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Chicken erythrocyte polynucleosomes which are soluble at physiological ionic strength and contain linker histones are highly enriched in β-globin gene sequences

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ABSTRACT

Mature chicken erythrocyte polynucleosomes which are soluble at physiological ionic strength are enriched in β-globin DNA sequences. Vitellogenin chromatin, which is not expressed in this tissue, is found in aggregation prone, salt insoluble chromatin. There is a direct correlation between the size of soluble fragments and the degree of globin gene enrichment, with the largest fragments being most highly enriched. The highly globin enriched (about 50 fold) polynucleosomes contain significantly elevated levels of acetylated histones H4, H2A.Z, and H2B, and ubiquitinated (prefix "u") histones H2A and H2B (with a significant relative increase of uH2B over uH2A). These polynucleosomes were complexed with histones H1 and H5 but at a lower level than that found in unfractionated chromatin.

INTRODUCTION

The biochemical basis for the differences between transcriptionally competent and repressed chromatin has been extensively investigated (see 1,2 for reviews). It is now clearly established that most genes that are potentially expressed within a given tissue are in a DNAase I sensitive conformation relative to other genes. Various aspects of chromatin structure have been associated with the active and/or DNAase sensitive states including the presence of high mobility group (HMG) proteins, histone acetylation and ubiquitination, DNA under-methylation and the presence of certain histone variants. The transcriptionally competent state has been described as being dynamic, in opposition to the stability of the repressed state. The H1-histones probably play a significant role in the stability of transcriptionally repressed chromatin (3).

Most models of chromatin function imply a hierarchy of

chromatin structures (2). In such a hierarchy, cell-lineage dependent patterns of gene expression are, in part, determined by a distinctly assembled chromatin for transcription competent genes. We have recently demonstrated that the transcriptionally competent chicken β-globin gene chromatin but not the transcriptionally inactive vitellogenin gene chromatin is soluble as polynucleosomes in 0.1 M KCl (4). The selective partitioning of these structurally different chromatins has allowed the further purification of the β-globin chromatin. The present work describes the isolation and characterization of a rare class of chromatin, highly enriched in competent β-globin gene sequences, and soluble at physiological ionic strength.

MATERIALS AND METHODS

Isolation and digestion of nuclei

Red blood cells from adult white leghorn chickens were collected in 75 mM NaCl, 25 mM EDTA, washed of the buffy coat and stored at -70°C. Nuclei were isolated by washing 4 times in 10 volumes of RSB (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM PMSF (phenylmethane sulfonyl fluoride)) including 0.25% NP-40 in the first two washes. Nuclei were resuspended in the digestion buffer (1 M hexylene glycol, 10 mM PIPES (piperazine-N,N' bis[2-ethane sulfonic acid]), pH 7.0, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1% thiodiglycol, 1 mM PMSF) to a DNA concentration of 2.5 mg/ml and incubated with 25 A_{260} units/ml micrococcal nuclease (Pharmacia) at 37°C for 30 min. Digestion was stopped with the addition of EGTA to 10 mM and nuclei were collected by centrifugation. All buffers contained 30 mM Na-butyrate.

Salt precipitation and gel filtration of soluble chromatin

Digested nuclei were resuspended into an equal volume of 10 mM EDTA, pH 7.2, 1 mM PMSF, and left on ice for 2 hours to release chromatin fragments into solution. Insoluble nuclear debris was removed by centrifugation (12,000 g x 20 min). 80.3 ± 3.8% (n=4) of the total nuclear 260 nm absorbing material was recovered in this way. This material (SEDTA) was diluted to a 260 nm absorbance of 30 units/ml by the addition of 10 mM EDTA; and NaCl was added with vigorous stirring to 0.15 M from a 4 M

stock. The resulting precipitate was collected immediately by centrifugation (12,000 g x 20 min). The supernatant (10.8 ± 0.4% of SEDTA, n=4) was concentrated against polyethylene glycol and applied to a 2.5 x 110 cm Biogel A5m column in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.15 M NaCl and run at a flow rate of approximately 50 ml/hr. 3.75 ml fractions were collected.

Electrophoresis and blotting

Pooled column fractions were dialyzed against water and lyophilized. For SDS 15% PAGE analysis of proteins, equivalent amounts of the dried powder were dissolved directly into reducing SDS sample loading buffer (5). For acetic acid/urea/Triton X-100 (AUT) 15% polyacrylamide gel electrophoresis the lyophilized samples were prepared as described (5).

