isocitrate or C-2 of citrate to near planarity (and perhaps also by deforming bond angles from 109 to 120°) so that a proton is readily transferred to a base on the enzyme [where it is not exchanged with solvent while substrates are present on the enzyme (Rose & O'Connell, 1967)], or by twisting and bending aconitate at the appropriate carbon to induce reaction with water (presumably assisted by proton transfer to a base on the enzyme). A similar mechanism has been deduced for fumarase from isotope effect studies by J. S. Blanchard and W. W. Cleland (unpublished experiments) and from inhibition by the carbanion of 3-nitro-2-hydroxypropionate as a transition-state analogue by Porter & Bright (1980). It thus appears that these enzymes use geometric deformation, accompanied by suitable acid–base chemistry, to accomplish catalysis.

It is interesting to note that the protonated forms of the nitro analogues which have only the two carboxyl groups ionized still bind as well or only 10 times weaker than the corresponding substrates. Thus, the nitro group, which has no net charge (although the oxygens have partial negative charge and the nitrogen has positive charge), provides the same or nearly the same binding strength as an ionized carboxyl group. The same is probably true for a protonated carboxyl group, since the V/K values for the substrates and the pK_i for tricarballylate do not decrease at the pH where one of the carboxyls of these molecules becomes protonated (J. V. Schloss, unpublished experiments). The failure of the pK_i value for the nitro analogue of citrate to decrease at pH 4.5, in fact, suggests that one of its carboxyl groups may also be protonated without decreased binding. It appears that both of the carboxyl groups which become aci-acids in reaction intermediates may be bound not to lysines or arginines but by hydrogen bonds to formally neutral groups on the enzyme.

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Chicken Reticulocyte Nuclear Antigen: Its Identification and Relation to Transcriptive Activity in Erythropoietic Cells†

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ABSTRACT: Antibodies to chicken reticulocyte dehistonized chromatin were produced. These antibodies can distinguish between chromatin from reticulocytes and erythrocytes and have no activity with chicken liver chromatin. The antibodies will also differentiate between reticulocytes and erythrocytes by the horseradish peroxidase bridge localization technique. The nonhistone protein components of the antigenic complex are very tightly bound to the DNA, removable by extraction with sodium dodecyl sulfate but not 2.5 M NaCl–5 M urea. Our data indicate that the antigen complexes could actually be present in both reticulocytes and erythrocytes but that chromatin condensation causes a structural masking of the complexes which coincides with the known decline in transcriptive activity of the erythrocyte.

Avian species, in contrast to mammalian species, retain their cell nuclei in erythrocytes through all stages of developmental and cellular differentiation. In the reticulocyte the nucleus is actively involved in transcription of globin and other messenger RNA. The erythrocyte nucleus in comparison is pycnotic and shows very little, if any, transcriptive activity (Barrett et al., 1974).

It has been proposed that nonhistone chromosomal proteins are involved in the regulation of transcription and the differentiation of cells during development (Baserga, 1974; Stein et al., 1974; Jeter & Cameron, 1974; Chiu & Hnilica, 1977). Specifically, we and others have shown that certain nonhistone chromosomal protein–DNA complexes have antigenic activity(s) and that the antibodies induced by the nonhistone–DNA complexes are able to distinguish between the tissue of origin and other tissues including tumor tissue (Chytil & Spelsberg,

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1971; Wakabayashi & Hnilica, 1973; Zardi et al., 1973; Chiu et al., 1974). In most cases, the work has been done with antigens to tumor tissue, for example, hepatoma (Chiu et al., 1975c; Fujitani et al., 1978) or large bowel tumor (Chiu et al., 1979, 1980).

Hardy et al. (1978) have demonstrated that chicken reticulocyte dehistonized chromatin can elicit a specific antibody in rabbit. Here we present further evidence that specific nonhistone chromosonal protein–DNA complexes exist in the chicken reticulocyte which enable us to immunologically distinguish between chicken reticuloctye, erythrocyte, chicken liver, and other rat tissue. We propose that these antigens are present in both reticulocytes and erythrocytes but are structurally masked in erythrocyte chromatin. The nuclear proteins responsible for immunospecificity of these two different states may be at least partially involved in cellular differentiation of chicken erythroid cells.

