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Cell-Specific Antigens in Chicken Erythroid Nuclei: Species Specificity†

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ABSTRACT: Antisera raised to dehistonized chicken reticulocyte chromatin were tested for their cell and species specificity. Quantitative microcomplement fixation and immunohistochemical localization revealed the presence in chromatin of erythroid cell-specific nonhistone protein antigen(s). The antigenic specificity was shown to depend on the association of the antigenic protein(s) with deoxyribonucleic acid (DNA). Although the antisera were exceptionally cell specific, they cross-reacted with erythroid cells of other avian species. The extent of cross-reactivity was found to approximate the phylogenetic distances of the tested avian species. Erythroid cells from fish and amphibians were not reactive. Reconstitution experiments of partially purified chicken reticulocyte chromosomal nonhistone protein antigens with DNAs isolated from several vertebrate species showed that the species specificity of the antigenic complexes is determined principally by the species origin of the nonhistone proteins. Our results show that a cell-specific chromosomal nonhistone protein(s) has undergone evolutionary change and the relative immunological differences observed are consistent with the accepted phylogenetic distances of the species examined.

Production of antibodies against cellular antigens represents one of the most powerful tools for probing macromolecular diversity and specificity. While most of the tissue antigens described in the literature are of extracellular or cytoplasmic origin, the presence of specific antigens in the cell nucleus has only recently been appreciated.

First notions that chromatin of eucaryotic cells may contain cell- or tissue-specific antigens were pointed out by Henning et al. (1962) and Messineo (1961), who studied the immunological properties of nucleohistones isolated respectively from calf thymus and leukocyte nuclei. Development of techniques for isolation of chromatin permitted detailed studies on its biological and structural properties. Using dehistonized chromatin from chick oviduct, Chytil & Spelsberg (1971) elicited antibodies which specifically recognized chromatin from this tissue. The immunological specificity of chromosomal complexes was confirmed by Wakabayashi & Hnilica (1973), who found that maligant growth significantly altered the antigenic properties of rat liver chromatin. A similar observation was also made by Zardi et al. (1973), who reported the antigenic properties of chromatin from SV40-transformed WI 38 cells to be different from those of the nontransformed controls. According to Wakabayashi et al. (1974), the anti-

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genic specificity of dehistonized chromatin preparations reflected stereospecific interactions between the DNA and some chromosomal nonhistone protein(s).

Since these initial observations, antibodies to chromosomal nonhistone proteins have been used to detect cell (Yeoman et al., 1976; Briggs et al., 1978; Campbell et al., 1979), species (Okita & Zardi, 1974; Zardi, 1975; Tsutsui et al., 1976, 1977), and tumor (Chiu et al., 1974, 1975, 1977; Yeoman et al., 1976; Fujitani et al., 1978; Davis et al., 1978) specific nuclear or chromatin antigens. Antisera have been raised to whole chromatin (Zardi et al., 1973; Okita & Zardi, 1974; Zardi, 1975), to nonhistone proteins which have been freed of other nuclear components (Yeoman et al., 1976; Silver & Elgin, 1976, 1977; Alfageme et al., 1976; Plagens et al., 1976; Cantarow & Stollar, 1977; Bustin et al., 1978), or to dehistonized chromatin in which the histones and some nonhistone proteins have been removed by treatment with solutions of high ionic strength containing urea (Chytil & Spelsberg, 1971; Wakabayashi & Hnilica, 1973; Tsutsui et al., 1976; Chiu et al., 1975; Briggs et al., 1978). Some of these antisera have been used in immunohistochemical experiments to show considerable selectivity in nuclear (Chiu et al., 1977; Briggs et al., 1978; Campbell et al., 1979), nucleolar (Davis et al., 1978), or chromosomal (Silver & Elgin, 1976, 1977; Tsutsui et al., 1977; Mayfield et al., 1978) localization. The association of specific antisera with either active or inactive bands of polytene chromosomes in *Drosophila* as shown by Elgin and associates (Silver & Elgin, 1976, 1977; Mayfield et al., 1978) is especially impressive.

In the present study, the immunological specificity of chromosomal nonhistone protein antigens in erythroid cells was investigated by immunolocalization and complement fixation assays. Our results show that a chromosomal nonhistone protein or proteins, unique to the nucleated erythroid cell of birds, have undergone evolutionary change and that the immunological differences are consistent with the accepted phylogenetic differences.

