Differential Salt Fractionation of Active and Inactive Genomic Domains in Chicken Erythrocyte*

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We have utilized the Sanders salt fractionation technique (Sanders, M. M. (1978) J. Cell Biol. 79, 97-109) to analyze the products of micrococcal nuclease digestion of adult chicken erythrocyte nuclei. By dot-blot hybridization with specific gene probes, it is found that nucleosomes from the globin gene domain, including a region extending to about 10 kilobase pairs 5' to the β gene are selectively enriched in the fractions eluted at low salt. In contrast, a single copy sequence located at about 10 kilobase pairs 5' to the β gene was concentrated in the less salt-soluble fractions. The vitellogenin and ovalbumin genes, which are never expressed in erythroid tissues, are also concentrated in the less salt-soluble fractions. Some more generally expressed genes (histone H4, thymidine kinase) appear to be more uniformly distributed. The low salt fractions are depleted in H3/H5, enriched in high mobility group 14 and 17, and contain somewhat more highly acetylated histones.

The special structural and/or compositional features that characterize a particular chromatin state have so far eluded characterization. One of the problems in obtaining this information has been the lack of a general and reliable method which would allow the separation of active and inactive regions.

The salt fraction technique developed by Sanders (1978) shows promise for such an application. The technique is based on Sanders' observations that if care is taken to prevent removal of Mg2+ during nuclear isolation and nuclease digestion (which can be accomplished by arresting digestion with EGTA rather than EDTA), the differential condensation of native chromatin is undisturbed even under conditions where the chromatin is cleaved by micrococcal nuclease. The ionic perturbation of digested nuclei prepared under these conditions by increasing concentrations of NaCl gradually dissociates subpopulations of nucleosomes differing in their packing interactions.

Davie and Saunders (1981) have extended these observations to show that the nucleosomes soluble at the lowest salt concentrations were highly enriched in transcriptionally competent gene sequences, as assayed by distribution of the label after salt fractionation of nick-translated calf thymus nuclei.

We think that more definitive criteria to unambiguously establish the relationship between transcriptional activity and differential solubility of the chromatin fractions should come from investigating the selective distribution of expressed genes among low and high salt fractions. The present communication shows the result of such investigation in adult chicken erythrocytes.

Chicken erythroid nuclei provide distinct advantages for such a study, since gene expression in this system has been extensively studied (see, for example, Stalder et al. (1980), Groudine and Weintraub (1982)). However, it should be noted that transcriptional activity is exceedingly low in mature erythrocyte nuclei; we are not, therefore, investigating active genes. Nevertheless, it has been shown that many of the characteristics of transcriptional activity (DNase I sensitivity and hypersensitive sites, for example) are retained in those genes which had been active at early stages of erythroid development (Groudine and Weintraub, 1982). It is perhaps preferable to refer to regions of the chicken genome that have been transcribed in early erythroid cells, but are no longer transcribed in mature erythrocytes, as “transcriptionally competent.”

EXPERIMENTAL PROCEDURES

Cells and Nuclei—Blood was obtained from White Leghorn chickens by bleeding through the neck vein and collected in 75 mM NaCl,

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1 The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; HMG, high mobility group protein; SDS, sodium dodecyl sulfate; kb, kilobase pairs.
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25 mM EDTA, pH 7.5, 30 mM Na butyrate (Rahbani et al., 1978).

The blood was filtered through several layers of cheesecloth and centrifuged at 5000 rpm in JS-42 (Beckman rotor) for 10 min to pellet the blood cells. The pellet was washed three times with three volumes of the same buffer as the cells were collected in until all theuffy coat was removed. Packed red blood cells were frozen at −70 °C in 56-ml portions.

Erythrocyte nuclei were isolated in RSB buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 0.25% Nonidet P-40 in the presence of 30 mM Na butyrate, 15 mM β-mercaptoethanol, and 0.4 mM PMSF as in Weisbrod (1982)).

