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Preparation of Membrane-Free Chromatin Bodies from Avian Erythroid Cells and Analysis of Chromatin Acidic Proteins†

Roger Harlow and Julian R. E. Wells*

ABSTRACT: A primary objective, realized in this study, was the preparation from avian erythroid cells of chromatin free of contaminating membrane, as a prerequisiste to the study of chromatin acidic proteins from cells throughout the maturation pathway. Cells are lysed in saponin (S), washed in Nonidet-P40 (N), and plasma membrane removed by high-speed rotating knives (K). Purified SNK nuclear bodies are recovered free of membrane after centrifugation through 2.35 *M* sucrose. The chromatin acidic proteins from such preparations of the three major avian erythroid cell types were studied. Reticulocyte SNK chromatin was compared with reticulocyte chromatin derived from saponin lysis of cells and subsequent dispersion in EDTA solutions (Harlow et al. (1972), *Cell Differ. 2,* 341). The dispersed preparation has a lower acidic protein/DNA ratio, but the pattern of these proteins is more complex, presumably due to the contaminating membrane. In examining SNK acidic proteins throughout the maturation pathway it is clear that there are quantitative and qualitative differences. In the dividing erythroblast, the pattern of proteins is complex and the amount relative to DNA is 1.25:1.0. For nondividing, but transcriptionally active reticulocytes and also for transcriptionally inactive erythrocytes, the pattern is very much simpler, being dominated by three bands visible on sodium dodecyl sulfate polyacrylamide gels. The ratios of chromatin acidic proteins in these preparations relative to DNA are 0.69:1.0 and 0.36:1.0, respectively. These results, obtained with purified populations of cells from a single cell line, indicate a strong positive correlation between the transcriptional activity of the cell and both the amount and complexity of non-histone proteins associated with chromatin. The correlation does not indicate whether the proteins are the cause or result of increased transcription.

Current interest in the acidic proteins found in isolated eukaryote chromatin stems from the search for factors within the chromatin that may control gene-specific transcription. Histones are unlikely to possess enough specificity for this task, and chromosomal RNA as defined by published preparative procedures (Dahmus and McConnell, 1969; Mayfield and Bonner, 1971) is a product of RNA degradation (Artman and Roth, 1971; Tolstoshev and Wells, 1974).

Cytochemical staining of fixed preparations suggests that there are acidic proteins associated with the genetic material of eukaryotes in vivo (Swift, 1964; Zirkin, 1973), but it is by no means certain that these are adequately represented by the acidic protein populations found in isolated chromatin. The term chromatin is a very loose one, since isolation methods vary widely, and nonspecific loss or addition of components can occur. Some preparations consist essentially of whole nuclei, hypotonically disrupted (Paoletti and Huang, 1969; Sadgopal and Bonner, 1970), whereas others

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involve extensive washing of homogenized nuclear material and the removal of membrane, either by centrifugation through concentrated sucrose (Elgin and Bonner, 1970; Hill et al., 1971) or by treatment with nonionic detergents (Hancock, 1973; Kamiyama et al., 1972; Seale and Aronson, 1973). Thus, many chromatin preparations contain membrane material (Jackson et al., 1968; Tata et al., 1972) and cytoplasmic proteins (Johns and Forrester, 1969) or ribonucleoprotein-particle proteins (Li and Wang, 1971; Bhorjee and Pederson, 1973). Both the amount (Dingman and Sporn, 1964; Marushige and Ozaki, 1967; Levy et al., 1972) and pattern (Hill et al., 1971; Seale and Aronson, 1973; Vidali et al., 1973; LeStourgeon and Rusch, 1973) of chromatin acidic proteins change with transcriptional changes in the cell or in subfractions of the chromatin (Frenster, 1965; Dolbeare and Koenig, 1970). In view of the problems of nonspecific contamination just described, the significance of such correlative results is uncertain.

In the avian erythroid series, purified populations of cells at different maturation stages and with defined transcriptional activities may be obtained: from early dividing erythroblasts (highly active in DNA, RNA, and protein synthesis) through reticulocytes (nondividing but active in RNA and protein synthesis) to mature erythrocytes (inactive in macromolecular synthesis). During maturation the decline in transcriptional activity is accompanied by progressive condensation of the chromatin.

In this paper we discuss (1) the changes in transcription during avian erythroid maturation, (2) the isolation of chromatin acidic proteins from these cells and the contribution of membrane proteins to isolated chromatin, (3) the preparation of membrane-free chromatin bodies, and (4) the analysis of acidic proteins from these preparations in relation to the transcriptional changes occurring during erythroid maturation.

Materials and Methods

Domestic chickens (Whiteleghorn x Australorp pullets), 4–6 months old, were obtained from Royal Park Poultry, South Australia, and maintained on "growers granules" and water. Male albino rats (Wistar) were maintained on mouse cubes and water.

Acrylamide and *N,N'*-methylenebisacrylamide were from Eastman Organic Chemicals, New York; bovine serum albumin and phenylhydrazine-HCl were from Sigma Chemical Co.; Eagle's complete medium and sodium heparin were from Commonwealth Serum Laboratories, Victoria, Australia; hydroxylapatite was from Clarkson Chemical Co., Miss.; Nonidet P-40 was obtained from B.D.H. Chemicals Ltd., Poole, England; and saponin (white) was from Merck. All other chemicals were of analytical reagent grade unless otherwise specified.

Erythroid Cells. Chickens were made anaemic by a course of daily injections with phenylhydrazine-HCl, and cells at different stages of maturation obtained from the circulation or bone marrow as pure populations, by fractionation on discontinuous albumin gradients. The exact procedures have been described elsewhere (Williams, 1972). Erythroblasts were obtained from the marrow of anaemic birds, on albumin layers of density 1.059 g/cm^3. Reticulocytes were obtained from the circulation of highly anaemic hens as a population of cells with buoyant density less than 1.071 g/cm^3, and mature erythrocytes were the circulating red cells of healthy hens.

