Biochem. J. (1989) 263, 179–186 (Printed in Great Britain)

Chromatin structure of erythroid-specific genes of immature and mature chicken erythrocytes

Genevieve P. DELCUVE and James R. DAVIE
Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3

The β-globin and histone H5 genes are transcriptionally active in immature chicken erythrocytes and potentially active in mature erythrocytes. In both immature and mature erythrocytes, the majority of these erythroid-specific gene sequences are located in two chromatin fractions: the low-salt-insoluble residual nuclear material and the 0.15 M-NaCl-soluble oligo- and poly-nucleosomes. These salt-soluble chromatin fragments are enriched in hyperacetylated species of H4 and H2B, ubiquitinated and polyubiquitinated species of H2A and H2B and are depleted of linker histones H1 and H5. The competent, transcriptionally inactive embryonic ε-globin gene, which is part of the DNAase I-sensitive β-globin domain, is highly enriched in the 0.15 M-NaCl-soluble polynucleosome fraction but not in the insoluble nuclear material. The repressed vitellogenin gene shows no enrichment in either of these fractions. These results suggest that only those genes that are expressed or have the potential for expression are enriched in the low-salt-insoluble nuclear material of immature or mature erythrocytes. The enrichment of active genes in the low-salt-insoluble residual nuclear material of immature erythrocytes is not dependent on on-going transcription, the presence of RNA or changes in the amount of acetylated histone species. Our results are consistent with the hypothesis that active and potentially active genes are insoluble because of the presence of preinitiation transcription complexes.

INTRODUCTION

Although most active genes are packaged into nucleosomes, they are organized into a less condensed chromatin structure than inactive genes. This is reflected by their increased susceptibility to digestion by various nucleases, especially DNAase I. Although the biochemical basis for the difference between expressed and repressed gene chromatin has been extensively investigated, a complete description of these two entities has not been achieved (for reviews see Reeves, 1984; Gross & Garrard, 1987). Analysis of chromatin regions separated by salt extraction has been an informative approach to determine the composition of transcriptionally active chromatin. Transcriptionally active gene chromatin has a soluble and/or insoluble nature, that is, active genes are located in two types of chromatin fragments, those that are soluble in 0.15 M-NaCl and/or 2 mM-$MgCl_2$ and those that co-fractionate with the insoluble residual nuclear material (Cohen & Sheffery, 1985; Rose & Garrard, 1984; Strätling *et al.*, 1986; Strätling, 1987; Ridsdale & Davie, 1987; Zhang & Nelson, 1988; Ridsdale *et al.*, 1988; Nickel & Davie, 1989*a*).

The nature of the insoluble transcriptionally active chromatin is unknown. It has been suggested that transcriptionally active genes are insoluble because of the presence of the transcription complex (Roberge *et al.*, 1988). Another explanation is that the expressed gene sequences are attached to the internal nuclear matrix (for review see W. G. Nelson *et al.*, 1986). It should be noted that the immature and mature chicken erythrocytes lack an internal nuclear matrix (Lafond & Woodcock, 1983; Lafond *et al.*, 1983).

The chicken erythrocyte is a popular system to study histone modifications and the structure of transcriptionally active and potentially active gene chromatin. Although there is general agreement that the β-globin gene is expressed in immature erythrocytes, there are conflicting reports as to whether the β-globin gene is expressed in mature chicken erythrocytes. Gariglio *et al.* (1981) reported that, although RNA polymerase II molecules are 'poised' at the 5′ end of the β-globin gene, the gene is not expressed in mature cells. In contrast, Affolter *et al.* (1987) demonstrated that the β-globin and histone H5 genes are transcribed in mature erythrocytes, with the transcriptional activity of the histone H5 gene in mature cells being comparable to that of immature cells.

We have demonstrated that the 0.15 M-NaCl-soluble polynucleosomes of mature chicken erythrocytes are highly enriched in DNAase I-sensitive, β-globin, histone H5 and ε-globin gene sequences (Ridsdale & Davie, 1987; Ridsdale *et al.*, 1988). The ε-globin gene, which is no longer expressed in mature or immature erythrocytes and is part of the DNAase I-sensitive, β-globin domain (Stalder *et al.*, 1980; Wood & Felsenfeld, 1982), is classified as competent chromatin. It is not known whether transcriptionally active, potentially active and competent chicken erythroid genes of mature and immature erythrocytes are enriched in the low-salt-insoluble nuclear material. To address this question, we examined the partitioning of erythroid-specific (ε-globin, β-globin and histone H5) genes among various fractions of