Nuclease Digestions and Chromatin Salt Fractionations—Nuclei were resuspended in a buffer similar to that of Wray and Stuhblefield (1980): 1 M bexylene glycol, 10 mM piperazine N,N'-bis(2-ethanesulfonic acid), pH 7.0, 2 mM MgCl₂, 1 mM CaCl₂, 1% thiodiglycol, 30 mM Na butyrate, 0.4 mM PMSF, and the DNA concentration was adjusted to 2 μg/ml. Digestions with micrococcal nuclease (Sigma) were performed either at 37°C for 10 min with 100 A₂₆₀ units/ml of enzyme or at 15 °C for 20 min and 90 min with 200 A₂₆₀ units/ml and halted by adding 2 mM EGTA, pH 7.5, on ice.

Digested nuclei were collected by centrifugation at 3000 rpm (Sorvall SS-34). The supernatant was centrifuged again at 10,000 rpm for 20 min; this second supernatant is termed $S_0$. The nuclei were then sequentially extracted with solutions containing 0.1 M hexylene glycol, 10 mM piperazine N,N'-bis(2-ethanesulfonic acid), pH 7.0, 2 mM MgCl₂, 25 mM KCl, 0.25 μM succrose, 10 mM EDTA, pH 7.5, on ice. After incubations, nuclei were again collected by a low speed centrifugation, and the supernatants were recentrifuged at 10,000 rpm for 20 min, yielding supernatants $S_{0.05}$, $S_{0.1}$, $S_{0.2}$, $S_{0.4}$, and a final pellet (P).

DNase I (Worthington) digestions were performed in RSB buffer at a nuclear concentration of 1 mg/ml of enzyme at 15 °C for 20 min. Digestions with micrococcal nuclease (Sigma) were performed either at 37 °C for 30 min with 50 mM Tris-HCl, pH 7.0, 2 mM MgCl₂, 25 mM KCl, 0.25 μM succrose, 10 mM EDTA, pH 7.5, on ice. After incubations, nuclei were again centrifuged at a low speed centrifugation, and the supernatants were recentrifuged at 10,000 rpm for 20 min, yielding supernatants $S_{0.05}$, $S_{0.1}$, $S_{0.2}$, $S_{0.4}$, and a final pellet (P).

Protein Analysis and Polyacrylamide Gel Electrophoresis—The various fractions were dialyzed against 3 mM (NH₄)₂CO₃ and samples corresponding to 1.0 A₂₆₀ were lyophilized. An aliquot of each sample was analyzed on a long 15% polyacrylamide–SDS mini slab gel (15 cm long x 10 cm x 0.1 cm) to allow good separation of the proteins HMG 14 and 17. The distribution of absorbance among the fractions is presented for the transcriptionally competent adult β-globin gene and the for the transcriptionally competent adult β-globin gene and the

| Fraction  | Total $A_{260}$ | HMG 14 | HMG 14/ DNA % |
|-----------|----------------|--------|---------------|
| 15 °C, 20 min | $S_0$ | 1.4 ± 0.1 | 0 | |
| S₀.05 | 2 ± 0.2 | 11.2 ± 2.0 | 6.47 |
| S₀.1 | 2 ± 0.3 | 14.7 ± 3.0 | 4.24 |
| S₀.2 | 7 ± 5 | 37.2 ± 3.2 | 7.06 |
| S₀.4 | 22 ± 4 | 36.9 ± 1.4 | 0.42 |
| P | 66 ± 2 | 0 | |
| 15 °C, 90 min | $S_0$ | 5 ± 0.5 | 0 | |
| S₀.05 | 4 ± 0.5 | 13.8 ± 2.6 | 3.19 |
| S₀.1 | 3 ± 0.3 | 22.2 ± 3.9 | 5.14 |
| S₀.2 | 7 ± 5 | 31.3 ± 2.1 | 7.09 |
| S₀.4 | 24 ± 3 | 32.6 ± 2.1 | 0.40 |
| P | 57 ± 2 | 0 | |
| 37 °C, 10 min | $S_0$ | 6 ± 0.7 | 0 | |
| S₀.05 | 4 ± 0.7 | 17.4 ± 1.6 | 6.08 |
| S₀.1 | 3 ± 0.6 | 25.2 ± 6.2 | 9.09 |
| S₀.2 | 7 ± 2 | 23.4 ± 4.4 | 3.88 |
| S₀.4 | 25 ± 4 | 34.0 ± 5.7 | 0.64 |
| P | 55 ± 2 | 0 | |
inactive vitellogenin gene. This same pattern is observed for all digestion times examined. Further evidence that the fractionation procedure is related to transcriptional activity comes from the fact that the β-globin gene fractionates with the high salt fractions in calf thymus chromatin where this gene is not transcribed (not shown). However, while there are marked enrichment and depletions of specific sequences in these fractions, there is also a distinct overlap. Thus, the fractionation is not absolute. Whether this is due to technical or biological causes remains to be determined.