Chromatin Preparations. ERYTHROID SOLUBLE CHROMATIN. For erythroid cells, chromatin was prepared from saponin-lysed cells by a modification of the method of Dingman and Sporn (1964), as published in detail previously (Harlow et al., 1972). Briefly, cells were lysed by mixing with 2 volumes of 0.5% saponin in isotonic sucrose (0.294 *M* sucrose–1 m*M* $MgCl_2$–1 m*M* potassium phosphate (pH 6.8) and the resulting nucleated ghosts were washed by resuspension and centrifugation (2000*g*, 5 min) in successive 10-ml aliquots of isotonic sucrose (two washes); 0.08 *M* NaCl–0.02 *M* EDTA (pH 6.3) (six washes); and 0.147 *M* NaCl (two washes). The pellet at this stage appeared nearly free of hemoglobin, being off-white in color, and consisted of nucleated ghosts with some cytoplasmic inclusions (Results). The pellet was resuspended in 0.2 m*M* EDTA (pH 7.2) (15 ml/1 ml of original cells) and sheared in a Dounce all-glass homogenizer (Type B; Kontes Glass Co., N.J.) with 30 complete strokes. The chromatin was recovered as a gelatinous pellet by sedimentation for 30 min at 38,000*g* and was resuspended in 0.01 *M* Tris-HCl (pH 8.0) (10 ml/ml of original cells) with 20 strokes of the Dounce homogenizer. The resulting suspension was termed crude chromatin (CC).[1]

In some experiments, crude chromatin was processed further by sedimentation through a 1.7 *M* sucrose cushion (Marushige et al., 1968). The preparation was layered onto a solution of 1.7 *M* sucrose–0.01 *M* Tris-HCl (pH 8.0) in cellulose nutrate tubes, the interface mixed gently, and the preparation centrifuged at 52,000*g* in a SW25.1 rotor for 3 hr at 4°. The amount of chromatin per tube was the equivalent of 0.5 ml of original cells/25-ml tube. After sedimentation, the upper flocculent layer consisting largely of membrane material (M) was aspirated, leaving a transparent gelatinous pellet termed "pure chromatin" (abbreviated X).

ERYTHROID CHROMATIN AS NUCLEAR BODIES. To prepare erythroid chromatin free of membrane contamination it proved necessary to employ the following method.

Extensively washed "saponin ghosts" prepared as described above (after the second wash in 0.147 *M* NaCl) were washed once more in 0.147 *M* NaCl–3 m*M* $CaCl_2$ to remove EDTA, and then in 0.5% Nonidet P-40–3 m*M* $CaCl_2$ to remove outer nuclear membrane. The ghosts were then resuspended in the homogenizing solution described by Zentgraf et al. (1971) (0.01 *M* Tris (pH 7.0), 3% gum arabic (purified; Kuehl, 1964), 0.4 *M* sucrose, and 4 m*M* 1-octanol) with the addition of 3 m*M* $CaCl_2$ to prevent "clumping" of the nuclei, and homogenized for 30 sec at 48,000 rpm in a Sorvall Omnimixer with continuous cooling in an ice bath. The homogenate was made to 10 ml in homogenizing solution, mixed, and centrifuged (1500*g*, 3 min), and the pellet washed in a further 10 ml of homogenizing solution. The pellet was then thoroughly resuspended in 13 ml of 2.35 *M* sucrose–3 m*M* $CaCl_2$ and centrifuged for 40 min at 39,000 rpm in a Beckman SW41 rotor. The final pellet consisted of chromatin bodies free from all membrane contamination (see Results) and still dispersible. Since the preparation involved saponin lysis, Nonidet P-40 detergent, and homogenization with knives these preparations are referred to as "SNK" nuclear bodies.

RAT LIVER CHROMATIN. Chromatin was prepared

[1] Abbreviations used are: albumin, bovine serum albumin; CC, crude chromatin; M, membrane from crude chromatin; X, pure chromatin derived from crude chromatin; SNK, nuclear bodies prepared by saponin lysis, Nonidet-P-40 detergent washing, and rotating knives homogenization of whole cells; SEM, standard error of mean.

from rat liver essentially according to the method of Marushige et al. (1968) with the following modifications. Livers were excised and placed in ice-cold 0.024 *M* EDTA–0.075 *M* NaCl (pH 8.0) and homogenized in a Teflon–glass homogenizer (40 ml of solution/10 g of liver; clearance 0.02 in; 1600 rpm; 10 strokes). After filtration through two layers of butter-muslin and sedimentation for 15 min at 1500*g* the crude nuclear pellet was washed by similar homogenization and centrifugation in 40 ml of EDTA–NaCl solution and two 40-ml changes of 0.05 *M* Tris-HCl (pH 8.0). The gelatinous pellet was resuspended in 0.05 *M* Tris-HCl (pH 8.0) (40 ml/10 g of liver aliquot) using a small-clearance Teflon–glass homogenizer (0.03 in; 1600 rpm; 30 strokes). After sedimentation at 10,000*g* for 15 min this step was repeated once and the final pellet homogenized again under the same conditions, but at 5 ml/10 g of original liver. This preparation was termed "crude chromatin" (CC). Layering in 5-ml aliquots onto 25-ml cushions of 1.7 *M* sucrose–0.01 *M* Tris-HCl (pH 8.0) and centrifugation as described for erythroid crude chromatin generated a membrane layer (M) and pure chromatin pellet (X).

Electron Microscopy. Electron microscopy of nuclei was performed on stained sections prepared by fixing a pellet of nuclei for 30 min in 2% glutaraldehyde and 0.18 *M* cacodylate (pH 7.2) and post-fixing in 1% osmium tetroxide in the same buffer. After dehydration in graded acetone washes, the pellet was embedded in Araldite and sectioned with a glass knife. Sections were picked up on carbon-coated grids and stained for 3 min with lead citrate. Examinations were made in a Siemens Elmiskop microscope at 80 kV with a 50-μ objective aperture.

Chromatin Acidic Proteins. Chromatin acidic proteins were isolated from chromatin by a minor modification of the hydroxylapatite chromatography method of MacGillivray et al. (1971). Columns of hydroxylapatite (1.5 × 10 cm) were poured under gravity and washed at room temperature with 50 ml of buffer 3 (0.5 *M* sodium phosphate (pH 6.8) in 5 *M* urea–2 *M* sodium chloride) and 50 ml of buffer 1 (0.001 *M* sodium phosphate (pH 6.8) again containing urea–sodium chloride). The chromatin sample, thoroughly dialyzed against buffer 1, was sonicated to reduce viscosity (Dawe "Soniprobe" 1130A; 2 × 20 sec treatments; probe immersed; setting 8; 3–4 mA). After dilution to 10 A_{260} units/ml, 100 units were applied to the column and the unbound histone fraction eluted with buffer 1. The acidic proteins were eluted with buffer 2 (0.15 *M* sodium phosphate (pH 6.8)–5 *M* urea–2 *M* sodium chloride), and the DNA and some residual acidic protein with buffer 3. The acidic proteins were recovered as a white powder after exhaustive dialysis against glass-distilled water followed by freeze-drying. Histones were extracted from chromatin by hydroxylapatite chromatography or by acid extraction (0.2 *N* HCl).

Polyacrylamide Gel Analysis. Polyacrylamide gel analysis of proteins was performed in two systems; at low pH, in 15% gels (Panyim and Chalkley, 1969) or in the presence of dodecyl sulfate either in the single gel system of Weber and Osborn (1969) or using the discontinuous system described by Laemmli (1970). These gels were stained with Coomassie Brilliant Blue according to Fairbanks et al. (1971).

[^{14}C]Histone was obtained by incubation of erythroblasts with [^{14}C]lysine (50 μCi/ml of packed cells) in Eagle's minimum essential medium minus lysine, for 60 min at 37°. The histones were extracted from washed nucleated ghosts prepared as described above, with 0.2 *N* HCl.