Abbreviations used: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PMSF, phenylmethanesulphonyl fluoride; u, ubiquitinated; MHS, micrococcal nuclease hypersensitive site.

immature and mature chicken erythrocyte chromatin. In addition, we analysed the histone composition of the chromatin fractions of immature chicken erythrocytes. We found that the β-globin and histone H5 genes, but not the competent ε-globin gene, are enriched in the low-salt-insoluble nuclear material of mature and immature erythrocytes. The selective partitioning of the β-globin and histone H5 genes with the insoluble nuclear material was not dependent on on-going transcription, the presence of RNA or the amount of acetylated histone species. Our results are consistent with the idea that the association of a preinitiation transcription complex with β-globin and histone H5 gene chromatin accounts for the insolubility of these genes. In immature chicken erythrocyte, the 0.15 M-NaCl-soluble oligo- and poly-nucleosomes, which are enriched in active and competent genes and depleted in repressed genes, are enriched in modified histone species, including hyperacetylated species of histones H2B and H4, ubiquitinated histones H2A and H2B and polyubiquitinated species of histone H2A and depleted in linker histones, H1 and H5.

MATERIALS AND METHODS

Isolation of immature and mature chicken erythrocytes

To obtain immature chicken erythrocytes [40% early and mid-polychromatic erythrocytes and 55% late polychromatic erythrocytes (Williams, 1972)], adult White Leghorn chickens were treated with phenylhydrazine hydrochloride (Ferenz & Nelson, 1985). Red blood cells from treated and untreated chickens were collected in 75 mM-NaCl/25 mM-EDTA/30 mM-sodium butyrate (except for cells incubated without butyrate), washed of the buffy coat and stored at −70 °C. For some experiments, immature erythrocytes were incubated at 37 °C for 60 min in Swim's S-77 medium with or without 10 mM-sodium butyrate, or the cells were incubated at 37 °C for 90 min in Swim's S-77 with or without the transcription inhibitors actinomycin D (0.04 μg/ml) and/or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (75 μM) (Ericsson *et al.*, 1986).

Fractionation of chromatin fragments

Nuclei were isolated and digested as described previously (Ridsdale & Davie, 1987), except that 10 mM-iodoacetamide was added to the RSB buffer. Digested nuclei (fraction T) were resuspended in an equal volume of 10 mM-EDTA (pH 7.5)/1 mM-PMSF and left on ice for at least 30 min to release chromatin fragments into solution. The soluble (S_E) and insoluble (P_E or low-salt-insoluble nuclear material) fractions were separated by centrifugation at 12000 *g* for 10 min. NaCl was added to the S_E fraction to a final concentration of 0.15 M, and after centrifugation at 12000 *g* for 10 min, an insoluble (P_{150}) and a soluble fraction (S_{150}) were obtained. The S_{150} fraction was applied on to a Bio-Gel A-5m column to produce four fractions F_I, F_{II}, F_{III} and F_{IV} (Ridsdale & Davie, 1987). RNAase digestion of the P_E fraction of immature erythrocytes was done in 10 mM-EDTA (pH 7.5)/1 mM-PMSF by incubation at 37 °C for 2 h with 200 units of RNAase T/ml and 200 μg of RNAase A/ml. After centrifugation at 12000 *g* for 10 min, an insoluble fraction (P_{RE}) and a soluble fraction were obtained. A mock digestion was done under the same conditions but RNAases were omitted. This produced the insoluble fraction P_{ME}.