Since, as shown in Fig. 1, mononucleosomes are the predominant species found in the lowest salt-eluted fractions, it might be thought that the enrichment of the globin genes in these fractions is due to the micrococcal nuclease sensitivity of the globin chromatin. However, Bloom and Anderson (1979) have shown that the globin chromatin in mature chicken erythrocytes is no longer preferentially cleaved by micrococcal nuclease. We have examined this question by separating DNA from the entire nuclear digests and blot-hybridizing it to β-globin and vitellogenin probes (Fig. 2B). In those experiments, we observe no evidence for preferential cutting of the globin chromatin or differences in the rate that both active and inactive genes are digested by micrococcal nuclease (compare A and C for the extent of cleavage of vitellogenin and β-globin genes, respectively). We conclude that while digestion to small nucleosome sizes is probably required for elution of competent globin genes from nuclei at the lowest ionic strengths, other features of these nucleosomes must be involved so as to preferentially select them for elution.

Salt Fractionation Defines a β-Globin Chromatin Domain—The various salt cuts from a typical nuclear elution experiment were tested by specific probes isolated from different regions of the β-globin locus (Fig. 3). Both the adult β5 globin gene as well as the embryonic β5 globin sequences fractionate with the low salt fractions. Moreover, flanking sequences as far as 8 kb 5' to β5-globin are also enriched in these fractions. However, a single-copy sequence (see Southern blot in Fig. 3) located slightly further upstream (about 10 kb from β5-globin) fractionates in the high salt fractions with the inactive vitellogenin and ovalbumin sequences. On the basis of these results, it seems that the salt fractionation procedure defines a boundary of the β-globin chromosomal domain whose 5' border is located about 10 kb 5' to the β5-globin gene. Experiments are currently in progress to define the 3' border of the domain and to see whether the domain borders change during the switch from embryonic to adult globin synthesis. It will also be of interest to see whether the domain is also established in red cell precursors which have not yet commenced the synthesis of Hb.

Previous results have defined a β-globin chromosomal domain in adult erythrocytes using DNase I sensitivity where it was shown that an intermediate level of DNase I sensitivity

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**Fig. 1. Electrophoretic analysis of the DNA present in the various salt-eluted chromatin fractions.** DNA from the various salt-eluted fractions was isolated and analyzed in a 4% polyacrylamide gel. Lanes 1, 2, 3, 4, and 5, DNA in fractions S 0.05, S 0.1, S 0.2, S 0.4, and remaining pellet (P) after fractionation, respectively, from a digest at 15 °C for 20 min; lanes 6, 7, 8, 9, and 10, DNA in same fractions as above eluted from a digest at 15 °C for 90 min; lanes 11, 12, 13, 14 and 15, DNA in same fractions as above eluted from a digest at 37 °C for 10 min.

**Fig. 2. Enrichment of competent globin sequences in the fractions eluted at low salt concentration.** A, dot blots from the different fractions (the 0.5 fraction represents the nuclear pellet after the previous extractions) were probed with the indicated nick-translated probes (Weintraub, 1983). For most experiments, the same dot blots were hybridized with a variety of different probes. The same pattern was obtained at all digestion times. B, micrococcal nuclease digestion of globin and vitellogenin chromatin. DNA was isolated from total micrococcal nuclease digests and electrophoresed on a 1% agarose gel and blotted hybridized to (A) vitellogenin probe or (C) a β-globin probe. (B) shows the ethidium bromide-stained gel. Markers are λ-HindIII fragments and pX174 HaeIII fragments. The first lane is a digest at 15 °C for 20 min; the second lane is a digest at 15 °C for 90 min; and the third lane is a digest at 37 °C for 10 min.
as a consequence of the salt but not of the nuclease digestion. Thus, the two seemingly independent assays (DNase I sensitivity and NaCl fractionation) define the same putative domain boundary. Similar results using nuclease digestion have also been reported for the active ovalbumin domain in chick oviduct (Lawson et al., 1982).