Measurement of RNA Synthesis. Cells were incubated in Eagle's Basal Medium (80%) plus dialyzed rabbit serum (20%). The medium was buffered with 0.01 *M N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid to pH 7.4 and isotonicity adjusted with solid NaCl. The whole medium was sterilized by filtration through 0.45-μ Millipore filters. Cells (4×10^7/ml of medium) were incubated in the presence of 5 μCi of [^{3}H]uridine (31 Ci/m*M*)/ml. Duplicate samples of 10^6 cells were taken at various times and processed to determine incoporation of radioactivity into 5% Cl_3CCOOH-insoluble material.

Analytical Methods. DNA was estimated by the diphenylamine reaction (Burton, 1956) using avian erythrocyte DNA as standard (Marmur, 1961), and assuming ϵ_{260} 6500 for DNA-phosphate. Protein was estimated according to Lowry et al. (1951) using albumin as standard ($A_{278.5}$ = 6.61 at 10 mg/ml; Peters and Blumenstock, 1967). Total cholesterol was determined as described by Clark et al. (1968), and phospholipid as described by Gehrlach and Deuticke (1963).

Results and Discussion

Transcription in Avian Erythroid Cells. During avian erythroid maturation, progressive condensation of the genetic material is accompanied by a pronounced decrease in levels of transcription, as measured by [^{3}H]uridine incoporation (Figure 1a). Erythroblasts are highly active, reticulocytes show a fourfold lower initial rate of incorporation, and erythrocytes are essentially inactive. There has been a report of RNA synthesis in mature avian erythrocytes (Madgwick et al., 1972), but in our hands, incorporation of [^{3}H]uridine in these cells is consistently at background levels compared with the earlier cells.

Isolation of EDTA particles (Pemberton et al., 1972) from the polysomes of reticulocytes prelabeled with [^{3}H]uridine reveals that there is essentially no rRNA synthesis, whereas a 10S RNA species is labeled (Figure 1b). This RNA has been translated in cell-free systems into α and β chicken globin chains (J.R.E. Wells and A. Scott, unpublished results). Thus, chromatin from reticulocytes and erythrocytes represent templates that are, respectively, competent for and unavailable for globin mRNA transcription. By comparing chromatin acidic proteins of the two cell types, changes in their composition may be correlated with transcriptional differences. Further information may be gained by considering similar comparisons with erythroblasts. It must be emphasized that such studies cannot alone establish a causal connection between chromatin components and the gene-specific control of transcription. They are, however, an essential first step in testing such hypotheses.

Isolation of Chromatin Acidic Proteins. In choosing a preparative method for the acidic proteins of isolated erythroid chromatin, the use of acid was avoided. Although this was the original operation defining histones and "non-histone" proteins, there has been a report that acid treatment of chromatin can cause significant histone binding to the chromatin (Sonnenbichler and Nobis, 1970) and further that structural alterations in the acidic proteins may also occur (Spelsberg et al., 1973). For its simplicity and rapidity, a modification of the hydroxylapatite chromatography method of MacGillivray et al. (1971) was chosen, in which chromatin is dissociated in 5 *M* urea–2 *M* sodium chloride and separated into histone, acidic protein, and DNA fractions by elution from a single column with stepwise in-

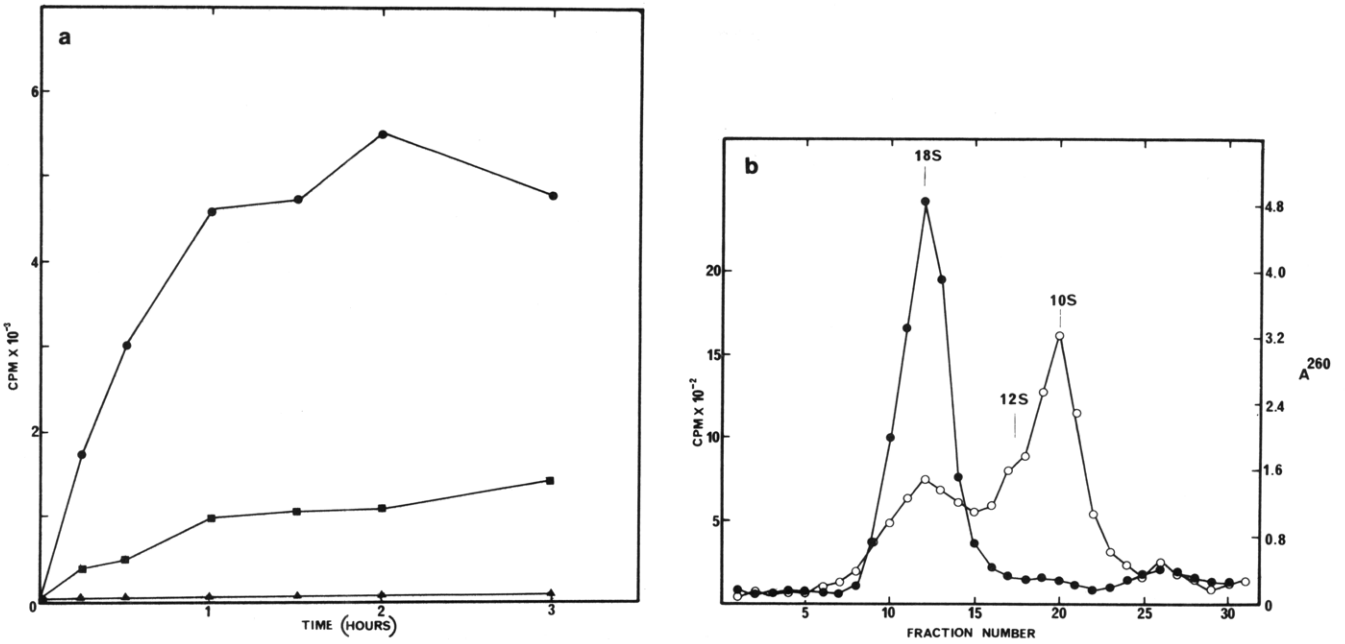

FIGURE 1: RNA synthesis in avian erythroid cells. (a) The procedures used were those of Williams (1971). Cells were incubated with [^{3}H]uridine at 1 μCi/2 × 10^8 cells. Samples were removed at the times shown and lysed and the Cl_3CCOOH-insoluble material was collected on filters and counted. (●) Erythroblasts; (■) reticulocytes; (▲) erythrocytes. (b) Reticulocytes were incubated with [^{3}H]uridine as described and the 20S–30S region isolated from sucrose gradients of EDTA-treated polysomes (Pemberton et al., 1972). RNA derived from this region was then centrifuged on a dodecyl sulfate–sucrose gradient (SW41 rotor, 40,000 rpm, 14 hr) and fractions were collected. (●) A_{260}; (○) cpm.

