by a diffuse band of between 1000 and 2000 base pairs with the mean value of ~1300 base pairs (Figure 6). The fractions of DNA fragments resulting from the Bio-Gel A-50 columns (Figure 4) did not exhibit any specificity in terms of the

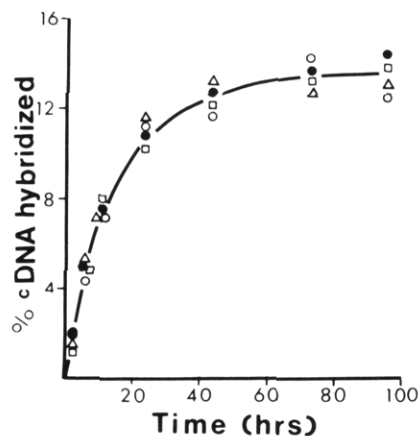

FIGURE 7: Hybridization of globin cDNA to DNA preparations isolated from total erythrocyte nuclear DNA (●), DNA from micrococcal nuclease resistant material (○), DNA from nuclease-solubilized material (□), and DNA from peak 1 or peak 2 (△) of the nuclease-solubilized material (15 min) separated by gel filtration as shown in Figure 4. Only one symbol is used for these two DNA preparations since their hybridization kinetics were essentially identical.

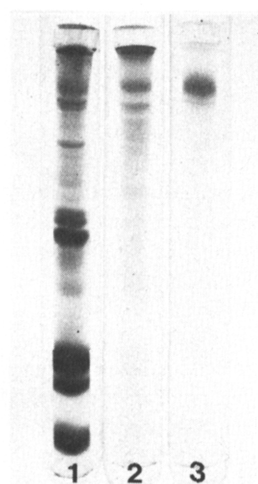

FIGURE 8: Polyacrylamide gel electrophoresis (in the presence of $NaDodSO_4$) of the residual chromosomal nonhistone proteins of chicken erythroid chromatin. Lane 1, total proteins of chicken erythrocyte chromatin; lane 2, proteins remaining with DNA after dehistonization of the immunologically active 200 mM potassium phosphate–5 M urea (pH 7.8) pellets; lane 3: immunologically active (after reconstitution with chicken DNA) protein fraction of erythroid chromatin. This fraction was obtained by preparative polyacrylamide gel electrophoresis.

distribution of nucleotide sequences complementary to the chicken reticulocyte globin mRNA (Figure 7). Addition of purified chicken DNA to fractions 2 and 3 of Figure 4 did not affect their immunological activity.

Partial Characterization of Protein Component(s). The solubility of the protein(s) comprising the immunologically active complexes was investigated by a series of extractions of erythrocyte chromatin with buffered urea of increasing ionic strength. Subsequent extractions of chromatin with 5 M urea in 15, 100, and 200 mM potassium phosphate buffers, pH 7.8, removed most chromosomal proteins. However, as determined by complement fixation, these extracts contained very little immunological activity, indicating that the bulk of the active nonhistone protein–DNA complexes have remained with the 200 mM potassium phosphate pellet. This pellet consisted of histones and high molecular weight nonhistone proteins. Removal of the histones with 2.5 M NaCl–5 M urea–sodium succinate buffer, pH 5.0, yielded a chromosomal nonhistone