DNA preparation and DNA hybridization

The percentage of DNA in each fraction was determined by diphenylamine assay (Giles & Myers, 1965). Chromatin samples were made up to 2.2% sodium *N*-dodecylsarcosine/0.16 M-NaCl/10 mM-EDTA/0.5 mg of Pronase/ml and incubated at 37 °C overnight. Afterwards they were digested with 100 units of RNAase T/ml and 100 μg of RNAase A/ml, extracted with phenol/chloroform and ethanol-precipitated. DNA was run on 1%-agarose gels containing 1 μg of ethidium bromide/ml, transferred on to nitrocellulose and hybridized to radiolabelled probes as described (Thomas, 1980). Washes were done as previously described (Maniatis *et al.*, 1982). Quantification of DNA sequences was done by slot blot analysis. The DNA was made to 0.3 M-NaOH, incubated at 37 °C for 30 min, neutralized by the addition of an equal volume of 2 M-ammonium acetate, pH 7.0, and various amounts were applied on to nitrocellulose using a Schleicher and Schuell slot blotting manifold. Each slot was rinsed with 10 × SSC (20 × SSC = 3 M-NaCl/0.3 M-sodium citrate). After hybridization to radiolabelled probes (Thomas, 1980), the autoradiograms were scanned with a densitometer. The relation between the signal and the amount of DNA loaded was linear, showing that the intensity of the signal was directly proportional to the amount of hybridizable DNA sequence. The intensity of the hybridization signal per μg of DNA was determined for DNA which was isolated from total unfractionated chromatin (T) and the chromatin fractions. The values obtained for the DNA of each chromatin fraction were divided by the value for total DNA, determining the partitioning ratio of DNA sequences among the different chromatin fractions. If the partitioning ratio was > 1, the analysed DNA sequence was enriched in that chromatin fraction, but if the partitioning ratio was < 1, the analysed DNA sequence was depleted. The percentage of a DNA sequence in a chromatin fraction was obtained by multiplying the partitioning ratio of a sequence by the percentage of total DNA in that fraction (this value was determined by diphenylamine assay). The cloned DNA probes used were: pCBG14.4, an unique intronic sequence of the adult β-globin gene; pCBG18.7, an embryonic ε-globin sequence (Villeponteau *et al.*, 1982); pChV2.5B/H, which contains the gene coding for histone H5 and some flanking sequences on both sides (Ruiz-Carrillo *et al.*, 1983); and pVTG412, which recognizes the 5′ region of the vitellogenin gene (Burch & Weintraub, 1983).

Protein electrophoresis and Western blotting

Chromatin fractions were extracted with 0.2 M-H_2SO_4. After centrifugation at 12000 *g* for 10 min, the supernatants were dialysed against 0.1 M-acetic acid and twice against water. Polyacrylamide-gel electrophoresis was performed as described (Davie, 1982). Ubiquitin protein conjugates were detected by the use of a polyclonal anti-ubiquitin antibody in Western-blotting experiments (Nickel *et al.*, 1987).

RESULTS

Transcriptionally active and potentially active genes are preferentially located in the low-salt-insoluble residual nuclear material and 0.15 M-NaCl-soluble oligo- and poly-nucleosomes