For electrophoresis and blotting of DNA equivalent amounts of the lyophilized material were dissolved into DNA sample loading buffer containing 0.2 volumes of 10% SDS. These samples were loaded onto 1% agarose mini gels with SDS to 0.1% added to the Tris-acetate buffer. After electrophoresis the gels were stained for 45 min in 1 ug/ml ethidium bromide. Southern transfer was carried out as in Maniatis et al. (6). Hybridization of the filters was performed as described by Thomas (7). Similar results were obtained with DNA prepared as follows: dialysed fractions were made to 2.2% sodium N-lauryl-sarcosine, 0.16 M NaCl, 25 mM EDTA and 0.5 mg/ml pronase and incubated at 37°C overnight. One-twentieth volume of a stock of mixed RNAase (5000 U/ml RNAase T and 5 mg/ml RNAase A) was added and incubation continued for two hours. Pronase was readded and incubation continued for a further two hours before extraction with phenol/chloroform and ethanol precipitation. The cloned DNA probes used were: pCBG 13 which contains the 5' end of the adult β-globin gene and some flanking sequences, and pCBG 18.7, an embryonic ε-globin sequence (8). Both probes were acquired from H. Martinson. pVTG 421, obtained from H. Weintraub, recognizes the 5' region of the chicken vitellogenin gene (9).

Quantitation of Data

Southern blots were prepared from agarose gels loaded with varied quantities of each fraction and hybridized. Each lane on

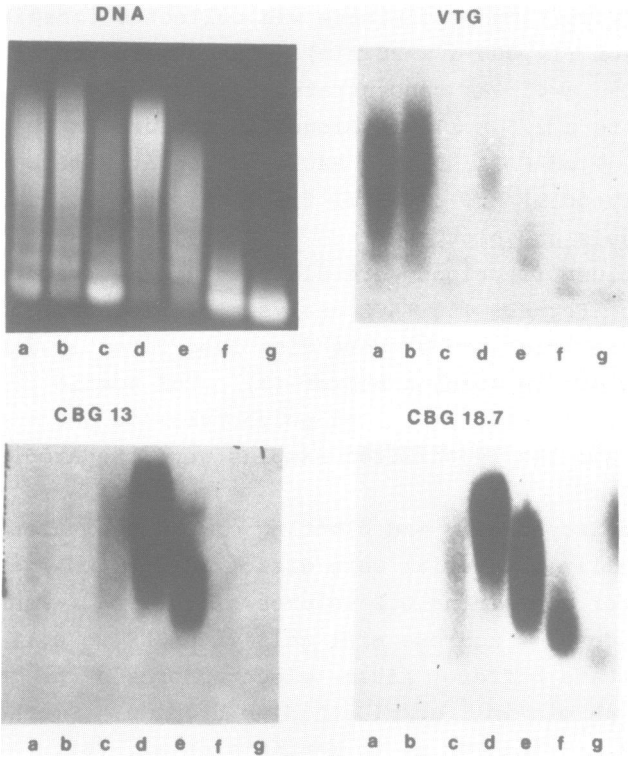

Figure 1 Polynucleosomes that are soluble at physiological ionic strength are highly enriched in β-globin sequences. DNA fragments isolated from unfractionated, EDTA released, chromatin (SEDTA, lane a), 0.15 M NaCl precipitate (aggregation prone chromatin, lane b), 0.15 M NaCl supernatant (aggregation resistant chromatin, lane c) and Biogel A5m column fractions I, II, III and IV (lanes d, e, f and g, respectively) were electrophoretically resolved on a 1% agarose gel. After transfer to nitrocellulose filters the DNA was hybridized to the indicated sequences (CBG 13, β-globin; CBG 18.7, ε-globin; VTG, vitellogenin).

the autoradiograms was scanned with a densitometer and the areas under the curves integrated to quantitate the extent of hybridization. The relation between signal and the amount loaded was essentially linear.

Protein content was measured by densitometric scanning of Coomassie blue stained polyacrylamide gels.

TABLE I
β-Globin sequence distribution in fractionated chromatin.