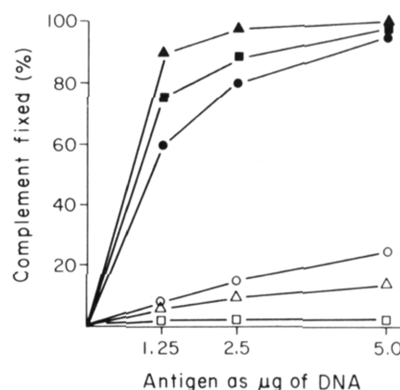

FIGURE 9: Complement fixation of protein fractions from chicken erythrocyte chromatin shown in Figure 8. Pellet after extraction of chromatin with 5 M urea–200 mM potassium phosphate buffer, pH 7.8 (●); the above pellet after dehistonization (lane 2 in Figure 8) (■); protein fraction (lane 3 in Figure 8) isolated by preparative electrophoresis of proteins from the dehistonized 5 M urea–200 mM potassium phosphate fraction (lane 2 in Figure 8) and reconstituted with purified chicken DNA (▲); the above protein fraction (lane 3 in Figure 8) without DNA and also DNA alone (□); proteins (after reconstitution with chicken DNA) recovered from the upper (high portion of polyacrylamide gel above the M_r 90 000 protein band (○); proteins (after reconstitution with chicken DNA) recovered from the lower (low M_r) portion of polyacrylamide gel under the M_r 90 000 protein band (△). The immunoassays were performed in the presence of antiserum to dehistonized chicken reticulocyte chromatin (1:100 dilution).

Table I: Amino Acid Composition of Antigenic Protein (M_r 90 000) of Chicken Erythroid Chromatin

amino acid[a]	recovery[b] (mol/100 mol recovered)	amino acid[a]	recovery[b] (mol/100 mol recovered)
lysine	6.9	alanine	7.4
histidine	2.2	cysteine	ND[c]
arginine	6.2	valine	6.9
aspartic acid	9.2	methionine	ND[c]
threonine	6.8	isoleucine	3.8
serine	9.5	leucine	11.6
glutamic acid	11.5	tyrosine	0.7
proline	3.4	phenylalanine	3.8
glycine	5.3		

[a] Tryptophan was not determined. [b] No corrections were made for hydrolytic losses. [c] ND, not detected.

protein fraction which could be dissociated from the DNA by extraction with a 2% buffered $NaDodSO_4$ solution (Figure 8). These proteins, when reconstituted with chicken DNA, were highly active in complement fixation assays with chicken reticulocyte antiserum (Figure 9). Preparative electrophoresis revealed that the immunological activity resided with a relatively prominent protein band of M_r ~90 000 (Figures 8 and 9). The somewhat diffuse appearance of this electrophoretic band indicates that more than one protein species may be present in this fraction. Positive staining of this protein fraction with periodic acid–Schiff reagent (Zacharius et al., 1969) suggested that it may consist of glycoproteins. Conceivably, the variable extent of glycosylation of this 90 000 M_r protein may explain the diffuse appearance of this protein band in gels stained with Coomassie blue (Figure 8). The amino acid composition of the 90 000 M_r protein fraction is shown in Table I.

Discussion

The antigenicity of nuclear nonhistone proteins from higher animals has been established by several investigators. Nuclear

nonhistone antigens, soluble in 0.6 M NaCl, designated as NAg 1, 2, 3, A, B1, and B2 were isolated, purified, and characterized by Yeoman et al. (1976, 1978). Antisera of considerable specificity were also obtained by immunizing animals with isolated nucleoli or nucleolar extracts (Davis et al., 1978; Marashi et al., 1979), and antibodies to electrophoretically separated nuclear protein antigens were used in selective immunolocalization experiments on *Drosophila* chromosomes by several investigators (Alfageme et al., 1976; Silver & Elgin, 1978; Mayfield et al., 1978).

The system described in this paper differs from the aforementioned antigens in that it requires for its immunological specificity the interaction of chromosomal nonhistone proteins with DNA. Based on our experiments with partially purified antigens, the formation of immunologically active complexes involves considerable interaction specificity (Wakabayashi & Hnilica, 1973; Chiu et al., 1974; Wang et al., 1976). Within a given species, the immunological cell or tissue specificity of antisera to dehistonized chromatin preparations depends on the cellular origin of the protein component (Chiu et al., 1974). Differentiation (Chytil & Spelsberg, 1971; Spelsberg et al., 1973; Chytil et al., 1974; Hnilica et al., 1978; Hnilica & Briggs, 1979) as well as neoplasia (Wakabayashi & Hnilica, 1973; Chiu et al., 1975, 1977) changed the cell and tissue specificity of chromosomal nonhistone protein–DNA complexes as evidenced by complement fixation assays.