Mature and immature chicken erythrocyte nuclei were

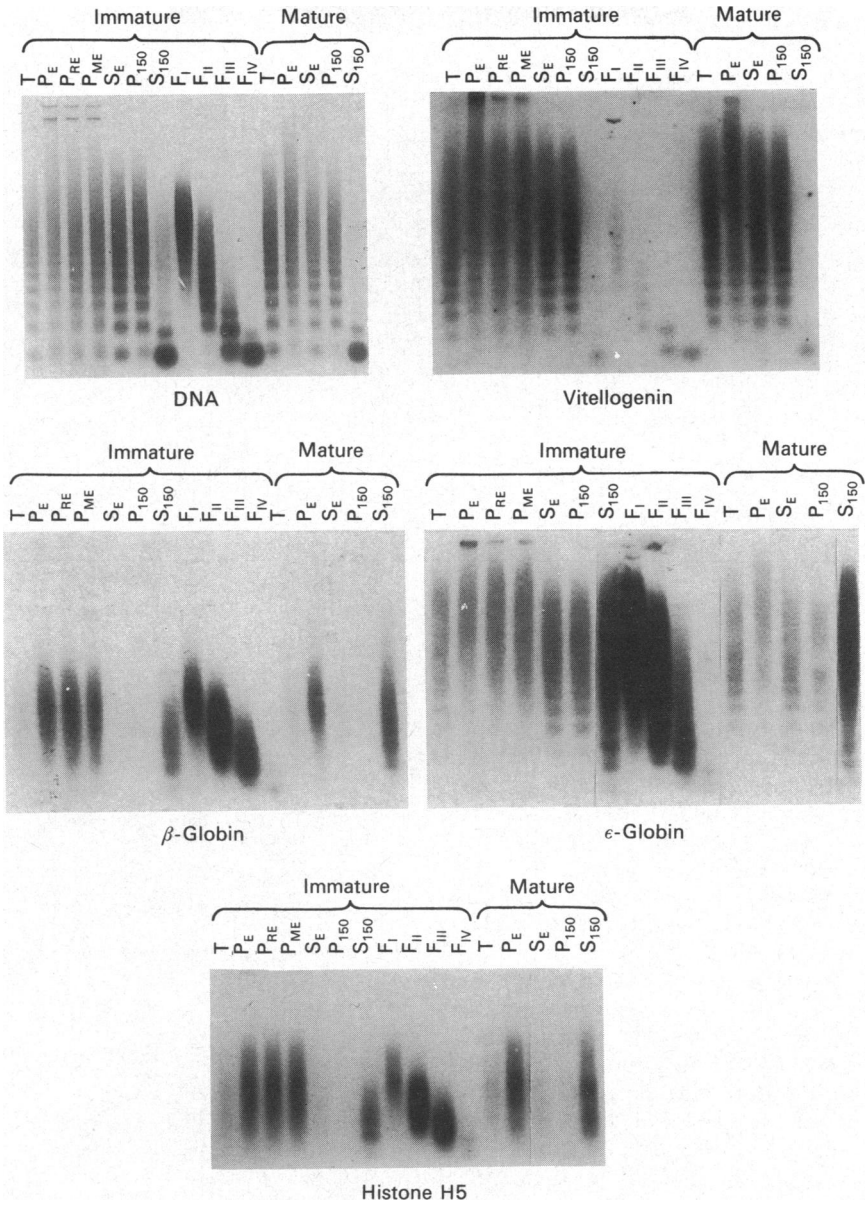

Fig. 1. DNA sequence distribution among the different chromatin fractions

DNA (8 μg) isolated from each chromatin fraction was run on 1% agarose gels, stained with ethidium bromide (DNA) or transferred on to nitrocellulose and hybridized to ^{32}P-labelled probes as indicated. P_{RE} and P_{ME} are DNA samples isolated from the low-salt-insoluble nuclear material fraction P_E that was treated or mock-treated with RNAase, respectively.

incubated with micrococcal nuclease, and the fragmented chromatin was fractionated by a low-salt procedure as described in the Materials and methods section. The repressed vitellogenin and competent ϵ-globin DNA sequences show a typical nucleosomal ladder like that of bulk DNA (Fig. 1). In contrast, the histone H5 and β-globin DNA sequences, which are active in immature erythrocytes and potentially active in mature cells, exhibit a non-discrete continuum of DNA lengths (Fig. 1), which is typical of genes that are expressed or have the potential for expression (Cohen & Sheffery, 1985).

For both immature and mature erythroid chromatin, the histone H5 and β-globin genes are enriched in fractions P_E (the low-salt-insoluble nuclear material) and S_{150}, the 0.15 M-NaCl-soluble chromatin (Fig. 1 and Table 1). The β-globin and histone H5 DNA sequences are enriched to similar extents in both mature and immature erythrocyte insoluble nuclear material (P_E), with an enrichment of 4.7–5.6-fold. However, around 10% of the DNA remains associated with the insoluble nuclear material of immature erythroid nuclei, compared to 5% with fraction P_E of mature erythroid nuclei (Table 1). Thus, the proportion of the β-globin and histone H5 gene chromatin in the insoluble residual nuclear material is greater in immature cells, in which these genes are expressed, than in mature cells, in which these genes are potentially active (Table 1). β-Globin and histone H5 DNA sequences of mature and immature erythrocytes are also enriched in the 0.15 M-NaCl-soluble oligonucleosomes and polynucleosomes (Fig. 1).

In both immature and mature erythrocytes, repressed vitellogenin and competent ϵ-globin DNA sequences are

Table 1. DNA sequence distribution among the different chromatin fractions

The percentage of DNA in each chromatin fraction was determined by diphenylamine assay and the percentage of each sequence was determined by hybridization of slot blots and densitometric analysis of the autoradiograms. Each value represents the mean ± S.E.M. from two to six measurements.

Source of chromatin	Fraction	DNA (%)	Histone H5 (%)	β-Globin (%)	ε-Globin (%)	Vitellogenin (%)
Mature chicken erythrocytes	Total	100	100	100	100	100
	P_E	4.9±0.3	27.4±3.8	23.0±0.5	6.0±2.3	6.6±1.3
	S_E	95.1±0.3	72.3±8.2	66.7±13.4	88.9±14.6	95.3±0.0
Immature chicken erythrocytes	Total	100	100	100	100	100
	P_E	9.9±0.8	51.4±9.9	47.8±7.2	14.4±0.7	11.2±0.5
	S_E	90.1±0.8	46.6±6.8	42.1±5.3	90.4±1.2	93.1±10.4
	P_{150}	78.3±3.3	9.8±4.0	13.7±4.0	35.3±14.1	90.1±5.6
	S_{150}	11.8±4.1	38.1±9.6	33.5±9.2	50.7±8.3	0.8±0.4
	F_I	0.5±0.1	1.9±0.1	2.9±0.1	17.6±1.8	0.1±0.1
	F_{II}	1.3±0.4	12.6±3.0	14.2±1.6	21.9±0.9	0.2±0.1
	F_{III}	1.6±0.3	8.8±1.9	9.2±1.3	5.1±0.8	0.2±0.0