Materials and Methods

Animals. The chickens used were male Leghorns, ~2 kg each. To obtain reticulocytes, we made the chickens anemic by bleeding 15 mL of blood from the wing vein, followed by 5 days of intramuscular injections of phenylhydrazine at 10 mg/kg. All blood used was collected in cold (4 °C) SSC[1] (0.14 M NaCl–0.014 M sodium citrate, pH 7.0) with 0.01% heparin. Erythrocytes were obtained by bleeding nonanemic chickens. The percentage of reticulocytes was determined by examination with new methylene blue. All erythrocyte preparations contained less than 5% reticulocytes, and the reticulocyte preparations were more than 95% reticulocytes.

Antibodies to chicken reticulocyte dehistonized chromatin were raised in male New Zealand rabbits which were given injections in the footpad and back of up to 1 mg of chromatin (measured as DNA) per injection mixed with Freuds' adjuvant. After 5–6 weeks of injections the animals received an ear vein boost of 0.33 mg of dehistonized chromatin and were bled 7 days later.

Isolation of Nuclei. Both erythrocytes and reticulocytes were prepared in the same manner. All procedures were performed at 4 °C. Blood in SSC and heparin was centrifuged at 500*g* for 10 min. Serum and buffy coat (containing white cells) of pellet were removed by aspiration. Cells were washed twice more with SSC.

Cells were lysed by the addition of 4 volumes of 5 mM $MgCl_2$ for 60–90 s. Immediately following lysis, 1 volume of 1.5 M sucrose was added and the crude nuclei were removed by centrifugation at 3000*g* for 10 min. The crude nuclear pellet was thoroughly homogenized in 0.25 M sucrose–0.5% Triton X-100–TKMC [50 mM Tris-HCl (pH 7.4)–24 mM KCl–5 mM $MgCl_2$–0.2 mM $CaCl_2$] and centrifuged at 3000*g* for 10 min to remove membrane ghosts. The nuclear pellet was washed and centrifuged with TKMC until the supernatant became colorless. The nuclear pellet, free of membrane ghosts, was centrifuged in 1.7 M sucrose–TKMC at 17000*g* for 30 min.

Preparation of Chromatin. All procedures were performed at 4 °C. Nuclear pellets were homogenized gently with a loose Teflon pestle in 10 mM Tris-HCl (pH 7.9)–1 mM ethylenediaminetetraacetic acid (EDTA). This was allowed to stand with occasional gentle homogenization for 30 min. The homogenate was centrifuged at 2000*g* for 10 min. The pellet was gently homogenized in 0.01 × SSC and allowed to hydrate for 30 min and then centrifuged at 12000*g* for 10 min. The pellet was washed twice more with 0.01 × SSC. The final chromatin pellet was stored at −25 °C until use. All calculations concerning chromatin are standardized according to micrograms of DNA as measured in 5 M urea at 260 nm. The purified chromatin was used to prepare dehistonized chromatin according to the procedure of Chytil & Spelsberg (1971).

For isolation of nonhistone proteins tightly bound to DNA, the chromatin was suspended in 50 mM Tris-HCl (pH 8.0)–2.5 M NaCl–5 M urea (3 mL of buffer per mg of chromatin) and homogenized occasionally for 3–5 h. The chromatin was then centrifuged at 100000*g* for 36–48 h. The pellets were suspended in a minimum volume of 2% sodium dodecyl sulfate ($NaDodSO_4$)–0.1% β-mercaptoethanol–0.01 M sodium phosphate (pH 7.0) (Elgin & Bonner, 1972) and dialyzed 2–3 days against several changes of the same buffer. The dialysate was centrifuged for 48 h at 100000*g*. The top two-thirds of the resulting supernatant contained the tightly bound nonhistone proteins free of DNA. $NaDodSO_4$ was removed from the proteins according to the method of Weber & Kuter (1971). Before the proteins could be tested in the complement fixation test, they were reconstituted to chicken DNA, 0.4 part of protein to 1 part of DNA. The proteins and DNA were dialyzed overnight against a decreasing salt gradient from 2 M NaCl–5 M urea to 1 M NaCl–5 M urea and then against another gradient with the final solution of 0.01 × SSC. The chromatin was dialyzed for an additional 1 h in a fresh solution of 0.01 × SSC and then used in the complement fixation test.