**Fractionation of Constitutively Competent Genes Expressed in All Tissues**—The various NaCl fractions were also probed with both adult (αβ) and embryonic (α*) α-globin genes in addition to a chicken thymidine kinase gene and a chicken histone H4 gene. As with the β-locus, both of the α-globin genes partition primarily to the low salt fractions (Fig. 4A). Surprisingly, the H4 and thymidine kinase genes fractionate with the inactive high salt fractions (Fig. 4A). The results for the histone genes are ambiguous, since this probe detects a number of genes in chicken DNA and some may prove to be inactive. However, the thymidine kinase gene is present as a single copy, and these red cells retain thymidine kinase-associated hypersensitive sites (Fig. 4B). These results show that salt fractionation of nuclei is not based in a simple way upon past transcriptional activity per se. One possibility is that only monomer nuclease associates with tissue-specific expression are preferentially eluted from nuclei at low NaCl concentrations. Alternatively, it is possible that the fractionation defines genes which have been transcribed at a high rate. Finally, it is conceivable that genes such as thymidine kinase become preferentially sequestered into the high salt fraction by virtue of an association with histone H5 (see below) late during erythroid cell maturation. Clearly a further analysis of other tissues and additional specific genes is required.

**Protein Composition of the Chromatin Fractions**—Having established that the fractions differ in their contents of transcriptionally competent and inactive gene sequences, we ask whether there are accompanying differences in the composition and/or modification of the associated chromosomal proteins.

For this purpose, proteins from each fraction were analyzed in a 15% polyacrylamide-SDS gel (Fig. 5A). As a control, we also examined the protein content of the various fractions eluted from “mock” digested nuclei (not shown). This control identifies those proteins which are solubilized from chromatin as a consequence of the salt but not of the nuclease digestion.

We find, in agreement with other laboratories, that in the absence of nuclear digestion: all HMG 1 and 2 are released from nuclei at 0.05 and 0.1 M NaCl (Albright et al., 1980); HMG 14 and 17 elute between 0.2 and 0.4 M NaCl (Nicolas et al., 1983); H1 and H5 are respectively extracted at 0.4 and

![Fig. 3. A large β-globin chromosomal domain defined by NaCl fractionation.](image)

![Fig. 4. Fractionation of the chicken thymidine kinase (TK) gene with the high salt fraction.](image)

![Fig. 5. Differences in protein composition among the various fractions.](image)
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0.6 mM NaCl, together with a small amount of core histones (Kuan and Walker, 1980).

Fig. 5A shows the protein composition of the fractions eluted from digested nuclei. All fractions contain core histones in equimolar amounts (Fig. 5A, lanes b–h). The slight apparent increase in the content of histone H3 in fraction S 0.05 (lanes b and d) is due to micrococcal nuclease which comigrates with this histone in SDSs gels. The fractions soluble at low salt (lanes b–e) are, however, depleted of histones H1 and H5 and highly enriched in HMG 1, 2, 14, and 17 relative to the core histones as compared to the fractions which elute at higher ionic strengths (lanes f and g).

Several additional proteins that migrate in the H1/H5 region of the gel are also found in the low salt fractions (lanes b–e). However, their migration is distinct from that of H1 or H5 on SDS gels, and they co-elute with HMG 1 and 2 from mock digested nuclei. Hence we cannot say whether or not all these proteins are actually associated with the low salt eluted nucleosomes.