not enriched in the insoluble nuclear material (P_E; Fig. 1 and Table 1). The repressed vitellogenin DNA sequences are located in the 0.15 M-NaCl-insoluble chromatin (P_{150}) and 0.15 M-NaCl-soluble mononucleosomes (S_{150}), but these sequences are under-represented in the 0.15 M-NaCl-soluble polynucleosomes. The competent ε-globin DNA sequences are enriched in the 0.15 M-NaCl-soluble polynucleosomes of S_{150} chromatin (Fig. 1 and Table 1).

The immature erythroid chromatin fragments that are soluble in 0.15 M-NaCl (fraction S_{150}) were fractionated by gel exclusion chromatography on a Bio-Gel A-5m column, obtaining four fractions F_I, F_{II}, F_{III} and F_{IV} of decreasing DNA lengths (see Fig. 1, DNA). Competent ε-globin sequences are highly enriched in fraction F_I polynucleosomes, while active histone H5 and β-globin DNA sequences are enriched to the greatest extent in fraction F_{II} (Fig. 1 and Table 1). The repressed vitellogenin DNA sequences were depleted in the 0.15 mM-NaCl soluble oligo- and poly-nucleosomes present in fractions F_I, F_{II} and F_{III} (Fig. 1 and Table 1). Since the 0.15 M-NaCl-soluble oligo- and poly-nucleosomes are highly enriched in competent and active DNA sequences and depleted in repressed DNA, these chromatin fractions will be the most informative as to the composition of transcriptionally active and competent chromatin.

The 0.15 M-NaCl soluble polynucleosomes of immature erythrocytes are enriched in modified histone species

Goldknopf *et al.* (1980) reported that immature chicken erythrocyte chromatin had a greater amount of ubiquitinated histone H2A (uH2A) than did mature erythrocytes. We determined whether the amount of uH2B in immature erythrocyte chromatin was also elevated. Histones of immature (lane T in Fig. 2) and mature (lane T_M in Fig. 2) erythrocyte nuclei were electrophoretically resolved on an acetic acid/urea/Triton/15%-polyacrylamide gel, and the amount of uH2A and uH2B was determined. As reported previously, the amount of uH2A is greater in immature than in mature erythrocyte histones (approx. 5-fold). This is also found for uH2B, with the amount of this modified histone being approx. 5-fold greater in immature erythrocyte chromatin.

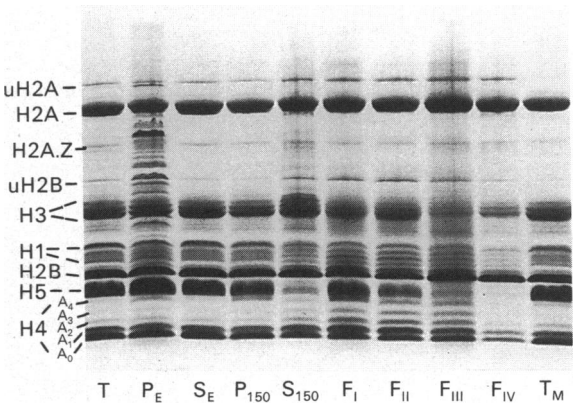

Fig. 2. Analysis of the proteins present in the different chromatin fractions

Acid-soluble proteins (9 μg) isolated from each chromatin fraction were electrophoretically resolved on an acetic acid/6.7 M-urea/0.375% Triton X-100 (AUT)/15%-polyacrylamide minislab 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. The basis by which the modified histone species were identified is described in Ridsdale & Davie (1987). All of the protein samples were from chromatin fractions of immature erythroid nuclei, except for T_M which is acid-soluble proteins isolated from mature erythroid nuclei.