Complement Fixation Test. The complement fixation technique was essentially the method of Wasserman & Levine (1961) modified as described by Chiu et al. (1975b,c). Antibodies to chicken reticulocyte chromatin were used at dilutions of 1:150 or 1:200.

Horseradish Peroxidase Bridge Antigen Localization. This is basically the procedure of Chytil (1978). Whole blood smears were made on slides and dried for 30 min, fixed for 10 min in acetone, and then incubated for 2 h at 4 °C in 1% H_2O_2 in acetone to remove exogenous peroxidases. The slides were washed 3 times in cold PBS [0.1 M sodium phosphate (pH 7.2)–0.14 M NaCl] for 10 min and then drained. Slides were placed in a humid chamber at room temperature and incubated 30 min with a drop of antibodies or control sera at a 1:200 dilution. They were washed 3 more times for 5 min each in cold PBS and then incubated with horseradish peroxidase labeled goat antirabbit γ-globulin (diluted 1:20; from Cappel) for 30 min at room temperature in a wet chamber. The cold PBS washes were repeated 3 times. The slides were stained with a diaminobenzidine solution for 12 min. After being stained, the diaminobenzidine solution was inactivated by adding 120 mL of bleach per 300 mL of staining solution. Slides were washed again in PBS and distilled water and dehydrated with 50, 70, 95, and 100% alcohol washes, 5 min each. They were clarified for 5 min in xylene, mounted, and photographed.

Results

The production of antibodies able to distinguish between reticulocyte and erythrocyte chromatin is critical to our experiments. Reticulocyte chromatin was dehistonized by the method of Chytil & Spelsberg (1971) and used to induce specific antibodies in rabbits. The chromatin is dehistonized because the presence of large quantities of histone could induce preferential antibodies to histones. This is especially important

[1] Abbreviations used: SSC, 0.14 M sodium chloride–0.014 M sodium citrate, pH 7.0; TKMC, 50 mM Tris-HCl (pH 7.4)–24 mM KCl–5 mM $MgCl_2$–0.2 mM $CaCl_2$; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PBS, 0.01 M sodium phosphate (pH 7.2)–0.14 M NaCl.

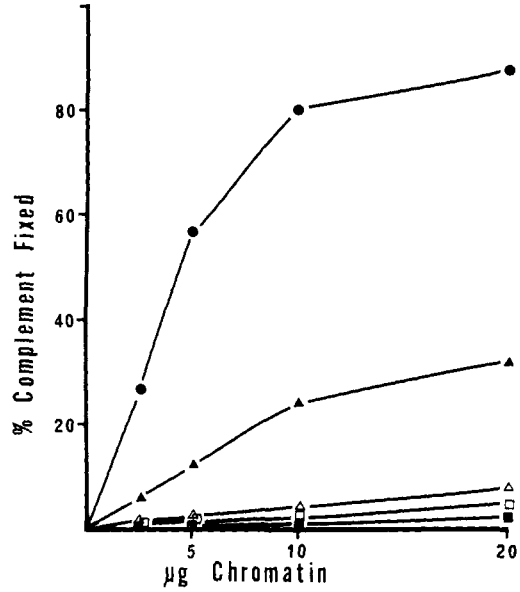

FIGURE 1: Affinity of antibodies against dehistonized chicken reticulocyte chromatin for various chicken and rat chromatins. The amount of antigen–antibody complex formed determines the percent of complement fixed. Chromatins were isolated from chicken reticulocyte (●), chicken erythrocyte (▲), chicken liver (△), rat large bowel transplantable tumor (□), and Morris hepatoma 7777 (■).