Materials and Methods

Isolation of Nuclei and Chromatin. The following species were used as a source of nucleated erythrocytes: chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), bobwhite quail (*Colinus Virginianus*), greylag goose (*Anser anser*), Peking duck (*Anas platyrhynchos domesticus*), red-eared turtle (*Chrysemys scripta*), bullfog (*Rana catesbeiana*), and catfish (*Ictalurus punctatus*). Erythrocytes were obtained either by cardiac puncture or by decapitation. For reticulocyte experiments, chickens were made anemic by phenylhydrazine injections (10 mg/kg of body weight for 5 days) and the blood was taken 24 h after the last injection. All blood samples were collected in 0.15 M NaCl and 15 mM sodium citrate in 0.01% heparin.

Nuclei were isolated by the method of Evans & Lingrel (1969) with the addition of 0.5% Triton X-100 to remove membrane ghosts. The nuclei were finally purified by centrifugation at 17000*g* through 1.8 M sucrose for 30 min. The isolation of chromatin was described previously (Spelsberg & Hnilica, 1971). Our more recent experiments involve nuclei prepared by nitrogen cavitation (Shelton et al., 1976) with essentially identical results.

Antisera and Immunoassays. Reticulocyte chromatin was dehistonized by solubilization in 2.5 M NaCl, 5 M urea, and 100 mM sodium phosphate buffer, pH 6.0. After being stirred at 4 °C for 6 h, the solution was centrifuged at 100000*g* for 36 h. The pellets containing nonhistone protein–DNA complexes were resuspended in 2 mM Tris–HCl buffer, pH 7.5, and stirred overnight at 4 °C. After small amounts of undissolved particulate material were removed by low-speed centrifugation, the supernatant was used to immunize New Zealand white rabbits following the schedule described by Chytil & Spelsberg (1971). Blood was obtained by marginal ear vein bleeding 7 days after the booster injection. All sera were heat-inactivated at 56 °C for 30 min.

The quantitative microcomplement fixation technique of Wasserman & Levine (1961) was used to test the immunological activity of chromatin preparations. Washed sheep red blood cells (GIBCO Diagnostic) were activated with antisheep red blood cell serum (Capell Laboratories). Guinea pig serum complement (Capell Laboratories) was titrated to give 100% cell lysis of the activated sheep red cells after 30 min of incubation at 37 °C. The chromatin concentration ranges were tested by incubating various chromatin dilutions 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 a 30-min incubation at 37 °C the extent of red cell lysis was determined spectrophotometrically at 413 nm. In some tests a standard amount of chromatin was mixed with varying dilutions of antiserum in order to determine titers to be employed in subsequent testing. All assays were tested for anticomplementarity. For immunoabsorbtion, reticulocyte or erythrocyte chromatin was used as an absorbent in a ratio of 200 μg of DNA to 1 mL of a 1:50 diluted antiserum. After incubation for 16 h at 4 °C, the chromatin and bound antibody were removed by centrifugation at 17000*g* for 15 min and the supernatants used in complement fixation assays.

Immunocytochemistry. The location of antigens was performed by using the horseradish peroxidase–antihorseradish peroxidase (PAP) soluble reagent of Sternberger (1974). Dry blood smears were fixed in acetone for 10 min at 4 °C and then incubated with antiserum diluted 1:200. Primary antibody was linked to the detector (PAP) through a sheep antirabbit γ-globulin bridge (Miles Corp.). The PAP complex was finally reacted with diaminobenzidine and H_2O_2 (Chytil, 1977).

Reconstitution Experiments. Antigenic proteins were dissociated from their complexes with DNA in dehistonized chromatin by 2% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol in 10 mM sodium phosphate buffer, pH 7.0. The dissociated DNA was sedimented at 100000*g* for 48 h. The protein-containing supernatants were collected and dialyzed, and the sodium dodecyl sulfate was removed by chromatography on Dowex AG1 × 2 resin (Weber & Kuter, 1971). The DNA pellets were further purified by phenol–0.1% hydroxyquinoline extractions and ethanol precipitation. DNA was mixed with the nonhistone proteins at ratios of 0.4, 0.2, or 0.1 mg of protein to 1 mg of DNA in 2.5 M NaCl, 5 M urea, and 50 mM Tris-HCl buffer, pH 8.0, and the NaCl was slowly removed by gradient dialysis against buffered 5 M urea, Finally, urea was removed by dialysis against 1.5 mM NaCl and 0.15 mM sodium citrate.