The chicken erythroid chromatin system employed in our recent experiments has several advantages. It represents a uniform population of cells which undergo progressive maturation accompanied by considerable condensation and heterochromatinization of the nuclear content. Additionally, the erythroid nuclei are engaged in the transcription of only one principal mRNA species. In contrast to some other tissues (e.g., rat liver), antibodies to dehistonized chicken erythroid could be obtained relatively easily with most rabbits responding to the immunological challenge. These antisera did not cross-react with chromatins of other cell types, but they recognized erythrocyte chromatins of other avian species related to domestic fowl (Krajewska et al., 1979).

Since dehistonization causes almost complete relaxation of chromatin structure, it can be anticipated that immunization of rabbits with dehistonized chromatin will elicit antisera mainly to antigens which were not denatured by the concentrated solutions of urea and NaCl used for dehistonization. Our finding that intact reticulocyte chromatin was less reactive in the complement fixation assays than dehistonized chromatin agrees with the assumption that many of the antigenic sites recognized by our antisera become either altered or inaccessible during the assembly of nucleosomes and chromatin supercoiling. Further condensation of chromatin during the final stages of erythroid maturation resulted in almost complete masking of the determinants recognized by the reticulocyte antiserum. This decrease in activity could be significantly reversed by treating isolated nuclei or chromatin with polyanions or hydrazine derivatives. It is well documented in the literature that exposure of nuclei or chromatin to dextran sulfate, heparin, or other polyanions produces their marked decondensation accompanied by a significant increase in transcriptional activity (Chambon et al., 1968; Kraemer & Coffey, 1970; Berlowitz et al., 1972; Kitzis et al., 1976; Taylor & Cook, 1977). Although not entirely understood, chromatin activation with polyanions most likely results from the displacement, but not complete dissociation, of the H1, the core histones (Ansevin et al., 1975; Kitzis et al., 1976; Taylor & Cook, 1977), and perhaps other proteins as well. The chromatin activation effect of hydrazine derivatives has not been described in the literature and may result from chemical modification of proteins which maintain the supercoiling of chromatin. It is noteworthy that, although the histones are necessary for reforming the principal chromatin structure, the extent to which chromatin condensation (as determined by its immunological reactivity) will proceed seems to depend on the presence of some chromosomal nonhistone proteins (Figure 2).

The nucleosomal organization (beads on a string) of chromatin provides information only about its most essential, first-order structure. Electron microscopy and other analytical methods show the nucleosomes are further organized into more complicated structures of which detailed architecture is not yet understood. Dehistonization and partial deproteinization of metaphase chromosomes shows the presence of a protein-rich "scaffold" from which DNA extends in the form of 10–30 μm long (30–90 kilobases) loops (Paulson & Laemmli, 1977). This observation, supported by the findings of other investigators (Benyajati & Worcel, 1976; Igo-Kemenes et al., 1977; Wu et al., 1979a,b; Renz, 1979), indicates that DNA in chromatin and chromosomes may be organized in domains of variable length from a few to tens and perhaps hundreds of kilobases. These domains can be cleaved with some selectivity from the chromosomal scaffold with DNase I and less specifically with micrococcal nuclease (Wu et al., 1979a,b; Adolph et al., 1977a,b). The exact manner in which these domains are connected and arranged to form meaningfully functional structures is not known. However, the experiments of Laemmli and co-workers (Paulson & Laemmli, 1977; Adolph et al., 1977a,b; Marsden & Laemmli, 1979) show that DNA loops of the individual domains are attached to a protein-rich backbone (scaffold). Extraction of HeLa metaphase chromosomes with polyanions such as dextran sulfate and heparin removes all the histones and most of the nonhistone proteins, leaving these characteristic structural cores or chromosomal scaffolds. These scaffolds were shown by the authors to consist of chromosomal nonhistone proteins and DNA. Micrococcal nuclease digestion of intact HeLa chromosomes prior to their extraction to remove histone and most nonhistone proteins did not affect substantially the structure of the scaffolds as seen under an electron microscope. This indicates that the scaffold may not be held together by DNA. Our observations on nuclease digestion and dehistonization of chicken erythroid chromatin as described in this paper are consistent with the localization of the immunologically cell-specific protein–DNA complexes within the chromosomal organizational unit such as scaffolds. Our observation that the nuclease-resistant fraction of erythroid nuclei or chromatin became actually enriched in the antigenic complexes as compared to the total chromatin points out that the antigenic complexes may be protected from the action of nucleases. This protection afforded either by structural features of the chromosomal scaffold or by protein–DNA association resulted in the accumulation of nuclease-resistant DNA fragments of 1 to 2 kilobases. There appears to be no correlation between these protected DNA fragments and the transcriptionally active genes (globin).