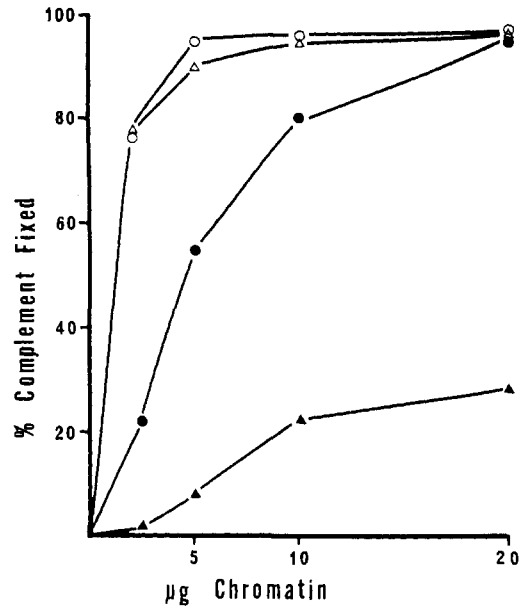

FIGURE 2: Effect of dehistonization on accessibility of nuclear antigens from chicken erythroid cells in the complement fixation test with antibodies to dehistonized chicken erythroid chromatins. Dehistonized erythrocyte chromatin (○); dehistonized reticulocyte chromatin (△); whole reticulocyte chromatin (●); whole erythrocyte chromatin (▲).

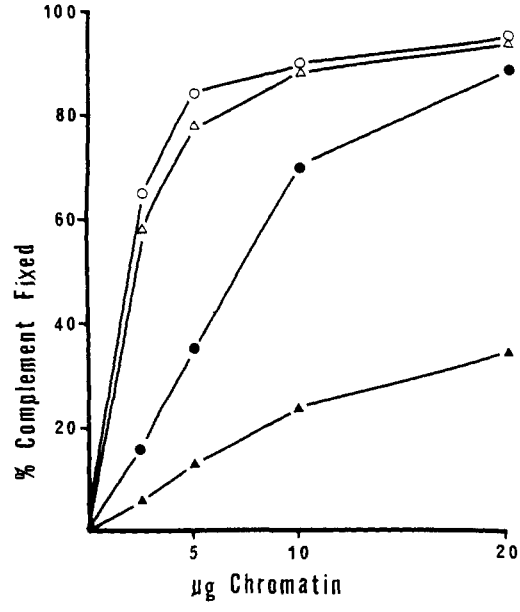

FIGURE 3: Effect of sonication on the accessibility of erythroid nuclear antigens in the complement fixation test with antibodies to dehistonized chicken reticulocyte chromatin. Sonicated reticulocyte chromatin (○); sonicated erythrocyte chromatin (△); intact reticulocyte chromatin (●); intact erythrocyte chromatin (▲).

for us to avoid since histone H5 is avian erythrocyte specific.

Figure 1 shows that by using the microcomplement fixation test the antibodies react strongly with reticulocyte chromatin, react very slightly with chicken erythrocyte chromatin, and have essentially no reaction when tested against chicken liver chromatin, chromatin from large bowel transplantable tumor in rats, or chromatin isolated from transplantable Morris hepatoma 7777 in rats. The reticulocyte chromatin always has at least 2–3 times as much antibody binding activity as the erythrocyte chromatin. Other data from our laboratory show that the reticulocyte dehistonized chromatin antibodies do not react with nucleated frog erythrocyte chromatin or Friend erythroleukemia cell chromatin (Pumo et al., 1980).

To further explore the nuclear antigenic relationship of the two blood cell types, we disrupted the chromatins from chicken reticulocytes and erythrocytes. When the chromatin is no longer intact, that is, after it has been exposed to dehistonization, it is not possible to distinguish between reticulocyte and erythrocyte chromatins. This data is shown in Figure 2. Following dehistonization, both reticulocyte and erythrocyte chromatins have a higher complement fixing capacity than intact reticulocyte chromatin. It was found that 2.5 μg of dehistonized chromatin from either erythrocyte or reticulocyte (measured as DNA) fixes 4 times as much complement as 2.5 μg of intact reticulocyte chromatin.