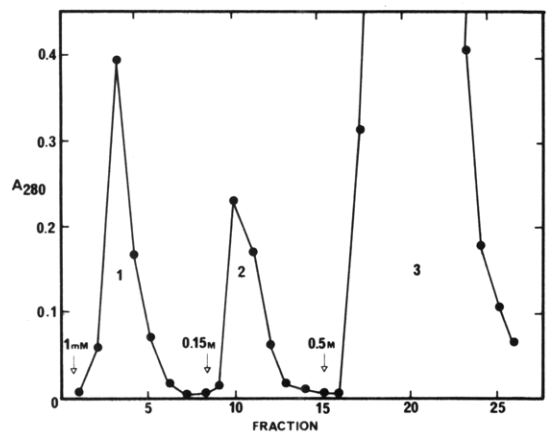

FIGURE 2: Hydroxylapatite fractionation of chromatin. Reticulocyte crude chromatin was prepared as described under Materials and Methods, and dissolved in 5 *M* urea–2 *M* NaCl–1 m*M* sodium phosphate (pH 6.8). After sonication and suitable dilution (Materials and Methods), 100 A_{260} units was loaded onto a column of hydroxylapatite (1.5 cm × 10 cm) and eluted with stepwise increases in phosphate concentration as shown.

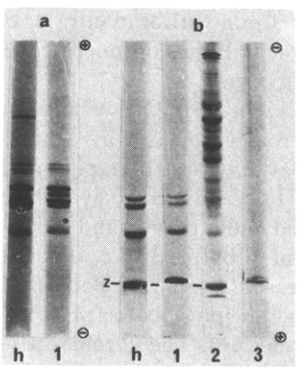

FIGURE 3: Analysis of hydroxylapatite chromatin fractions. Reticulocyte crude chromatin was fractionated on hydroxylapatite columns as described under Materials and Methods. Peak material from each buffer change was dialyzed exhaustively against water, freeze-dried, and analyzed on polyacrylamide gels (a) at low pH and (b) in presence of dodecyl sulfate (Laemmli, 1970). (h) Acid-extracted histone; (1, 2, 3) hydroxylapatite fractions.

creases in phosphate concentration.

In initial experiments with chicken erythroid chromatin, it was found that the phosphate step of 0.05 *M* used by MacGillivray et al. (1971) to define the acidic protein fraction left quite large amounts of acidic protein bound to the column (16% in the case of reticulocyte crude chromatin). Since native DNA is not normally eluted from hydroxylapatite until a phosphate concentration of 0.20–0.25 *M* is reached (Bernardi, 1969), it seemed likely that more acidic protein could be retrieved without eluting DNA by use of a phosphate concentration considerably higher than 0.05 *M*. By elution of chromatin from hydroxylapatite with a linear gradient of phosphate, it was found that the maximum practical concentration was 0.15 *M*. Figure 2 shows the elution profile obtained for reticulocyte crude chromatin using phosphate steps of 0.001 *M*, 0.15 *M*, and 0.5 *M* to define the histone, acidic protein, and DNA fractions, respectively. Peak 1 consisting of histone, comprises approximately 70% of the total chromatin proteins. In experiments in which ^{14}C-labeled histone prepared from erythroblasts by extraction with 0.2 *N* HCl was added to the chromatin prior to chromatography, 93.3% of the label emerged in this peak, and the rest in peak 2. Polyacrylamide gel electrophoresis of peak 1 material at low pH (Panyim and Chalkley, 1969) gives a pattern closely resembling that of histones extracted by mineral acid, with no contamination by other basic proteins (Figure 3a). The absence of contaminating acidic proteins was confirmed by electrophoresis on polyacrylamide gels containing dodecyl sulfate; the pattern of peak 1 components again closely resembles that of acid-extracted histone, and consists of only four bands, since separation in this system is based on size alone (Figure 3b).

Peak 2 contains about 28% of the total chromatin protein and electrophoresis on low pH gels fails to reveal any histone contamination, even at extremely high loadings (5 mg of protein/gel). However, electrophoresis on gels containing dodecyl sulfate shows a complex band pattern including species with widely differing molecular weights (Figure 3b). With the exception of one band (z) there appears to be little material in peak 2 with the same mobility as histone. Band z accounts for a significant proportion of the acidic protein in reticulocyte crude chromatin. It is possible that this band is due at least in part to contaminating histone in peak 2 and this point is considered subsequently (see Table II).

Peak 3 contains all the DNA present in the chromatin, which is eluted in 100% yield. A small amount (2–3%) of the total chromatin protein is retained by this peak, a considerably lower amount than that remaining after a 0.05 *M* phosphate step, as originally recommended (MacGillivray et al., 1971). Analysis of peak 3 on any of the acrylamide gel systems used gave no well-defined pattern, probably because of the large amount of DNA present, but the possibility remains that a unique class of protein is present here.

Membrane in Avian Erythroid "Soluble" Chromatin. In a previous study (Harlow et al., 1972) we reported that avian erythroid chromatin prepared from saponin-lysed cells and purified through a 1.7 *M* sucrose density barrier (Marushige and Bonner, 1966) still contained significant amounts of membrane. From analytical data on the purified preparation (X) and the membrane removed from it (M; see Materials and Methods) it was suggested that membrane might contribute a major percentage of the non-histone components of chromatin. Any conclusions about the contribution of membrane protein to chromatin acidic protein of pure chromatin depended on the equivalence of membrane types found in fractions M and X. Given such equivalence, one might expect similar species to be present in the avidic protein component of crude chromatin (CC) and the fractions derived from it, namely, membrane (M) and pure chromatin (X). These proteins were analyzed on polyacrylamide gels containing dodecyl sulfate and it is clear that the patterns are similar for CC, M, and X (Figure 4a). These findings support our previous conclusion that acidic proteins of chromatin prepared by classical procedures (Marushige and Bonner, 1966) may include species of nonchromosomal origin. Chromatin was also prepared from rat liver (Marushige and Bonner, 1966; Materials and Methods) and fractions equivalent to CC, M, and X were analyzed. Not only was a large amount of membrane found in fraction X (unpublished observations), but again the acidic protein component from all three fractions appeared to be remarkably similar (Figure 4b). It seems likely that sedimentation of crude chromatin through 1.7 *M* sucrose can give extremely variable results with regard to the amount of membrane removed.

Despite the consistency of the data presented on the membrane origin of chromatin acidic proteins in avian erythroid and rat liver chromatin, it was found that the degree of apparent similarity between the acidic proteins of erythroid crude chromatin, membrane, and pure chromatin was dependent on the gel system used. In Figure 4c the proteins are separated on discontinuous dodecyl sulfate polyacrylamide gels (Laemmli, 1970), and it is apparent that, despite similarities between mobilities of major bands n and o (48,000 and 43,000 daltons) and many less prominent species in the regions a–g and o–t, from preparations CC, M, and X, there now appear to be major species preferentially associated with pure chromatin (X). In particular, bands j (65,000 daltons) and z (14,500 daltons) are markedly enriched in this fraction. In this gel system, virtually all the protein enters the separating gel, and it is clearly a superior method for resolving all the acidic protein species.