Electrophoretic Analysis. Samples were dialyzed against 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 8 M urea, and 10 mM sodium phosphate buffer, pH 7.0. After dialysis the electrophoretic separation of proteins was performed by the method of Weber & Osborn (1969) with the addition of a 3% stacking gel to allow separation of proteins in the presence of DNA (Briggs et al., 1976). Gels were stained with Coomassie brilliant blue.

Results

In accordance with the initial observations of Henning et al. (1962), we have found that antisera to dehistonized chro-

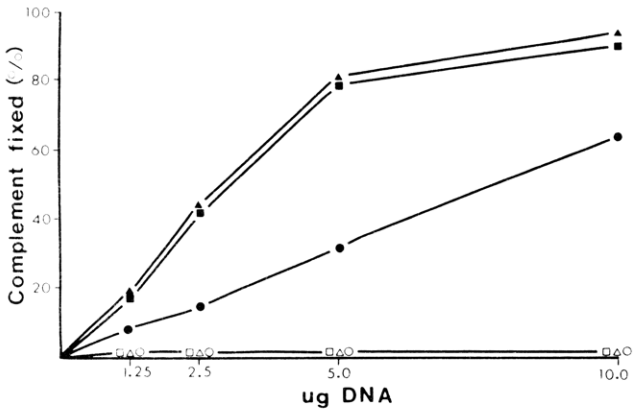

FIGURE 1: Complement fixation of various chromatins in the presence of antiserum (dilution 1:100) to dehistonized chicken reticulocyte chromatin: chicken reticulocyte (■), chicken erythrocyte (●), chicken liver (□), rat liver (△), and Novikoff hepatoma (○) chromatins and chicken erythrocyte chromatin treated with 400 μg of dextran sulfate (▲) per 1000 μg of DNA.

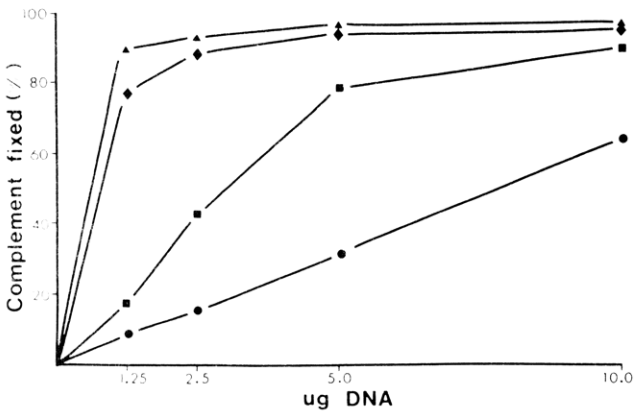

FIGURE 2: Complement fixation of chicken dehistonized and normal erythroid chromatins when reacted with antiserum diluted 1:100. Reticulocyte dehistonized (▲) and normal (■) chromatin; erythrocyte dehistonized (◆) and normal (●) chromatin.

matin are incomplete; i.e., they do not contain precipitating antibodies. Consequently, the specificities of the antisera raised to dehistonized chicken reticulocyte chromatin were assayed by quantitative microcomplement fixation (Wasserman & Levine, 1961) or immunolocalization (Chytil, 1977).

Cell Specificity of the Antisera. Although dehistonization of chromatin enhances its immunogenicity (Chytil & Spelsberg, 1971) and changes some of its antigenic determinants (Zardi et al., 1973; Zardi, 1975), the complement fixation assays, when performed with a purified IgG fraction of the antiserum, can utilize "native" chromatin preparations. The specificity of one of the antisera elicited to dehistonized chromatin of chicken reticulocytes is illustrated in Figure 1. Only native chromatin from chicken reticulocytes fixed the complement extensively. Chromatin from mature chicken erythrocytes was much less reactive with the reticulocyte antiserum. However, a brief treatment of erythrocyte chromatin with dextran sulfate resulted in the decondensation of the heterochromatic nuclear regions and a substantial increase of its complement-fixing ability. This indicates that the dextran sulfate treatment exposed antigenic sites previously inaccessible to or not recognized by the antibody. Chromatins isolated from chicken or rat liver and Novikoff hepatoma were all negative. Dehistonization of either reticulocyte or erythrocyte chromatins further increased their complement-fixing ability (Figure 2). These findings indicate that histones constrain the DNA in chromatin in a fashion which interferes with the