Almost identical results are achieved when the chromatins from both types of blood cells are mechanically sheared by sonication. The results are shown in Figure 3. Again, the intact chromatins show typical patterns of activity, but the disrupted chromatins show an increase in complement fixing activity over the standard level of reticulocytes. This data is similar to that shown in Figure 2; the immunoactivity of the disrupted chromatin from either reticulocytes or erythrocytes is almost fourfold higher than that of intact reticulocyte chromatin.

These data strongly suggest that the reticulocyte nuclear antigens are present in the erythrocyte but are masked in some way by the structural complexity of the intact erythrocyte. The erythrocyte chromatin is very condensed relative to the reticulocyte chromatin. When the native structure of the chromatin is drastically altered by removing the histones (Figure 2) or sonication (Figure 3), the nuclear antigen protein–DNA complexes present cannot be structurally hidden, hence the increase in measurable immunoactivity.

We have found that the nonhistone proteins required for the specificity of the antigenic nonhistone–DNA complexes are very tightly bound to the DNA. We extracted reticulocyte chromatin with 2.5 M NaCl–5 M urea to remove histone proteins and the majority of nonhistone proteins. We extracted the remainder of the tightly bound nonhistones with 2% Na-DodSO$_4$ (Elgin & Bonner, 1972). Very little evidence of antigenic activity was found in the high salt–urea extractable

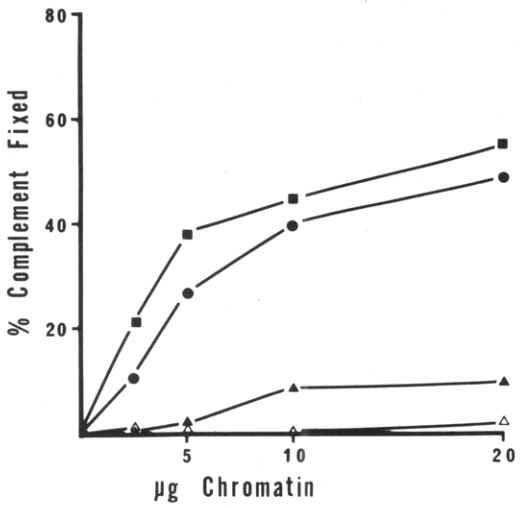

FIGURE 4: Nuclear antigenic activity in sodium dodecyl sulfate extractable (tightly bound to DNA) nonhistone chromosomal proteins from chicken reticulocytes. Proteins were reconstituted to chicken DNA before being assayed. Tightly bound nonhistone chromosomal proteins (■); whole chicken reticulocyte chromatin (●); whole erythrocyte chromatin (▲); chicken liver chromatin (△).

fractions; however, as shown in Figure 4, the fraction extracted with $NaDodSO_4$ binds slightly more complement than reticulocyte chromatin. In order to obtain immunoactivity, we removed $NaDodSO_4$ from the proteins by column chromatography through Dowex AG1-X2 resin (Weber & Kuter, 1971) since the presence of $NaDodSO_4$ can interfere with the complement fixation test. We reconstituted the proteins to chicken DNA before testing in the microcomplement fixation test because protein or DNA alone does not have immunoactivity; protein–DNA complexes are required (Pumo et al., 1980).