Fraction	Globin Sequences (% of SEDTA)	Enrichment Over Total
SEDTA	100	1.0 X
150 mM NaCl sup.	58	5.4 X
150 mM NaCl ppt.	42	0.5 X
Column fraction I	34	48.2 X

The extent of hybridization to the probe pCBG13 of the various chromatin fractions was determined as described in the "Materials and Methods" section. Enrichments are expressed relative to the SEDTA hybridization signal.

RESULTS

The micrococcal nuclease digestion of the mature chicken erythrocyte nuclei was of the extent to give a release of 6.0 ± 0.5% of the 260 nm absorbing material into the digestion buffer, most of which was acid soluble. The micrococcal nuclease digested nuclei were resuspended in 10 mM EDTA which released the majority of the chromatin (80.3 ± 3.8% (n = 4) of the total nuclear 260 nm adsorbing material). The solubilized chromatin was collected in the supernatant following centrifugation. The proportion of β-globin and vitellogenin sequences in the DNA remaining with the insoluble nuclear material in the pellet was found to be similar (not shown).

We have recently reported that β-globin and vitellogenin chromatin fragments differ in their solubility properties in 0.1 M KCl (4). β-globin polynucleosomes and vitellogenin mononucleosomes were aggregation resistant and salt soluble while vitellogenin polynucleosomes were aggregation prone and salt insoluble. Similar results were obtained when the chromatin fragments were fractionated by the addition of NaCl to 0.15 M (Figure 1). It should be noted that: 1) β-globin but not vitellogenin polynucleosomes are soluble in 0.15 M NaCl and 2) the predominant nucleosome species in the 0.15 M NaCl soluble fraction is the mononucleosome (see Figure 1, DNA , lane c). The β-globin sequences were enriched in the 0.15 M NaCl soluble chromatin fraction (Table I). Measurements of the extent of

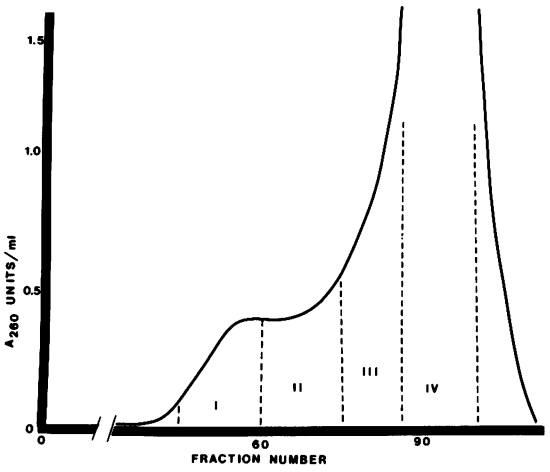

Figure 2 Gel exclusion chromatography of aggregation resistant chromatin fragments. Aggregation resistant chromatin fragments were loaded on a Biogel A5m column equilibrated with 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. The four pooled fractions (I, II, III, IV) are shown.

vitellogenin hybridization in the 0.15 M precipitate indicated that this fraction contained the same enrichment of these sequences as the EDTA released material (within the limits of the precision of the measurement technique).

In order to separate the globin enriched polynucleosomes from the bulk of the material the 0.15 M NaCl soluble fraction was subjected to gel filtration. The Biogel A5m column profile of the chromatin fragments soluble in 0.15 M NaCl and the four fractions pooled are shown in Figure 2. The major peak occurs at fraction number 93 at 3.830 A_{260} units/ml. The four pooled fractions (I, II, III and IV) contained 6.9, 10.4, 19.8 and 62.9%, respectively, of the 260 nm absorbing material loaded on the column. The fragment sizes in the column fractions are as follows: fraction I, larger than octamers; fraction II, dimers to about dodecamers; fraction III, mainly mononucleosomes; and fraction IV, only core sized mononucleosomes (Figure 1, DNA).