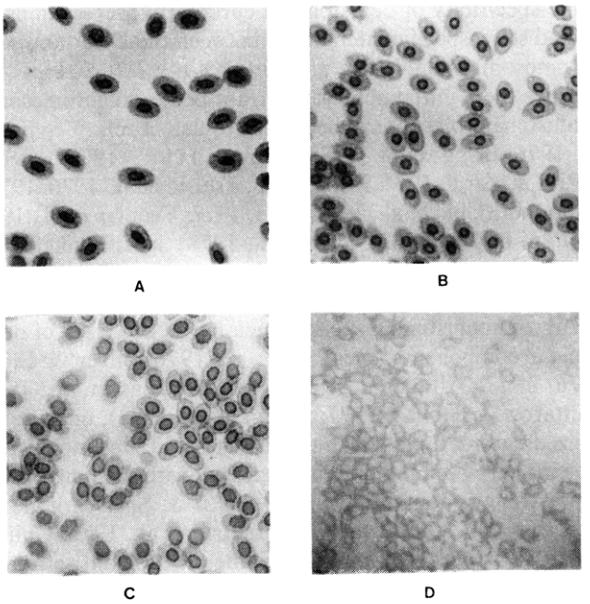

FIGURE 3: Immunocytochemical localization of the chicken reticulocyte chromatin antigen using the peroxidase–antiperoxidase reagent. Antiserum (diluted 1:200) was raised to dehistonized chicken reticulocyte chromatin and reacted with chicken reticulocytes (A), chicken erythrocytes (B), and chicken erythrocytes treated with hypotonic solution (C). No nuclear staining reaction was observed when smears of chicken erythroid cells were reacted with serum (diluted 1:200) collected from the rabbit before immunization (D).

recognition of all antigenic sites potentially available in dehistonized chromatin.

Immunocytochemical Localization. Evidence in the literature illustrates the exceptional value of the use of conjugated antibodies for the visualization of selected antigens within various cellular structures. For assurance that the antigenic complexes which were found to be specific for chicken erythroid chromatin are indeed of nuclear origin, the reacting antigen was localized in acetone-fixed blood smears by the peroxidase–antiperoxidase method of Sternberger (1974). This technique was chosen over the fluorescent conjugate method because of its superior sensitivity and potential application to electron microscopy. The antiserum clearly localized in both the reticulocyte and erythrocyte nuclei (Figure 3). The apparent concentration of staining in the peripheral area of the erythroid cell nucleus was diminished in nuclei swollen by prior exposure to hypotonic solutions (Figure 3C). This suggests that the antigen may be uniformly distributed throughout the cell nucleus and the peripheral nuclear staining of untreated cells is the result of heterochromatization. No immunocytochemical reaction was observed with erythroid cells incubated with rabbit serum obtained before immunization (Figure 3D). The cell-specific distribution of this chromosomal protein antigen was also demonstrated by the absence of the positive immunocytochemical staining reaction in the nuclei of the parenchymal cells in chicken liver and kidney tissue sections (data not shown). Results of the immunocytochemical localization experiments support the complement fixation data showing that the antigenic chromosomal complexes are cell (or tissue) specific. Additionally, they indicate that the antigens are localized in the cell nucleus.

Species Specificity. If the antigenic nuclear nonhistone protein–DNA complexes are indeed associated with the process of cell differentiation, as appears to be indicated by their exceptional cell specificity, it can be anticipated that these complexes will exhibit only limited species specificity. This notion is supported by the complement fixation assays shown

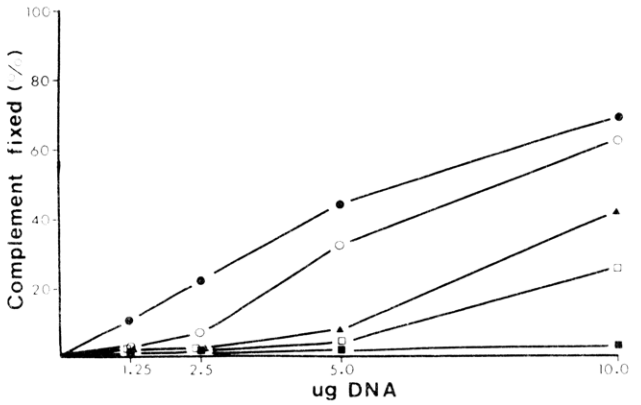

FIGURE 4: Complement fixation of erythrocyte chromatins isolated from various avian species in the presence of antiserum (dilution 1:100) to dehistonized chicken reticulocyte chromatin: chicken (●), turkey (○), quail (▲), goose (□), and duck (■).