It is obvious that specific antisera to chromosomal nonhistone proteins and their complexes with DNA are potentially useful tools for probing the chromatin structure on both the biochemical and ultrastructural level. However, the individual antigens must be well-characterized and available in workable quantities. In accord with these goals, we have identified a moderately acidic nuclear protein fraction which migrated in

NaDodSO$_4$–polyacrylamide gel electrophoresis with an M_r of ~90 000 and which binds rather tenaciously to chicken DNA as the one containing the protein component of the erythroid cell-specific chromosomal protein–DNA complex. The somewhat diffuse appearance of this fraction in polyacrylamide gels suggests either that it may contain more than one protein species or that its protein component may be chemically modified to a variable extent. Investigations in progress should elucidate the character of these immunologically specific protein–DNA complexes.

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References

Adolph, L. W., Cheng, S. M., & Laemmli, U. K. (1977a) *Cell (Cambridge, Mass.) 12*, 805–810.

Adolph, K. W., Cheng, S. M., Paulson, J. R., & Laemmli, U. K. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4937–4941.

Alfageme, C. R., Rudkin, G. T., & Cohen, L. H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2038–2042.

Ansevin, A. T., MacDonald, K. K., Smith, C. E., & Hnilica, L. S. (1975) *J. Biol. Chem. 250*, 281–289.

Bantle, J. A., Maxwell, I. H., & Hahn, W. E. (1976) *Anal. Biochem. 72*, 413–427.

Benyajati, C., & Worcel, A. (1976) *Cell (Cambridge, Mass.) 9*, 393–407.

Berlowitz, L., Kitchin, R., & Pallotta, D. (1972) *Biochim. Biophys. Acta 262*, 160–168.

Briggs, R. C., Rothstein, H., & Wainwright, N. (1976) *Exp. Cell Res. 99*, 95–105.

Burton, K. (1956) *Biochem. J. 62*, 315–323.

Bustin, M. (1978) *Cell Nucl. 4*, 195–238.

Chambon, P., Ramuz, M., Mandel, P., & Doly, J. (1968) *Biochim. Biophys. Acta 156*, 504–519.

Chiu, J. F., Craddock, C., Morris, H. P., & Hnilica, L. S. (1974) *FEBS Lett. 42*, 94–97.

Chiu, J. F., Hunt, M., & Hnilica, L. S. (1975) *Cancer Res. 35*, 913–919.

Chiu, J. F., Hnilica, L. S., Chytil, F., Orrahood, J. T., & Rogers, L. W. (1977) *J. Natl. Cancer Inst. 59*, 151–154.

Chytil, F., & Spelsberg, T. C. (1971) *Nature (London), New Biol. 233*, 215–218.

Chytil, F., Glasser, S. R., & Spelsberg, T. C. (1974) *Dev. Biol. 37*, 295–305.

Davis, F. M., Busch, R. H., Yeoman, L. C., & Busch, H. (1978) *Cancer Res. 38*, 1906–1915.

Evans, M. J., & Lingrel, J. B. (1969) *Biochemistry 8*, 3000–3005.

Friedman, E. Y., & Rosbash, M. (1977) *Nucleic Acids Res. 4*, 3455–3471.

Fujitani, H., Chiu, J. F., & Hnilica, L. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1943–1946.

Hardy, K., Chiu, J. F., Beyer, A. L., & Hnilica, L. S. (1978) *J. Biol. Chem. 253*, 5825–5836.

Hnilica, L. S., & Briggs, R. C. (1979) in *Cancer Markers: Developmental and Diagnostic Significance* (Sell, S., Ed.) pp 463–483, Humana Press, Inc., Clifton, NJ.