The DNA from the pooled column fractions was resolved on agarose gels and corresponding hybridization results are shown in Figure 1. The autoradiographic intensity of CBG 13 (β-

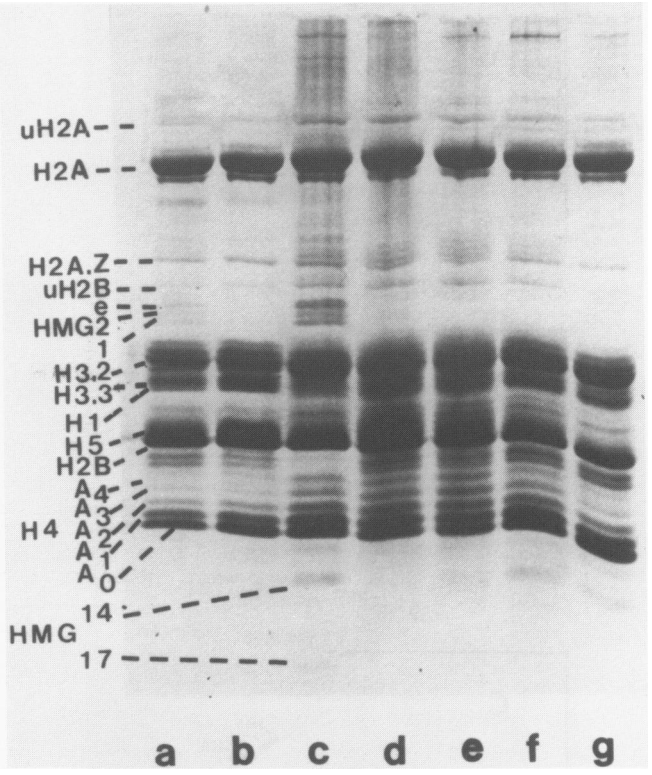

Figure 3 Analysis of the proteins present in the various fractions. Proteins isolated from unfractionated chromatin (SEDTA, lane a), 0.15 M NaCl precipitate (aggregation prone chromatin, lane b), 0.15 M NaCl supernatant (aggregation resistant chromatin, lane c) and Biogel A5m column fractions I, II, III, and IV (lanes d, e, f and g, respectively) were electrophoretically resolved on an acetic acid / 6.7 M urea / 0.375% Triton X-100 (AUT) 15% mini slab polyacrylamide gel. The gel was stained with Coomassie blue. A_0, A_1, A_2, A_3 and A_4 denote the un-, mono-, di-, tri-, and tetra-acetylated species of H4, respectively.

globin) hybridization for the column fraction I is well beyond the saturation level of the film when most of the other fractions are not yet visible. In order to better quantify the extent of hybridization in each of these fraction gels were loaded with varying amounts of chromatin and the autoradiograms were measured by densitometry as described in the "Materials and Methods". The column fraction I was found to be almost 50 fold

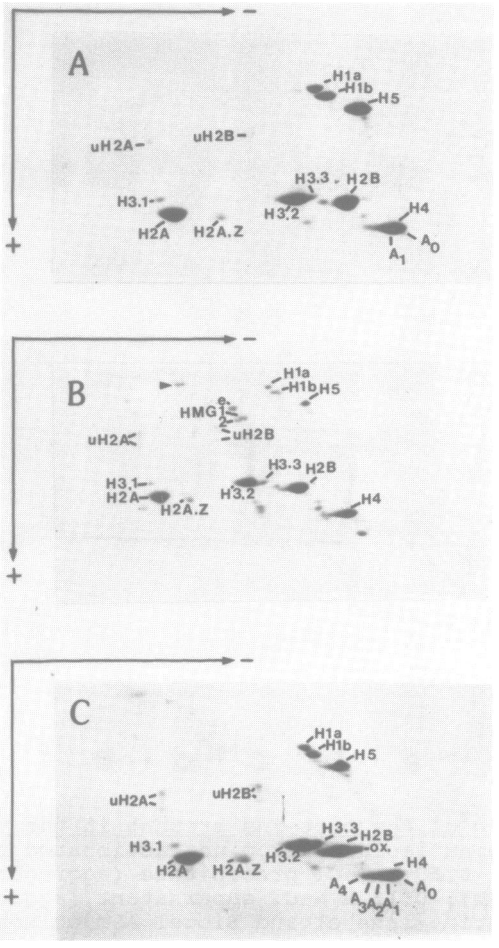

Figure 4 Two dimensional gel electrophoresis of proteins present in various chromatin fractions. Proteins from unfractionated chromatin (A), aggregation resistant chromatin (0.15 M NaCl supernatant (B), and polynucleosomes (column fraction I, C) were separated by two dimensional gel electrophoresis (AUT→SDS). The gels were stained with Coomassie blue. A_0, A_1, A_2, A_3, and A_4 denote the un-, mono-, di-, tri-, and tetra-acetylated species of H4, respectively.

enriched over total for β-globin sequences (see Table I). CBG 18.7 (ε-globin) (Figure 1) and CBG 14.4 (an intronic sequence of the β-globin gene) (not shown) give essentially the same pattern. Identical blots probed with vitellogenin show that the 0.15 M NaCl soluble material contains little of these sequences.