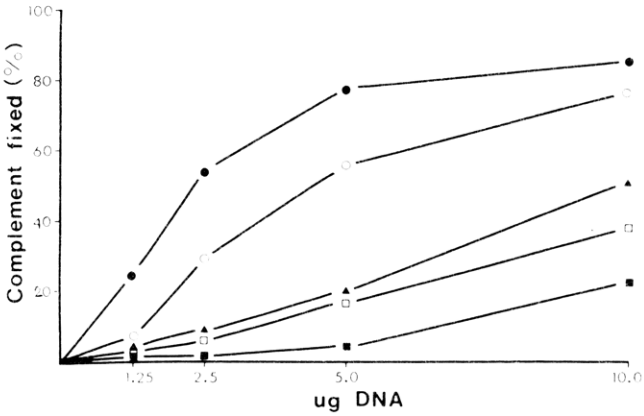

FIGURE 5: Complement fixation of dextran sulfate treated chromatins (400 μg/1000 μg of DNA) isolated from chicken (●), turkey (○), quail (▲), goose (□), and duck (■). Antiserum to dehistonized chicken reticulocyte chromatin was diluted 1:100.

in Figure 4. When antiserum to chicken reticulocyte dehistonized chromatin was employed for the detection of similar antigens in the erythroid cells of several other species, the closely related ones showed cross-reactivity. Partial decondensation of chromatin by exposure of the erythroid chromatins from chicken and other species to dextran sulfate increased the complement fixation activity but did not show closer similarities (Figure 5). No antigens related to those found in dehistonized chicken erythroid chromatin were detected in chromatins isolated from nucleated erythrocytes of animals representing different classes of lower vertebrates (turtle, bullfrog, and catfish), and immunocytochemical tests confirmed that the cross-reactivity of antisera to chicken erythroid chromosomal antigens is limited to birds phylogenetically related to domestic fowl (Figure 6).

The specificity of the antigens was further tested by immunoabsorption experiments. Absorption of the antiserum with chromatins isolated from duck, turtle, bullfrog (data not shown), or catfish had no effect on the reactivity of the antiserum against chicken reticulocyte chromatin (Figure 7). However, absorption of the antiserum with chromatin isolated from goose, quail, or turkey erythrocytes had some inhibitory effect on antiserum reaction, and absorption with chicken erythrocyte chromatin eliminated the antiserum immunological reactivity with reticulocyte chromatin all together (Figure 7).

Reconstitution Experiments. We have shown previously that the cell or tissue specificity of the chromatin antigens assayed in our experiments depends on the association of antigenic chromosomal nonhistone proteins with DNA of the same or closely related species (Wakabayashi & Hnilica, 1973; Wakabayashi et al., 1974; Chiu et al., 1975, 1977; Wang et al., 1976; Hardy et al., 1978). In view of these observations, we have compared the immunological specificity of the interactions between the residual chromosomal nonhistone proteins and DNA in the antigenic complexes. The proteins were dissociated from dehistonized chromatin preparations and separated from their DNAs. Purified chicken DNA was then reconstituted with the isolated residual nonhistone proteins from chicken, duck, or catfish. Only the homologous complex (chicken DNA + chicken nonhistone protein) gave a complement fixation comparable to the native chromatin (Figure 8A). On the other hand, residual nonhistone proteins from dehistonized chicken erythroid chromatin reconstituted with DNA from duck or catfish yielded fixation activity similar to that observed with the homologous complex (chicken DNA + chicken nonhistone proteins). This somewhat unexpected behavior was observed at all the tested protein/DNA reconstitution ratios. Results of these experiments are shown in Figure 8B. The absence of any complement fixation with chicken DNA or nonhistone proteins alone (parts A and B of Figure 8) demonstrates the requirement of both components to form an immunologically cell-specific complex.