Another approach used to identify the presence of reticulocyte-specific antigens was the horseradish peroxidase bridge technique. The results of the antigen localization procedure are shown in Figure 5. Frames A and B of Figure 5 clearly show that the antigens are accessible to antibodies in the nuclei of reticulocytes (Figure 5B), but not in their cytoplasm nor in the nuclei or cytoplasm of intact erythrocytes (Figure 5A). We reasoned that if our ideas concerning a structural masking of the nuclear antigen were correct, it should be possible to expose the antigens by treating otherwise intact erythrocytes with dextran sulfate. The dextran sulfate should induce a decondensation of the chromatin within the erythrocyte (Ansevin et al., 1975), allowing the antigens to become available for binding with antibodies which would be visible with the horseradish peroxidase bridge technique. Erythrocyte blood smears were incubated in a moist chamber with a solution of dextran sulfate (400 µg/mL); after the incubation the treated erythrocytes were prepared in the same manner as the reticulocytes and intact erythrocytes. Figure 5C illustrates that the dextran sulfate treatment allowed the antibodies to bind nuclear antigens. Although the level of binding is still less than the amount found in chicken reticulocytes, it is considerably greater than that of the intact erythrocytes in Figure 5A.

Discussion

In the cell nucleus, DNA interacts with histone and nonhistone proteins to form chromatin, which in eucaryotic organisms is the principal site of genetic endowment and its expression. A detailed knowledge of the chemical identity and function of chromatin's components is essential for the understanding and manipulation of cell differentiation and genetic expression. This realization intensified the research on DNA, histones, and especially chromosomal nonhistone proteins. Development of techniques for the isolation of chromatin opened the way for studies on the biological and structural aspects of this important macromolecular complex. It was realized that chromosomal nonhistone proteins are essential for transcriptional expression of selected genes (Paul & Gilmour, 1968; Gilmour & Paul, 1969; Spelsberg & Hnilica, 1970; Chiu et al., 1975b; Stein & Kleinsmith, 1975; Tsai et al., 1976). Chromosomal nonhistone proteins are very complex and physically heterogeneous (Stein et al., 1974; Jeter & Cameron, 1974; Chiu & Hnilica, 1977; Busch, 1978). Systematic analysis of each individual nonhistone protein present in chromatin is a foreboding task. However, elucidating specific properties which can be selectively associated with the individual protein species is essential for our understanding of chromosomal protein biochemistry and biology. The exceptional sensitivity and selectivity of antigen–antibody interactions have the potential of becoming a major tool in studies of chromatin function and structure.

Using antisera against dehistonized chromatin from adult rat liver, Chytil et al. (1974) showed gradual changes in the antigenicity of chromosomal nonhistone proteins in developing