The protein pattern of the fractions shown in Fig. 5A does not change regardless of the extent of digestion of the chromatin (compare lane b to lane d and lane c to lane e). Also, we have not detected histones or HMG proteins in the fraction S0.6 (not shown) consistent with the observation that no nucleosomes are released prior to salt fractionation of the nuclei. Whether the observed depletion of H1/H5 relative to the core histones in the low salt fraction reflects the in vivo composition of these nucleosomes is difficult to establish by these experiments. We do not think that this marked depletion is due to proteolysis since we do not observe characteristic H1 cleavage products (Garrard and Hancock, 1978) on the acrylamide-SDS gels (Fig. 5A). On the other hand, it is conceivable that the H1/H5 interaction sites on these nucleosomes are eliminated during digestion. That is, when the chromatin region is cleaved to monomers, there may be a rearrangement of the H1/H5 with these proteins dissociating from the small DNA fragments and reassociating with the preferred longer nucleosome strands (Renz et al., 1977; Thomas and Rees, 1983). The data on hand do not allow us to rule out this possibility. However, the observations that the trace amounts of H1 and H5 associated to fractions S 0.05 and S 0.10 do not vary appreciably regardless of the size of the chromatin fragments present in these fractions (compare protein patterns of Fig. 5A, lanes c and e, to the DNA sizes of Fig. 1, lanes 2 and 12) and that the differences in solubilization of H1 and mononucleosomes occur as well in digests that were carried to a limit monomeric stage (Sanders, 1978) suggest that the interaction, if any, of the lysine-rich histones with the nucleosomes eluted at low salt is different from their interaction with the other nucleosome subfractions. Further support for the idea that the globin chromatin may be devoid of H1/H5 comes from the recent work of Kimura et al. (1983) where they demonstrate that the solenoidal structure of the globin gene chromatin in mature erythrocytes is likely to be unfolded, possibly due to the absence of the linker histones H1/H5.

The nonhistone proteins HMG 14 and 17 are clearly enriched in the fractions soluble at 0.05 and 0.1 mM NaCl. As Table I shows, 35% of the total population of HMG 14 and 17 elute into these fractions, which carry only 6% of the total DNA; the low salt fractions are enriched in HMG 14 and 17 nearly 14-fold over the high salt fractions. Thus, there appears to be an enrichment of HMG proteins in the nucleosomes which preferentially contain transcriptionally competent DNA sequences, in agreement with earlier results (Weisbrod and Weintraub, 1979). Nonetheless, it should be noted that 65% of the HMG 14/17 population is found associated with the higher salt fractions (Table I). These results may support the view that these proteins are also bound to nucleosomes containing inactive DNA sequences (Nicolas et al., 1983). However, it is also possible that these HMGs could be associated with the rest of globin genes that elute at S 0.2 and to other active genes such as thymidine kinase or histone H4 which fractionate in the high salt cuts. The enrichment of HMG 14/17 in each of the higher salt fractions is difficult to assess as we have observed that these proteins begin to dissociate from chromatin at 0.2 mM NaCl (see above).

In order to search for histone modifications and variants, core histones from the various fractions were further analyzed by two-dimensional gel electrophoresis using polyacrylamide-SDS in the first dimension followed by acetic acid-urea-Triton X-100 gels in the second (Fig. 5B). The chicken erythrocyte histone variants have been identified and characterized by Urban et al. (1979). When we examine the various two-dimensional electrophoretic patterns, we do not find noticeable differences in the content of the different variants including the H3 variants and M1 (also called H2A.Z (Bail et al., 1983)). However, we find the distribution of the histone H4-modified species to vary among the salt fractions with the nucleosomes eluting at the lowest NaCl concentrations containing increased amounts of the hyperacetylated forms with respect to the other fractions (compare lane A to B or C). The slight enrichment of the acetylated H4 species with the fraction enriched in the globin genes has also been observed by Weisbrod (1982) and Goldsmith (1981).