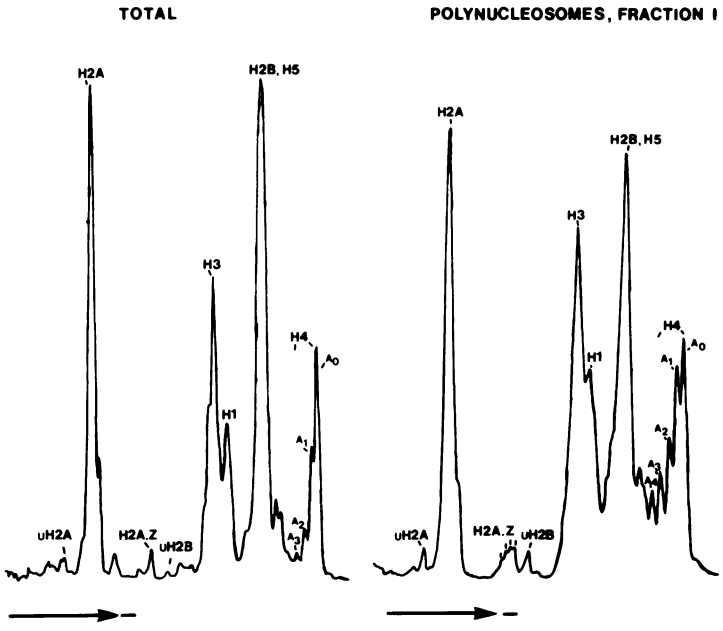

Figure 5 Densitometer scans of the AUT gel electrophoretic patterns. Proteins isolated from the unfractionated chromatin and the salt soluble polynucleosome (column fraction I) were resolved on AUT gels and stained with Coomassie blue. Densitometer scans of the electrophoretic patterns are shown. Modified species of H2A.Z are indicated by lines above the scan.

The protein composition of the various fractions were analyzed on AUT gels (Figure 3) and on two dimensional (AUT⟶SDS) gels (Figure 4). The column fraction I was highly enriched in the modified histone species including the acetylated species of H4 (Figure 3, lane d and Figure 5), H2A.Z (also called M1 and H2A.F (10, 11)) (Figure 5) and H2B (Figure 4; note that in panel C, H2B trails toward the anode on the AUT gel). We have made these assignments based on the following information: firstly, Sung et al. (12) demonstrated that, in mature erythrocytes, histone phosphorylation and methylation cease to occur; secondly, Nelson et al. (13) have shown that all of the erythrocyte nucleosomal histones, including some of the histone variants, can be acetylated; thirdly, Pantazis and Bonner (14) reported that mouse H2A.Z had three acetylated species but no phosphorylated forms; and, fourthly, the ubiquitinated histone

TABLE II

Comparison of the protein content of the salt soluble polynucleosomes (column fraction I) with total chromatin.

	Enrichment Over Total
uH2A	1.3 X
H2A.Z	2.8 X
uH2B	3.7 X
Acetyl H4	2.4 X
Linker histones	0.7 X

Protein content was determined by densitometry of AUT and SDS PAGE gels stained with Coomassie blue. The amount of the variant and modified H2A and H2B were compared as a percent of total H2A or H2B in each fraction. Acetylated H4 is compared as a weighted sum of total acetylation as described (16). Linker histones are compared as a ratio of the total of H1 and H5 and the total of core histone.

species have been identified as such with an anti-ubiquitin antibody (Nickel and Davie, manuscript in preparation). The enrichment of uH2B is especially striking since in this fraction its content is similar to that of uH2A (Figure 4, panel C and Figure 5). The content of uH2A in chromatin is typically three fold greater than that of uH2B (Figure 4, panel A and (15)). The column fraction I appears to be enriched in the histone variants H2A.Z and H3.3 (compare panel C to panel A in Figure 4 and Figure 5). We are concerned that the enrichment of H3.3 may be an artefact due to oxidation. In other analyses, however, where oxidation does not seem to be apparent, increased amounts of H3.3 were observed. Quantitation by densitometry of Coomassie stained gels was carried out for those proteins which could be sufficiently resolved by electrophoresis. The results are given in Table II.