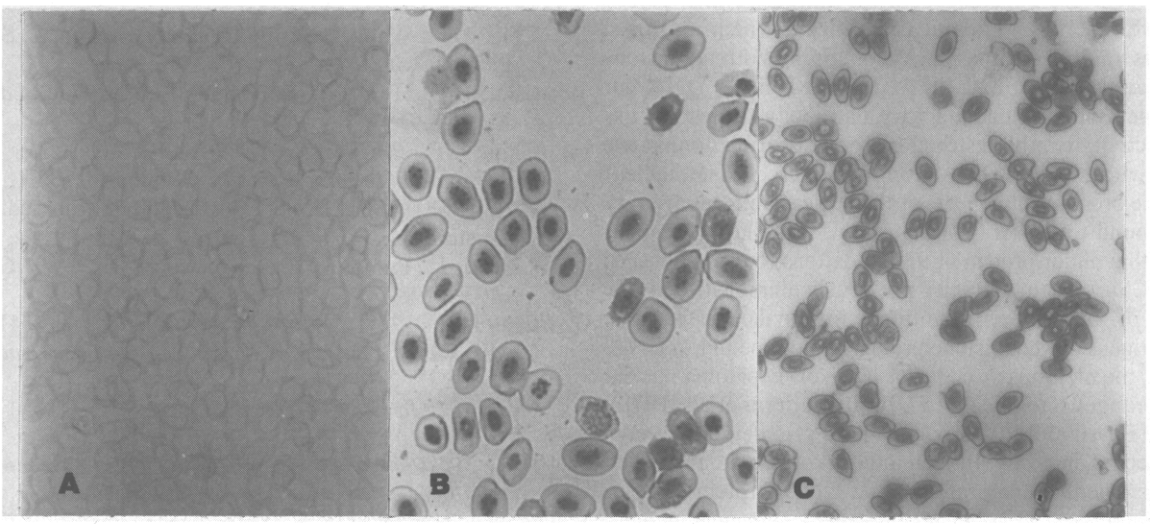

FIGURE 5: Horseradish peroxidase localization of chicken reticulocyte nuclear antigen. All localizations were performed on blood smears which were pretreated to remove endogenous peroxidases before being exposed to antibody made against chicken reticulocyte nuclear antigen. (A) Intact chicken erythrocytes. (B) Intact chicken reticulocytes. (C) Dextran sulfate treated chicken erythrocytes. Magnification was 960×.

embryonic and postpartum livers of rats. Similar changes were also seen in the immunoreactivity of oviduct chromatins of chicks stimulated with diethylstilbestrol as compared with the unstimulated, 7-day-old controls (Spelsberg et al., 1972). These data indicated pronounced dissimilarities between antigenic sites in chromatins derived from the respective tissues, suggesting a high chemical or conformational specificity in the chromosomal nonhistone proteins is responsible for this phenomenon.

Another example of the immunological tissue specificity of chromatin was observed in maturing chicken reticulocytes (Pumo et al., 1980). The present study has shown that the relative amounts of nuclear antigens in the erythroid cell remain essentially constant during development of the mature erythrocyte. The disappearance of nuclear antigenicity in native chromatin during maturation is due to the change in chromatin configuration. The maturation of the avian erythrocyte involves a programmed series of nuclear and cytoplasmic events in the differentiating cell that eventually leads to an almost complete cessation of its metabolic function. During the course of cellular inactivation, the nucleus becomes pycnotic, with condensation of the chromatin (Lucas & Jamroz, 1961), and its activity in DNA and RNA synthesis is severely diminished (Grasso et al., 1963; Cameron & Prescott, 1963; Seligy & Miyagi, 1969; Williams, 1972). When the configuration of the erythrocyte chromatin is drastically altered by removing the histones or sonication, a consequent increase in immunoactivity is realized. The antigenic proteins which are present cannot be structurally hidden—therefore, the increase in measurable immunological activity. Dextran sulfate, a polyanion, can also induce a decondensation of the erythrocyte chromatin and the chromatin within the erythrocyte cell, allowing the antigens to become available for binding with antibodies. However, one cannot also rule out the possibility that the elevation of apparent antigenicity when the structure of chromatin is altered might be due to interaction of other antibodies than those reacting with chromatin in the condensed state.

Hardy et al. (1978) raised an antibody against chicken reticulocyte dehistonized chromatin. This antibody fixed the complement only in the presence of reticulocyte chromatin; erythrocyte chromatin was essentially nonreactive. Neither dehistonized erythrocyte chromatin nor sonicated erythrocyte chromatin could react to their antibody. Therefore, our nuclear antigens are different from the antigens which Hardy et al. have reported. The nuclear antigens which we raised antibodies against are complexes containing both nonhistone proteins and DNA (Pumo et al., 1980). Neither DNA nor nonhistone proteins alone react with antibody. However, immunoreactivity can be restored by reconstituting nonhistone proteins with chicken DNA from any source. The nuclear nonhistone protein components of the nuclear antigens are very tightly bound to DNA. These proteins are a heterogeneous group with a major protein of 95 500 daltons (our unpublished data). Chiu et al. (1975b) previously demonstrated the presence of tumor-specific antigens in Novikoff hepatoma. These antigens are nonhistone proteins tightly bound to DNA. However, the molecular weights of Novikoff hepatoma nuclear antigens range from 45 000 to 60 000 (Fujitani et al., 1978). Recently Busch and his associates have also demonstrated a tumor-specific nucleolar antigen in Novikoff hepatoma. Their antigen is a nonhistone protein, not a DNA–protein complex (Marashi, et al., 1979).