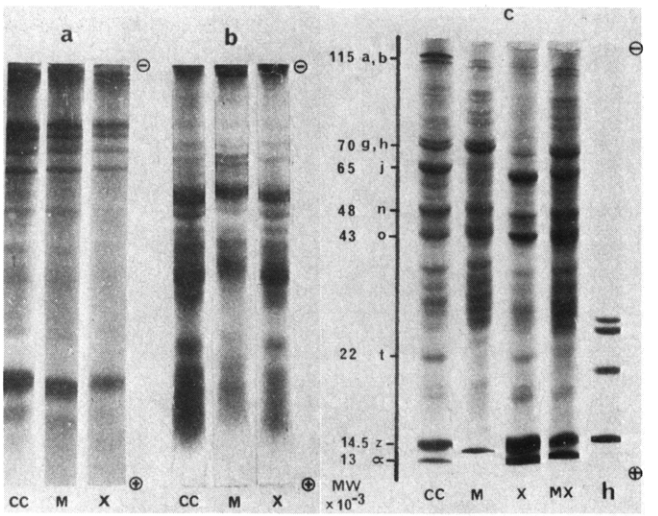

FIGURE 4: Acidic proteins of chromatins fractionated on 1.7 *M* sucrose. Crude chromatin (CC) from (a, c) chicken reticulocytes or (b) rat liver was fractionated on 1.7 *M* sucrose barriers (Harlow et al., 1972; Marushige and Bonner, 1966) to give membrane (M) and pure chromatin (X) fractions. Acidic proteins were extracted and compared; (a) chicken reticulocyte chromatin and (b) rat liver, both on continuous buffer, 7.5% polyacrylamide dodecyl sulfate gels (Weber and Osborn, 1969), (c) chicken reticulocyte chromatin fractions on discontinuous buffer, 10% polyacrylamide dodecyl sulfate gels (Laemmli, 1970). Molecular weights were determined by coelectrophoresis with six known protein standards, h, histone.

It is clear that saponin lysis of avian erythroid cells followed by subsequent exhaustive washing of the nuclear preparations, as used in earlier studies (Appels et al., 1972; Harlow et al., 1972) was not effective in removing plasma membrane, so that crude chromatin, despite extensive washing, was in fact derived from cell ghosts (Figure 5a). Particular emphasis has now been placed on the preparation of avian erythroid nuclei which, by the criterion of electron microscopy, are devoid of membrane.

Preparation of Chromatin as "Nuclear Bodies". Dingman and Sporn (1964) found it impossible to remove the plasma membrane from avian erythroid nuclei by Dounce homogenization in isotonic media, and our experience with several homogenization methods bears this out. The plasma membrane remains extremely resistant to shear even under hypotonic conditions, and we have found that phase-contrast microscopy is often inadequate to show membrane contamination, whereas membrane fragments adhering to nuclei are clearly visible by electron microscopy.

Hancock (1973) has described a method for complete lysis of cultured mouse cells using the detergent Nonidet P-40; all membrane structures break down in a 0.5% detergent solution, releasing the chromosomal material as membrane-free bodies with the form of whole nuclei. In the present study, it was found that while chicken reticulocytes are easily lysed by this method, just as they are by the detergent saponin (that is, the plasma membrane is rendered permeable to large molecules, and the cytoplasmic hemoglobin may be washed out), the plasma membrane is not removed. The addition of 3 m*M* calcium chloride was found to be necessary to prevent the nuclear pellet from coagulating during successive cycles of detergent treatment, and it is possible

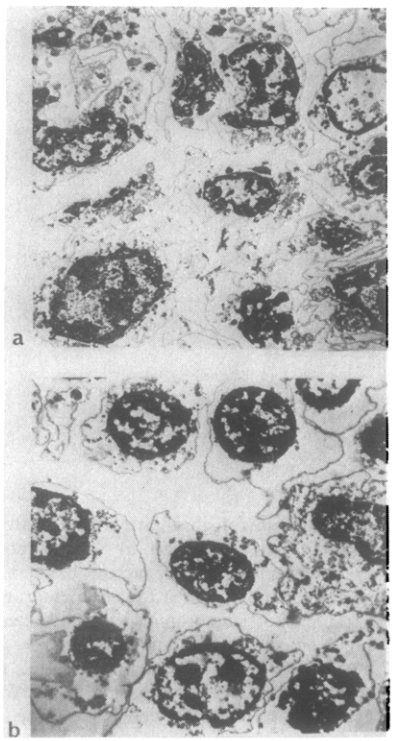

FIGURE 5: Nucleated reticulocyte ghosts produced by detergent lysis. (a) Cells lysed in 0.5% saponin–isotonic sucrose, extensively washed in EDTA–NaCl and 0.147 *M* NaCl (Materials and Methods); (b) cells lysed in 0.5% Nonidet P-40, as described by Hancock (1973), but with addition of 3 m*M* $CaCl_2$. Electron microscopy was performed as described under Materials and Methods. Magnification, ×4000.

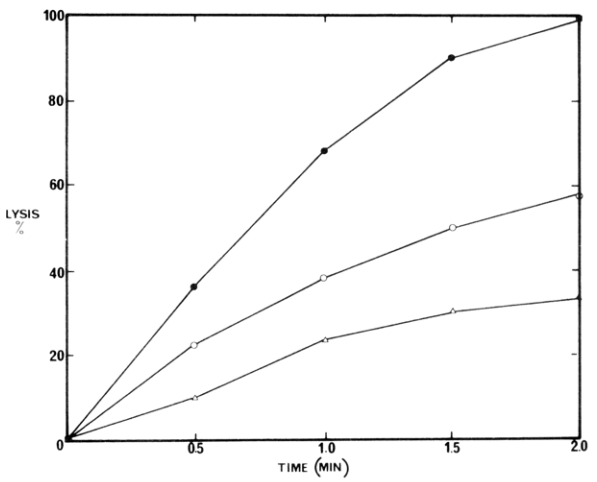

FIGURE 6: Lysis of cells with rotating blades. Reticulocytes were homogenized at various speeds in a Sorvall Omnimixer Micro attachment, using 0.4 *M* sucrose, 3% gum arabic, 4 m*M* 1-octanol, 0.01 *M* Tris-HCl (pH 7.0) at 5 ml/0.5 ml of packed cells. Samples were removed at various times and lysis was estimated by sedimenting the cells and ghosts and measuring A_{540} of the released hemoglobin; full lysis was measured in a separate sample treated with 0.5% Nonidet P-40 in the same final volume. (●) 22,000 rpm; (○) 15,000 rpm; (△) 7500 rpm.

that this modification prevents successful solubilization of the plasma membrane; even after three cycles of detergent treatment and sedimentation, it remains as a discrete structure surrounding the nucleus (Figure 5b). Visually these nucleated ghosts appear pink in color, indicating that much hemoglobin is still present; this is reflected in a high protein/DNA ratio (Table I). Significant amounts of phospholipid and cholesterol reflect the presence of plasma membrane, although phospholipid levels are slightly lower than those of crude chromatin, possibly indicating some membrane solubility in the detergent.