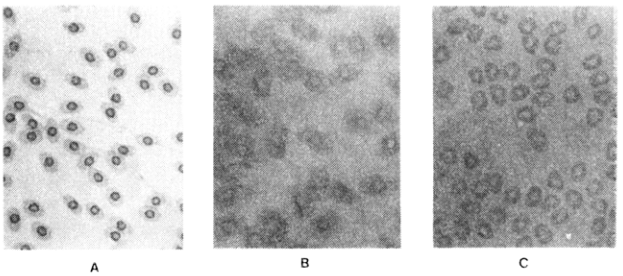

FIGURE 6: Immunocytochemical localization of antigen in the presence of antiserum to dehistonized chicken reticulocyte chromatin (dilution 1:200). Antiserum reaction with chicken (A), turtle (B), and catfish (C) erythrocytes.

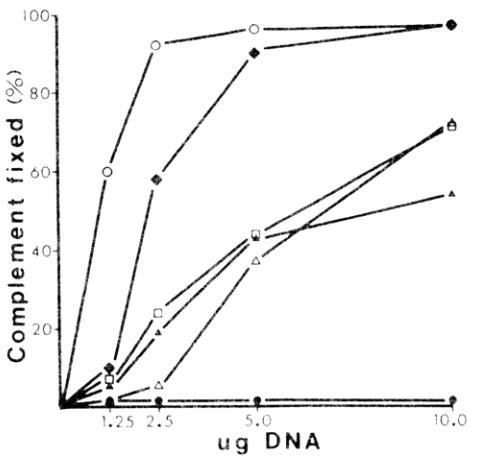

FIGURE 7: Complement fixation of chicken reticulocyte chromatin in the presence of antiserum (dilution 1:100) to dehistonized chicken reticulocyte chromatin absorbed with erythrocyte chromatins from the following species: chicken (●), turkey (▲), quail (□), goose (△), catfish (◆), and unabsorbed chicken reticulocyte antiserum (○).

Discussion

It is evident, mostly through the original studies of Stollar and his associates (Sandberg et al., 1967; Stollar & Ward, 1970), that histones are relatively poor antigens and that most fractions are not tissue or species specific. Some exceptions

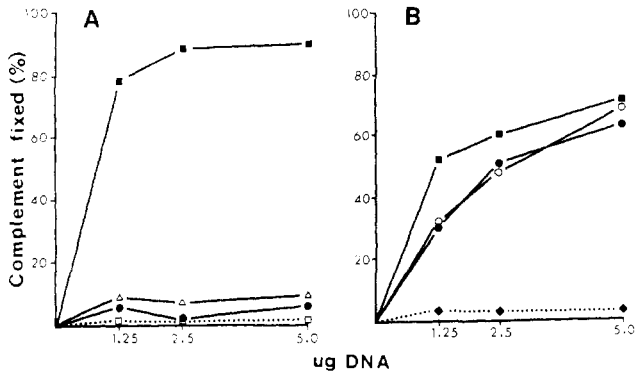

FIGURE 8: Complement fixation of reconstituted nonhistone protein–DNA complexes of nucleated erythroid cells in the presence of antiserum to dehistonized chicken reticulocyte chromatin. Panel A: chicken DNA reconstituted with nonhistone proteins dissociated by 2% sodium dodecyl sulfate from dehistonized chromatins of chicken reticulocytes (■) and duck (△) and catfish (●) erythrocytes. Panel B: chicken reticulocyte nonhistone proteins dissociated from dehistonized chromatin by 2% sodium dodecyl sulfate and reconstituted with chicken (■), duck (○), and catfish (●) DNA. Broken lines represent the reaction of chicken DNA (□) and chicken reticulocyte residual nonhistone proteins (▼) alone, without reconstitution of the nonhistone protein–DNA complex.

have been noted; e.g., the H1 histones are both chemically and immunologically heterogeneous (Bustin & Stollar, 1972; Bustin, 1976). Antisera to H1 histone subfractions showed little immunological correspondence between those of rat and calf thymus (Bustin & Stollar, 1973). Conversely, antisera to chicken erythrocyte specific histone H5 were found to cross-react extensively with H5 histones in nucleated erythroid cells of six different species of fish (Goetz et al., 1978). However, such antisera gave no positive immunological reaction with other chromatin components (Mura et al., 1974, 1978; Enea et al., 1978). Since our antisera to dehistonized chicken reticulocyte chromatin do not react with fish erythrocyte chromatin, we conclude that the involved antigens are not related to the H5 histone. This conclusion is further supported by our observations that isolated chicken erythrocyte histones do not react with our antisera and when added to dehistonized chicken reticulocyte chromatin they substantially decrease its immunological reactivity. The increased detection of antigen(s) following removal of histones from erythroid cell chromatin (Figure 2) also makes it unlikely that any of the histone proteins could be responsible for the immunological activities observed in our experiments. Additionally, preliminary data on the initial purification of the erythroid specific antigen show it to be a non-acid-extractable chromosomal protein of molecular weight 90 000.