DISCUSSION

Our results show that the Sanders technique allows the recovery of a fraction of chicken erythrocyte chromatin soluble at low ionic strengths which selectively contains transcriptionally competent globin genes. The region of the β-globin locus that is soluble at low concentrations of NaCl extends some 10 kb 5' to the first gene in the complex; the 3' border has not yet been mapped. This rather large chromosomal unit (greater than 30 kb) may define a domain of structure and function and, perhaps, of regulation. Interestingly, DNase I sensitivity defines a domain of about the same size which has a similar 5' border. It was previously hypothesized that such a domain may correspond to a closed loop of chromatin (Stalder et al., 1980) analogous to a lambbrush chromosome loop. That such a domain may have functional significance is suggested by recent experiments using microinjection of supercoiled DNA into frog oocytes. These experiments have shown that a closed chromatin topology is in fact required for continued transcription, possibly reflecting a requirement for DNA supercoiling (Harland et al., 1983).

The factors involved in the differential solubilization of the globin chromatin at concentrations of salt below 0.2 mM following micrococcal nuclease digestion are poorly understood. As the low salt fractions contain mainly mononucleosomes, the enrichment of the globin sequences in these fractions might be explained by their preferential sensitivity to micrococcal nuclease. However, this is not a sufficient explanation for our results, since we and others (Bloom and Anderson, 1979; Torres-Martinez and Ruiz-Carrillo, 1982) have demonstrated that the globin genes are not preferentially digested in adult erythrocytes. In fact, both the globin, vitellogenin, and ovalbumin gene sequences are digested at similar rates. But it is the mononucleosomes containing the globin sequences that are enriched in the low salt fractions.

Possible explanations for the differential fractionation of the sequences are the following. First, the presence of mainly
mononucleosomes in the low salt fraction may be a consequence of nuclear membrane permeability, that is, at low ionic strengths, only mononucleosomes have the freedom to leave the nuclei. Second, the mononucleosomes containing globin or ovalbumin sequences may be harbored in chromatin regions that have differences in macromolecular composition and levels of condensation. Thus, chicken erythrocyte mononucleosomes containing ovalbumin sequences may be contained in a chromatin region from which they are released with difficulty, even after nuclease cleavage. Such a difference in higher order structure might correspond to differences in protein content or composition. The protein analysis of the various fractions (Fig. 5, A and B) shows that the nucleosomes eluted at low salt concentrations differ in their content of H1/H5, HMG proteins 14/17, and H4 acetylated species. The contribution of these factors to the increased solubility is unknown at present.

Perry and Chalkley (1982) have recently reported that hyperacetylated chromatin has an increased solubility at 0.1 M NaCl in the presence of Mg" ions. However, we doubt that acetylation per se could be directly responsible for the elution of the globin nucleosomes at low salt because of the slight differences observed in hyperacetylated histones among the various fractions. Furthermore, recent observations of one of us demonstrated that the presence of hyperacetylated histones with a chromatin region was not sufficient requirement for that region to elute into the low salt fraction. In these experiments, late stage trout testis chromatin which contains high levels of the hyperacetylated histone species was fractionated according to the Sanders procedure. Very little of the chromatin material (0.1%) was found in the low salt fraction. Thus, this study suggests that factors other than histone acetylation must ultimately lead to the release or retention of the chromatin fragments from the nuclei at low ionic strength.

Since the role of the lysine-rich histones, H1/H5, in the precipitation/aggregation of chromatin fragments around physiological ionic strengths and in the retention of nucleosomes within the nucleus (Lawson and Cole, 1982) is well established, we believe that differences in the content or binding of H1/H5 between active and inactive regions are primarily responsible for the selective fractionation of the globin-containing nucleosomes. We do not wish to imply that the linker regions of these chromatin regions are bare, which would be incompatible with the observed lack of preferential micrococcal nuclease sensitivity of the globin gene. However, it is conceivable that the binding of H1/H5 to these regions could be modified, possibly by other alterations in the organization of these "active" nucleosomes. In this regard, H1 is thought to interact through both electrostatic and hydrophobic forces with the terminal portions of the chromatosome DNA as well as the core histones in the nucleosome (Boulikas et al., 1980). Alternatively, it is also possible that in transcriptionally competent regions H1/H5 could be modified or substituted by other proteins that perform some but not all H1 functions. Future experiments will be directed to test these hypotheses.

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