Table I: Composition of Reticulocyte Chromatin Preparations.[a]

Preparation	Protein	Histone	Acidic Protein	Cholesterol	Phospholipid
CC	2.71	1.59	0.78	0.205	0.613
X	1.55	1.36	0.30	0.080	0.102
Nonidet	4.13	2.74	1.39	0.203	0.774
Knives	4.20	2.21	1.98	0.085	1.49
NK	4.14	1.72	2.40	0.082	0.24
SNK	1.94	1.30	0.69	0.007	0.022

[a] Chromatin and nuclear preparations from reticulocytes were analyzed on hydroxylapatite columns and by lipid extraction (Folch et al., 1957) and estimation (Materials and Methods). Composition is expressed relative to DNA on a w/w basis (except for phospholipid; mole/mole of P) and values are the mean of at least two determinations. Symbols: CC, crude chromatin; X, pure chromatin; "Nonidet", cell ghosts as in Figure 5b; "Knives", nuclei produced by high-speed homogenization of whole cells and purified on 2.35 *M* sucrose as in Figure 7b; "NK", the same nuclei after washing with 0.5% Nonidet P-40–3 m*M* $CaCl_2$ as Figure 7c; SNK, nuclear bodies prepared as described under Materials and Methods (see Figure 7d).

Zentgraf et al. (1969) have described a method for preparing clean nuclei of chicken erythrocytes by homogenization with sharp rotating blades. The machine originally used was a Bühler homogenizer, and the optimal conditions involved homogenization of washed cells at 8000 rpm for a total of 90 sec, in a medium consisting of 3% gum arabic, 0.4 *M* sucrose, 4 m*M* 1-octanol, and 0.01 *M* Tris-HCl (pH 7.0). Sedimentation of the homogenate through concentrated sucrose yielded a final 50% of clean nuclei. Figure 6 shows the efficiency of the Sorvall omnimixer (a machine with similar blade geometry and speed capability) in homogenization of reticulocytes in this solution. To obtain full lysis (judged only by hemoglobin release) in 90 sec, a speed of at least 22,000 rpm was required. Furthermore, erythrocytes proved twice as resistant to lysis as reticulocytes. When avian reticulocytes were homogenized for 90 sec at 22,000 rpm, and the mixture sedimented over 2.35 *M* sucrose (which allows only membrane-free nuclei to penetrate), no pellet was observed. Examination of these lysates by electron microscopy showed that only whole cells and nucleated ghosts were present. In order to obtain the plasma membrane-free nuclei described by Zentgraf et al. (1969) a speed of 48,000 rpm was found to be necessary. At this speed, all hemoglobin was released into the supernatant within 90 sec, but only about 30% of the original DNA was recovered as membrane-free nuclei after sedimentation through 2.35 *M* sucrose, the remaining DNA being accounted for as nonsedimented nucleated ghosts. Examination of sections of such clean nuclei by electron microscopy showed little plasma membrane (Figure 7b). Despite the loss of much nucleoplasmic material (compare with nuclei of fixed intact cells; Figure 7a) these nuclei have a protein/DNA ratio of 4.2, considerably higher than that of "soluble" crude chromatin preparations (Table I), although the lipid content is much lower, and can be lowered still further by washing in Nonidet P-40 solution (Figure 7c). The extra protein present in these nuclei emerges in both the histone and acidic protein peaks during hydroxylapatite chromatography (unpublished results) and probably represents soluble

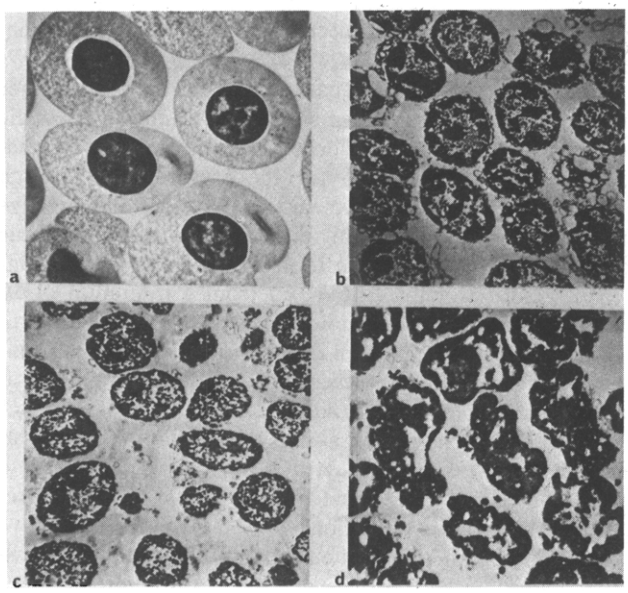

FIGURE 7: Nuclear preparations from avian reticulocytes. (a) Various nuclear preparations were examined by electron microscopy of thin sections in comparison with those of whole cells. (b) "Knives" nuclei prepared by homogenizing whole cells at high speed in the Sorvall omnimixer (48,000 rpm, standard medium, 30 sec), washing twice in the same medium, and sedimentation through 2.35 *M* sucrose (Materials and Methods). (c) Nuclei NK, produced by washing "Knives" nuclei once in 10 volumes of 0.5% Nonidet P-40–3 m*M* $CaCl_2$. (d) Nuclear bodies, SNK, prepared from saponin-lysed cell ghosts as described under Materials and Methods. Magnification ×4000.

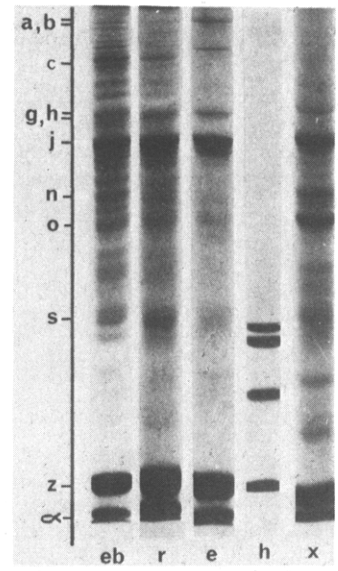

FIGURE 8: Chromatin acidic protein patterns during erythroid maturation. Cells from the three major cell types were used to prepare SNK chromatin bodies. These were fractionated on hydroxylapatite and the acidic proteins compared on discontinuous dodecyl sulfate polyacrylamide gels (Laemmli, 1970); eb, erythroblast; r, reticulocyte; e, erythrocyte; h, reticulocyte histone. Gel x shows acidic proteins from reticulocyte chromatin preparation X (Materials and Methods), obtained by saponin lysis, dispersion in EDTA solution, and sedimentation through 1.7 *M* sucrose.

nucleoplasmic and cytoplasmic protein. The acidic protein pattern of such preparations contains many bands not found in "soluble" chromatin preparations (Materials and Methods).