The content of the modified histone species diminished as the size of the chromatin fragments decreased (Figure 3, lanes d to g). For column fraction IV (Figure 3, lane g) the level of modified histone forms was similar to that in unfractionated chromatin (Figure 3, lane a). It can also be seen on the AUT gel (Figure 3) that the HMG proteins 14 and 17, which are enriched in the 0.15 M NaCl soluble fraction, are preferentially bound to smaller nucleosome oligomer-, monomer- and core particle sized species.

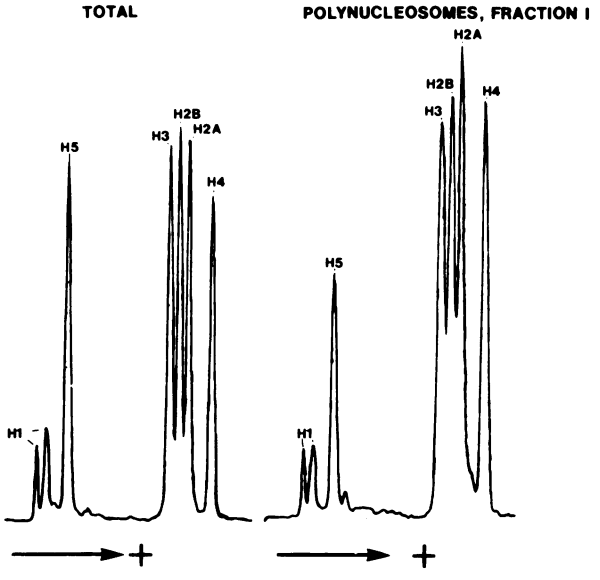

Figure 6 Densitometer scans of the SDS gel electrophoretic pattern. Proteins isolated from unfractionated chromatin (Total) and polynucleosomes (column fraction I) were resolved on SDS gels and stained with Coomassie blue. Densitometer scans of the electrophoretic patterns are shown.

A number of proteins which are present in the 0.15 M NaCl soluble fraction are absent from the first column fraction (compare panels B and C in Figure 4). This indicates that they are not bound to these polynucleosomes at this ionic strength. These proteins include the HMG proteins e, 1 and 2; and include a protein (shown by the arrow in Figure 4 panel B) which, to our knowledge, is heretofore undescribed and uncharactarized. This species has a mobility similar to H1a on SDS PAGE and is present in soluble chromatin prepared by different salt fractionation schemes (17,18). It is likely that this species has been misidentified as H1a leading to the suggestion of the enrichment of this histone in certain active-gene-enriched fractions (18). The peptide map of this protein indicates that it is not similar to any of the linker histones (not shown).

In experiments where the EDTA solubilized chromatin (SEDTA) polynucleosomes were obtained by gel filtration on Biogel A5m in low ionic strength and subsequently precipitated with 0.15 M

NaCl (inverting the order of salt precipitation and gel filtration), a similar degree of globin-sequence enrichment is found in the soluble polynucleosomes. Under these conditions, however, these proteins (HMG e, 1, 2 and others) are found in the supernatant fraction (not shown). This indicates that these proteins are not bound to chromatin fragments at physiological ionic strength but are bound under low ionic strength conditions and that their presence in salt soluble chromatin may be the result of indiscriminate extraction.

In order to compare the content of the linker histones of the polynucleosomes in the column fraction I, the histones isolated from this fraction and from total chromatin were electrophoretically resolved on a SDS polyacrylamide gel (Figure 6). The salt soluble polynucleosomes had a partial depletion in the linker histones, containing about 70% of the total complement of linker histones associated with unfractionated chromatin.

DISCUSSION

In this study solubilization of chromatin from micrococcal nuclease digested nuclei was optimized. The buffer used for micrococal nuclease digestion was designed so that no nucleosome sized material escapes from the nuclei. The solubilized nucleoprotein used represented about 80% of the total nuclear 260 nm absorbing material. Of the remaining 20%, nearly one-third (6.0 ± 0.5%) was released largely as nucleotides by the action of micrococcal nuclease. Even a conservative estimate of the amount of remaining insoluble debris would suggest that we are dealing with almost all of the nuclear DNA. In light of the work of Razin *et al.* (19) which suggests that the attachment of transcriptionally active genes to the insoluble nucleoskeleton is destroyed by EDTA treatment and our own hybridization of the DNA remaining after chromatin release by EDTA, we may also suggest that we are dealing with a nearly sequence-random solublized chromatin.