Hnilica, L. S., Chiu, J. F., Hardy, K., Fujitani, H., & Briggs, R. C. (1978) *Cell Nucl. 5*, 307–331.

Igo-Kemenes, T., Greil, W., & Zachau, H. G. (1977) *Nucleic Acids Res. 4*, 3387–3400.

Kitzis, A., Defer, N., Dastugue, B., Sabatier, M. M., & Kruh, J. (1976) *FEBS Lett. 66*, 336–339.

Kraemer, R. J., & Coffey, D. S. (1970) *Biochim. Biophys. Acta 224*, 568–578.

Krajewska, W. M., Briggs, R. C., & Hnilica, L. S. (1979) *Biochemistry 18*, 5720–5725.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 266–275.

Marashi, R., Davis, F. M., Busch, R. K., Savage, H., & Busch, H. (1979) *Cancer Res. 39*, 59–66.

Marmur, J. (1961) *J. Mol. Biol. 3*, 208–218.

Marsden, M. P. F., & Laemmli, U. K. (1979) *Cell (Cambridge, Mass.) 17*, 849–858.

Mayfield, J. E., Serunian, L. A., Silver, L. M., & Elgin, S. C. R. (1978) *Cell (Cambridge, Mass.) 14*, 539–544.

Mejbaum, W. (1939) *Hoppe-Seyler's Z. Physiol. Chem. 258*, 117–120.

Oka, A., & Takanami, M. (1976) *Nature (London) 264*, 193–196.

Paulson, J. R., & Laemmli, U. K. (1977) *Cell (Cambridge, Mass.) 12*, 817–828.

Ramsay-Shaw, B., Herman, T. M., Kovacic, R. T., Breudreau, G. S., & Van Holde, K. E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 505–509.

Renz, M. (1979) *Nucleic Acids Res. 6*, 2761–2767.

Shelton, K. R., Cobbs, C. S., Povlishock, J. T., & Burkat, R. K. (1976) *Arch. Biochem. Biophys. 174*, 177–186.

Silver, L. M., & Elgin, S. C. R. (1978) *Cell Nucl. 5*, 215–262.

Solner-Webb, B., & Felsenfeld, G. (1975) *Biochemistry 14*, 2915–2920.

Spelsberg, T. C., & Hnilica, L. S. (1971) *Biochim. Biophys. Acta 228*, 202–211.

Spelsberg, T. C., Hnilica, L. S., & Ansevin, A. T. (1971) *Biochim. Biophys. Acta* 228, 550–562.

Spelsberg, T. C., Mitchell, W. M., Chytil, F., Wilson, E. M., & O'Malley, B. W. (1973) *Biochim. Biophys. Acta 312*, 765–778.

Taylor, W. R., & Cook, R. T. (1977) *FEBS Lett. 78*, 321–325.

Tomizawa, J., Ohmori, H., & Bird, R. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1865–1869.

Wakabayashi, K., & Hnilica, L. S. (1973) *Nature (London), New Biol. 242*, 153–155.

Wang, S., Chiu, J. F., Klyszejko-Stafanowicz, L., Fujitani, H., & Hnilica, L. S. (1976) *J. Biol. Chem. 251*, 1471–1475.

Wasserman, E., & Levine, L. (1961) *J. Immunol. 87*, 290–295.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4411.

Wu, C., Bingham, P. M., Livak, K. J., Holmgren, R., & Elgin, S. C. R. (1979a) *Cell (Cambridge, Mass.) 16*, 797–806.

Wu, C., Wong, Y. C., & Elgin, S. C. R. (1979b) *Cell (Cambridge, Mass.) 16*, 807–814.

Yeoman, L. C., Jordan, J. J., Busch, R. K., Taylor, C. W., Savage, H. E., & Busch, H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3258–3262.

Yeoman, L. C., Woolf, L. M., Taylor, C. W., & Busch, H. (1978) *Biol. Markers Neoplasia: Basic Appl. Aspects [Int. Conf.]*, 409–418.

Zacharius, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969) *Anal. Biochem. 30*, 148–152.