Nuclei prepared by high-speed rotating blades only were still pink in color, and efforts to reduce the protein/DNA ratio to levels considered normal for chromatin were unsuccessful using washes with either 0.5% Nonidet P-40 or 0.024 *M* EDTA–0.08 *M* NaCl. Since efficient washing of the nucleated ghosts prepared by saponin lysis (Figure 5a) can easily be performed without "clumping" using EDTA–NaCl and isotonic NaCl, an attempt was made at removal of plasma membrane from highly washed nucleated ghosts by the same homogenization method used for whole cells. Ghosts were prepared and washed to the last EDTA–NaCl change as previously described (Harlow et al., 1972) and then washed twice with 10 volumes of 0.147 *M* NaCl–3 m*M* $CaCl_2$ to remove EDTA. At this stage the cell ghosts were suspended in 10 volumes of 0.5% Nonidet P-40-3–m*M* $CaCl_2$, which removes the outer nuclear membrane while maintaining the chromatin as a nuclear body inside the intact plasma membrane. The sedimented ghosts were then suspended in 10 volumes of homogenizing medium (3% gum arabic, 0.4 *M* sucrose, 4 m*M* 1-octanol, and 0.01 *M* Tris-HCl (pH 7.0)) to which 3 m*M* $CaCl_2$ had been added. Homogenization at 48,000 rpm for 30 sec followed by sedimentation through 2.35 *M* sucrose–3 m*M* $CaCl_2$ gave a dispersible white pellet containing over 95% of the input DNA. This preparation (SNK; saponin–Nonidet–knives nuclear bodies) is free of morphologically recognizable membrane under the electron microscope (Figure 7d) and consists of chromatin bodies having the form of whole nuclei, slightly swollen in size. These chromatin bodies can be prepared from all three major cell types of the avian erythroid series and all appear essentially free of membrane contamination as judged by electron microscopy.

Analysis of Chromatin from Nuclear Bodies. Parameters of various reticulocyte nuclear preparations are compared with those of the "soluble" crude chromatin (CC) and pure chromatin (X) in Table I. The very low levels of cholesterol and phospholipid in SNK support the observation that membrane is virtually absent in the preparation. The histone/DNA ratio is marginally higher for crude chromatin, but is very similar for pure chromatin and SNK. On the other hand, total protein/DNA is significantly higher for SNK than pure chromatin, and this is reflected in a 2.3-fold higher ratio of acidic protein/DNA. However, the increase is not explained by an increased number of protein species; in fact, the patterns of acidic proteins are comparable. Gels from these preparations on which equal amounts of protein were loaded are shown in Figure 8. Preparation X contains major bands n and o not prominent in SNK; these are probably of membrane origin (see Figure 4c), since previous data (Harlow et al., 1972) have shown that after centrifugation of crude chromatin over 1.7 *M* sucrose, at least 93% of the DNA is recovered in pure chromatin (X). Hence fraction M consists largely of membrane (the data show high ratios of protein and lipid relative to DNA in this fraction), but significant amounts of membrane are present in pure chromatin. It is concluded that the lower acidic protein content of preparation X relative to SNK preparations is due to the several homogenization and centrifugation steps involved in the former preparative procedure (Harlow et al., 1972).

Most of the staining material on gels of SNK acidic protein from reticulocytes and erythrocytes is accounted for by bands α (13,000 daltons), z (14,500 daltons), j (65,000 daltons), and g (70,000 daltons). Bands z and α require further comment since they migrate with the same mobility as a histone component and globin (respectively) in this gel system. Major acidic protein components with mobilities iden-

Table II: Amino Acid Composition of Band z and Histone from Dodecyl Sulfate Gels.[a]

Amino Acid	Mol %	
	Histone Band	Band z
Asp	5.9	5.7
Thr	6.0	6.3
Ser	7.6	7.3
Glu	8.9	11.0
Pro	3.4	4.0
Gly	10.3	9.1
Ala	11.9	11.9
Val	5.3	5.3
Met	0.6	0.7
Ile	4.2	4.2
Leu	8.0	8.7
Tyr	3.0	2.5
Phe	1.7	3.1
Lys	11.8	8.4
His	2.4	2.3
Arg	9.5	9.8

[a] Erythrocyte SNK chromatin was fractionated on hydroxylapatite and the peak 1 and 2 material run on discontinuous dodecyl sulfate polyacrylamide gels (Laemmli, 1970). Band z (peak 2) and the corresponding histone band (peak 1) were cut out of the gels and the protein was eluted by incubation for 8–12 hr in 0.1% sodium dodecyl sulfate. After exhaustive dialysis, samples were freeze-dried, acid hydrolyzed (6 *N* HCl, 16 hr, 100°), and sealed under vacuum in the presence of a trace of phenol. Analyses were carried out on a Beckman 120 C automatic amino acid analyzer fitted with a tenfold scale expansion device. Estimates were made on two separate preparations and are expressed as the mean. In the duplicate analyses the greatest variation for any residue was 1.3 mol % and in most instances the values were much closer than this.

tical with histone species are commonly observed in dodecyl sulfate gels (Elgin and Bonner, 1970; Hill et al., 1971). Because acidic protein preparations do not reveal bands on low pH gels it is assumed that histones are not present. We also fail to observe such bands even on overloaded gels, but this result is readily explained if aggregation of a histone component with the acidic proteins prevents penetration into the gel. Studies in which purified [^{14}C]histone is added to SNK chromatin prior to hydroxylapatite chromatography show that contamination of the acidic protein fraction (although relatively minor) can occur, and that this labeled material subsequently runs with the same mobility as band z on dodecyl sulfate gels (unpublished results). Amino acid analyses of band z derived from hydroxylapatite peak 2 and the corresponding histone band (Figure 8) derived from peak 1 material are shown in Table II. Technically the analyses were entirely satisfactory, since agreement between duplicate analyses were excellent and only peaks corresponding to expected amino acids were observed (this was not the case if gels slices were homogenized during extraction of the protein from the gel). With the exception of glutamic acid and lysine, there is very close agreement between values obtained for all residues in band z compared with the histone material. Despite this, the differences observed for glutamic acid and particularly for lysine (a difference of 3.4 mol %) cannot be ignored. It is quite possible that the bands analyzed consist of more than one protein species and further characterization of this material is being undertaken to determine how much (if any) of band z is due to histone.