Most chromatin soluble in 10 mM EDTA is precipitated in 0.15 M NaCl. Of the 10 - 11% that remains soluble, most is monomer and core sized particles. Size fractionation of this soluble material indicates that the largest polynucleosomes are the most

highly enriched in ε and β-globin gene sequences (Table I). This suggests a size dependency of chromatin solubility at physiological ionic strength: smaller fragments are soluble mainly as a function of their size while larger fragments are soluble because of other distinct properties. This can be seen easily by examination of the globin hybridization pattern to any of the fractions soluble in 0.15 M NaCl (see Figure 1). The degree of hybridization increases substantially toward the top of the gel, where the bulk DNA distribution decreases (see Figure 1). Differences in the size dependent efficiency of hybridization alone will probably not explain this.

A certain degree of caution must be exercised when interpreting the results of chromatin fractionation based on salt solubility because of the exchange of linker histones amongst chromatin fragments at even slightly elevated ionic strengths, and the preferred binding of these histones to longer oligonucleosomes (20 - 22). Komaiko and Felsenfeld (18), for example, found that the preferential solubility of globin chromatin was lost as a function of the degree of redigestion of the salt insoluble fraction when remixed with the soluble fraction. They concluded that the enrichment of globin sequences in the salt soluble chromatin was due to its micrococcal nuclease sensitivity and the loss of linker histones from the resultant smaller average sized fragments to larger ones. Jin and Cole (23) have directly addressed the problem of linker histone exchange and its possible influence on chromatin partitioning by salt induced precipitation. They have shown that H1 histone in HeLa cell chromatin does not substantially equilibrate between soluble and insoluble polynucleosome fractions (approximately octamer size and greater) and conclude that these chromatin fractions are inherently different. The results presented here also support the model of inherent differences in chromatin types giving rise to differences in solubility. Our finding that the largest salt soluble polynucleosome fraction is the most enriched in the globin gene, for example, runs counter to the argument that globin gene chromatin is soluble due to greater nuclease sensitivity and resultant smaller mean fragment size.

The globin enriched polynucleosomes were associated with hyperacetylated species of H4, H2A.Z and H2B, the ubiquitinated forms of H2A and H2B (particularly uH2B), and a partial depletion of H1 and H5. This chromatin fraction may also contain elevated levels of H2A.Z and H3.3 (Table II). No evidence was found for a depletion of any of the nucleosomal histones including H2A and H2B.

To what extent histone acetylation, linker histone depletion or other factors may contribute to the preferential solubility of the β-globin chromatin is unknown. Ferenz and Nelson (24) and Nelson et al. (13) demonstrated that the preferential solubility of β-globin was dependent on both the acetylated state of the β-globin chromatin and the presence of Mg^{+2}. Their results are in agreement with those of Perry and Chalkley (25) who demonstrated that, in the presence of Mg^{+2}, histone acetylation increased the solubility of chromatin fragments. Since we are fractionating chromatin in the absence of Mg^{+2}, histone acetylation may not be responsible for the selective solubility of the globin enriched polynucleosomes. We are inclined to ascribe the greatest contribution to determining the solubility of chromatin fragments in 0.15 M NaCl to the linker histones. Clearly, linker histones are required for NaCl-induced chromatin aggregation for, in their absence, chromatin fragments remain soluble (23, 26, 27). The partial depletion in histones H1 and H5 of the globin enriched polynucleosomes may explain why these chromatin fragments are soluble in 0.15 M NaCl. In vivo, histone acetylation, histone ubiquitination and reduced content of linker histones may all make important contributions to destabilizing higher order structure and to promoting solubility of the β-globin domain. Since acetylation/deacetylation (13, 24, 28), ubiquitination/deubiquitination (15, 29, 30), and, perhaps, the association of linker histones with various regions of chromatin are in a dynamic equilibrium, the maintenance of the altered structure of the β-globin domain should be considered as a dynamic process.

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