During the preparation of this manuscript, we noticed that Bekhor & Mirell (1979) and Chae and co-workers (Ross et al., 1979), working with chicken blood cells, reported a class of nonhistone proteins which remain bound to DNA in 2 M NaCl and postulated that these proteins include globin gene regulators. The proteins are only present in erythroid cells (erythrocytes and reticulocytes), not in oviduct cells. These proteins can specifically locate the globin gene sequences of DNA even in the presence of total genomic DNA. The finding of these two laboratories strengthens our speculation that the erythroid-specific nuclear antigens, nonhistone proteins tightly bound to DNA, are globin gene regulators. Immunological differences between chromosomal nonhistone proteins in different tissues indicate their probable involvement in cellular differentiation. Does their antigenic specificity merely reflect the specific biochemical phenotype maintained by differentiation or do the chromosomal nonhistone protein antigens play a more important and perhaps a decisive role in regulation of gene expression during organogenesis and differentiation? Although the limited experimental evidence presented here does not permit any definite conclusions, evidence (Chytil et al., 1974; Spelsberg et al., 1972; Chiu et al., 1974, 1975a) demonstrates that at least some of the nuclear antigens may regulate mechanisms by which cells determine their differentiated state.

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Separation of Membrane-Bound γ-Glutamyl Transpeptidase from Brush Border Transport and Enzyme Activities†

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ABSTRACT: A new population of membranes from rat renal cortex containing γ-glutamyl transpeptidase (γ-GTP) has been found. Membranes with γ-GTP can be separated from brush border as well as basal-lateral transport and enzyme activities. Free-flow or liquid curtain electrophoresis was used to separate membranes with γ-GTP from brush border membranes containing alkaline phosphatase (AP), 5′-nucleotidase, and the Na^+-dependent D-glucose carrier. The electrophoretic mobility of membranes with γ-GTP was almost identical with that of basal-lateral infoldings containing NaK ATPase. Separation of two membrane populations containing γ-GTP and NaK ATPase was accomplished by using high-resolution density gradient centrifugation with a modified colloidal silica medium (Percoll). Mixtures of renal membranes containing AP in addition to NaK ATPase and γ-GTP were resolved by Percoll density gradient centrifugation into three distinct populations with bouyant densities of 1.038, 1.030, and 1.058 g/cm^3, respectively. The purification of NaK ATPase was 7-fold, that of AP was 5-fold, and that of γ-GTP was 10-fold compared to that of the homogenate. Thus, centrifugation in fixed-angle rotors of renal membranes in reorienting Percoll density gradients not only has defined a unique population of membranes with γ-GTP but also is capable of separating brush borders from basal-lateral infoldings. The rapidity and ease of this centrifugation method make it suitable for the preparative isolation of these three membrane fractions. Although our studies do not establish the anatomical location of membrane-bound γ-GTP, they do define, on the basis of both net surface charge and bouyant density, a previously unrecognized population of membranes containing γ-GTP.

The vectorial movement of solutes across epithelial cells is a result of the polarity of the cellular surface membranes (Ussing & Thorn, 1973). For example, Na^+ cotransport of D-glucose occurs via a phlorizin-inhibitable carrier at the brush border surface of the renal proximal tubule (Kinne et al., 1975; Kinne, 1976). The Na^+ ions which accumulate intracellularly are removed by the NaK ATPase on the basal-lateral surface (Skou, 1972). The glucose exits from the interior of the cell by facilitated diffusion via a phloretin-inhibitable carrier also located on the basal-lateral surface (Novikoff, 1960). With the development of subcellular fractionation procedures, it is now possible to isolate brush border and basal-lateral membrane vesicle populations which exhibit these two different glucose transport systems. Brush border vesicles are enriched for AP[1] while basal-lateral vesicles are enriched for the NaK ATPase (Kinne et al., 1975; Kinne, 1976). These copurifications of enzyme markers with transport systems correlate well with anatomical localization by histochemical and immunocytochemical techniques (Novikoff, 1960; Ashworth et al., 1963).

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[1] Abbreviations used: AP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; FFE, free-flow electrophoresis.

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