Because band α (Figure 8) has precisely the same mobility as purified [^{14}C]avian globin chains (unpublished results), it is likely that a major proportion of this material is globin. These findings emphasize the problem of assigning chromosomal origins to species isolated as chromosomal acidic proteins.

Comparison of SNK preparations from the three major erythroid cell types shows a significant decrease in acidic protein content as maturation occurs; the ratio acidic protein/DNA falls from 1.25 for the erythroblast, through 0.69 for the reticulocyte, to a final 0.36 in the inactive erythrocyte (Table III). This difference appears to be due to a progressive decrease in the complexity of the population of acidic proteins (Figure 8) from a minimum of 30 species of widely varying molecular weights in the erythroblast, a less complex pattern in the reticulocyte, to the simple population found in the erythrocyte preparation, dominated by the three major bands j, z, and α. The significance of two bands in the region a–c in the erythrocyte preparation but absent in reticulocytes is not known. Band j is a major component of all acid protein preparations and requires further characterization.

It is difficult to know which (if any) of the bands visualised on dodecyl sulfate gels of chromatin acidic proteins from SNK preparations might be of significance in gene-specific transcription, but electron micrographs and analyses of cholesterol and phospholipid levels reported here give some degree of confidence that the proteins are of chromosomal origin in vivo. Most previously published studies of chromatin acidic proteins of nondividing avian erythroid cells show much more complicated band patterns (Shelton and Neelin, 1971; Vidali et al., 1973) probably due to membrane and possibly cytoplasmic contamination, since chromatin was prepared merely by saponin lysis of cells and extensive washing in isotonic sodium chloride solution (cf. Figure 5a). The patterns recently reported by Sanders (1974) are much simpler, approaching those obtained for reticulocyte SNK nuclei. On this basis, and considering the low protein/DNA ratios obtained (1.42–1.68), Sander's use of homogenization in 0.5% Triton X-100 followed by sedimentation through 2 *M* sucrose probably does remove much membrane material.

The nuclear bodies described here retain the form of whole nuclei, and as such provide a good starting point for the study of a number of aspects of chromatin composition and function. It is possible to obtain these preparations from all three major erythroid cell types, whereas in the past, preparation of dispersed chromatin from erythroblasts has been difficult due to nuclease action. Until recently, analyses of acidic proteins from avian erythroblasts had not been reported.[2]

There have been numerous publications on the preparation and properties of eukaryote chromatin, and the approach of Bhorjee and Pederson (1973) in which nuclear components are fractionated on the basis of size, and in which particular note is taken of chromatin contamination by nucleoli and heterogeneous nuclear RNA-containing particules, is noteworthy. We undertook the present study chiefly because of evidence that membrane contamination occurs in chromatin prepared from detergent-lysed cells,

[2] After submission of this manuscript, Ruiz-Carrillo et al. (1974) published data on non-histone proteins from dividing and nondividing duck red cells. In essence the results agree in that a complex population of non-histone proteins of molecular weight greater than 20×10^3 which are obvious in erythroblasts markedly decrease as maturation proceeds and there is a parallel decrease in the overall amounts of these proteins. Strict comparisons cannot be made, however, as different preparative and analytical methods have been used, but band "j" reported here is probably equivalent to band 9 of these authors.

Table III: Composition of SNK Chromatin during Erythroid Maturation.[a]

Cell Type	Protein DNA	Histone DNA	Acidic Protein DNA
Erythroblast	2.43 ± 0.15	1.17 ± 0.05	1.25 ± 0.11
Reticulocyte	1.94 ± 0.21	1.30 ± 0.23	0.69 ± 0.13
Erythrocyte	1.62 ± 0.20	1.28 ± 0.17	0.36 ± 0.03

[a] SNK chromatin bodies were prepared and analyzed as described under Materials and Methods from the three major cell types of the erythroid cell series. Values are expressed on a w/w basis, ±SEM (n = 3).

and therefore we were particularly concerned with the problem of preparing nuclei free of plasma membrane. Like Bhorjee and Pederson (1973) we do not suggest that any one preparative procedure is superior to others for all purposes, but we would like to emphasise several points. In our experience it is not always easy to judge adequately the presence of membrane contamination in nuclear or chromatin preparations by phase contrast microscopy; we have used the electron microscope, and would advocate the presentation of electron micrographs to show nuclear preparations. It is likely that different cell types require different techniques for the preparation of nuclei. For avian erythroid cells, we have found that high-speed homogenization of saponin-ghosts with rotating knives is superior to methods involving Dounce homogenization or French pressure cell treatment of whole cells, particularly with regard to membrane and cytoplasmic (hemoglobin) contamination. SNK nuclear bodies represent a preparation in which the extensively washed chromatin is maintained in the physical form of whole nuclei, and analyses have been carried out directly on it without sonication and subsequent centrifugation of the material, since losses of chromatin-associated components might occur. Despite the higher levels of chromatin-associated protein in SNK compared with preparation X, the pattern of proteins is simpler and for reasons already discussed is more likely to be representative of nuclear acidic proteins in vivo.

Three major bands dominate the pattern of SNK acidic proteins from reticulocytes and erythrocytes, although the pattern is much more complex in erythroblasts. The simplicity of acidic protein patterns observed in the more mature cells suggests that some of the major chromatin acidic proteins are structural elements, equivalent in this sense to the histones. Band z, for example, is similar to a major component seen in sea-urchin chromatin (Hill et al., 1971) (band 18), and to a band, α, common to chromatin preparations of various rat tissues (Wu et al., 1973). As already discussed, the characterization of band z requires further study.

The changes we observe in the pattern and amount of chromatin acidic proteins as avian erythroid cells mature are consistent with the hypothesis that this class of chromatin proteins contains gene-specific regulatory molecules. Equally, the observed changes might be results rather than causes of altered gene expression. Molecules involved in transcriptional regulation in eukaryotes may be present at levels of only a few molecules per cell, by analogy with prokaryote systems (Gilbert and Müller-Hill, 1966), and gel electrophoresis at the present sensitivity would be inadequate in detecting them, even assuming they were chromatin bound.

While the comparison of chromatin components from reticulocytes and erythrocytes can suggest a possible role in control of globin mRNA transcription, an assay for in vitro transcription of the globin gene is a more direct test of the role of such components. Gilmour and Paul (1973) have found that the RNA transcribed in vitro from chromatin of mouse hemopoietic cells contains globin mRNA sequences at the level of 1–2 parts in 10^5 (see also Axel et al., 1973). To determine which components are necessary for the initiation of transcription of HnRNA containing globin mRNA sequences will be more difficult.

In this study it is shown that the most active cell (erythroblast) contains more chromatin acidic protein and a greater complexity of protein species than the less active cells. If this result is of biological significance then fractionation of chromatin from *one* cell type into "active" and inactive" components may give further clues to the possible significance of chromatin-associated proteins in specific transcription.

Acknowledgments

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