The immunolocalization of the antigen(s) demonstrates its nuclear origin, and the concentration of the immunocytochemical reaction near the periphery of the erythroid cell nucleus suggests a possible association with the nuclear pore complex lamina fraction. However, the partially purified antigenic protein has a molecular weight of ~90 000, which is larger than the 75 000-dalton and smaller polypeptides known to comprise the envelope-associated material. In addition, antisera to the nuclear envelope associated complex do not show the cell-type specificity found in our experiments (Ely et al., 1978; Gerace et al., 1978; Krohne et al., 1978). The molecular weight of our antigenic protein component also limits the possibility of it being associated with the nuclear matrix or residual oligomeric polypeptides (Shelton, 1978), but some high molecular weight proteins have been found to be common to nuclear membrane and nonhistone protein preparations (Jackson, 1976). High molecular weight material has also been thought to derive from plasma membrane contamination (Shelton, 1973). The latter possibility is remote as both methods employed for nuclei isolation in our studies (detergent and nitrogen cavitation) yield nuclei free of plasma membrane ghosts as confirmed by the antigen immunocytochemical localization. The former possibility cannot be ruled out, but if true would suggest that the nuclear membrane components may have greater specificity than previously thought and may also include components with DNA binding properties. Our further studies are aimed at elucidating these possible relationships.

The dehistonization and dextran sulfate experiments which both resulted in a markedly increased complement fixation activity of the erythroid chromatin can be interpreted in that the histones affect the availability of antigen in chromatin. Whether this steric hindrance occurs at the nucleosome spacing or chromatin strand supercoiling is presently not known. Our preliminary experiments using chromatin digestion with nucleases indicate structural involvement more complex than the nucleosome level. The requirement of chromosomal nonhistone protein antigens for DNA to be immunologically specific suggests a specific recognition between the antigenic protein(s) and DNA. However, the specificity of these interactions appears to depend on the species origin of the protein rather than on that of the DNA. This differs from our earlier experience with higher vertebrates where the antigenic protein–DNA complexes specific for rat liver or Novikoff hepatoma exhibited much greater selectivity for homologous DNA (Wakabayashi & Hnilica, 1973; Chiu et al., 1975). Apparently, the antigenic protein–DNA complexes represent a complicated and unique interacting system which can be studied in detail once its components are isolated and characterized. This notion is further supported by our earlier observations (Hardy et at., 1978) that antisera to dehistonized chicken reticulocyte chromatin could be obtained which did not react immunologically with dehistonized chicken erythrocyte chromatin. As can be seen in Figure 2, antisera employed in this study did not exhibit such an exceptional specificity.

A comparison of the immunoreactivity of chromatins from nucleated erythroid cells of other species showed a wide variation in reactivities. No cross-reacting nuclear antigen was detected in nucleated erythrocytes of animals from different classes of vertebrates, but among the birds the varying levels of immunoreactivity were in agreement with the known taxonomic groupings (Welty, 1962). The relatedness of chicken and turkey antigens was expected since both species belong to Galliforms. Clearly, chicken should share more common determinants with those birds than with duck or goose, which belong to Anseriformes. It was somewhat surprising, however, that quail erythrocyte chromatin was less immunoreactive than turkey (both Galliformes) (Seligy et al., 1976). It should also be noted that tests of immunoreactivity by complement fixation, immunocytochemistry, and immunoabsorption experiments all supported the same restricted cell-type distribution as well as the same levels of immunological relatedness between the species examined. A similar degree of immunological relatedness between these avian species was previously demonstrated by Landsteiner & Von der Scheer (1940) with antisera to ovalbumin and more recently by Cantarow & Stollar (1977) with antisera to a nuclear enzyme, nicotinamide mononucleotide adenyltransferase.

Unlike the histones, our immunological studies of nonhistone proteins reveal a strict cell-type distribution of at least one protein group. The remarkable cell specificity of these an-

tigenic complexes points to their possible role in cellular differentiation and developmental processes. The discovery of some cross-reacting determinants in the same cell type of closely related species indicates a similar functional requirement of the protein in the same cell type of other species but that some evolutionary changes occurred in this chromosomal nonhistone protein antigen.

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