The 0.15 M-NaCl-soluble polynucleosomes of mature chicken erythrocytes are enriched in acetylated species of histones H2B and H4 and ubiquitinated species of histones H2A and H2B (Ridsdale & Davie, 1987). The 0.15 M-NaCl-soluble immature erythroid oligo- and poly-nucleosomes are also enriched in these modified histone species (see lanes F_I, F_{II} and F_{III} in Fig. 2). When the amount of the ubiquitinated histones of the 0.15 M-NaCl-soluble oligo- and poly-nucleosomes (lanes F_I, F_{II} and F_{III}) was compared to that of unfractionated chromatin (lane T), uH2A and uH2B were enriched approx. 1.7-fold and 3.2-fold, respectively. This enrichment is comparable to that observed for the 0.15 M-NaCl-soluble polynucleosomes of mature erythrocytes

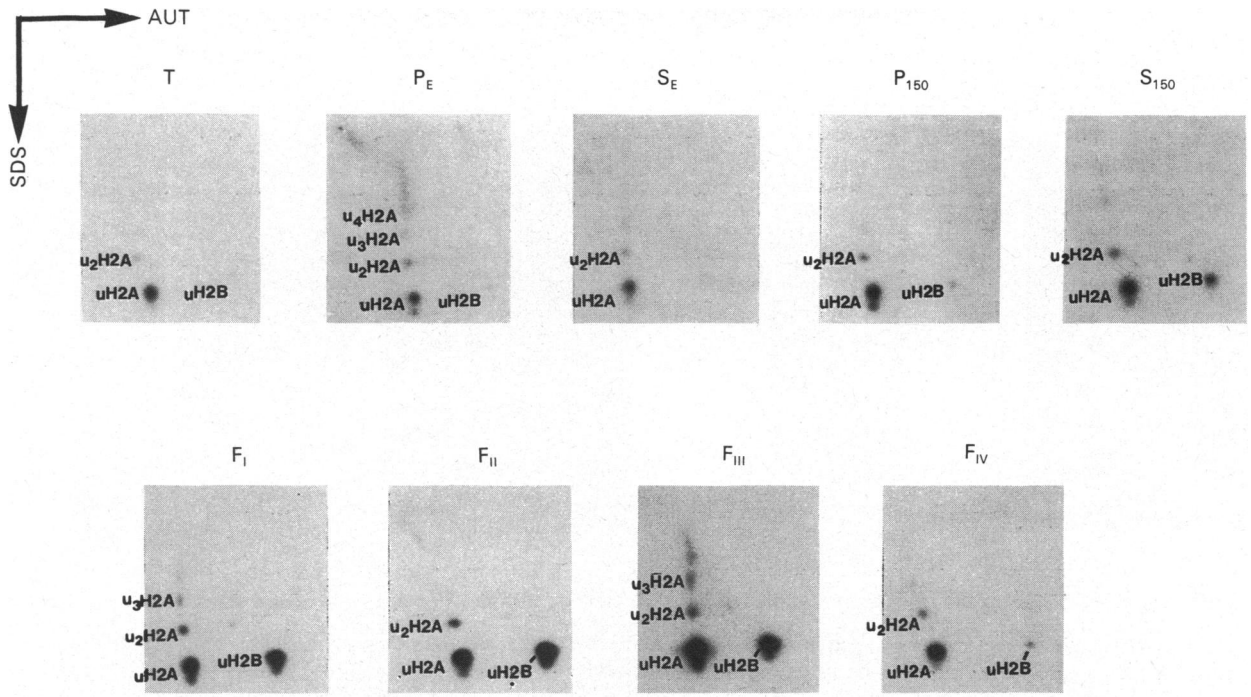

Fig. 3. Identification of ubiquitinated histone species in the different immature erythroid chromatin fractions

Acid-soluble proteins (9 μg) isolated from each immature erythroid fraction were electrophoretically separated on two-dimensional gels (AUT into SDS). The proteins were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with anti-ubiquitin IgG and ^{125}I-labelled protein A. The autoradiograms are shown. The polyubiquitinated histone species are labelled as u_2, u_3 and u_4, representing the attachment of two, three and four ubiquitins respectively.

(Ridsdale & Davie, 1987). However, it should be noted that the amount of ubiquitinated histones of the 0.15 M-NaCl-soluble polynucleosomes of immature erythrocytes is approx. 5-fold greater than the amount of uH2A and uH2B of 0.15 M-NaCl-soluble polynucleosomes of mature cells. None of the other chromatin fractions, including P_E, are enriched in acetylated histone species or uH2A. However, uH2B appears to be enriched in fraction P_E. The histones of immature erythrocyte chromatin fractions F_I and T were electrophoretically resolved on SDS/polyacrylamide gels (not shown). Analysis of the amount of linker histones H1 and H5 in the histones of these two chromatin fractions shows that fraction F_I has approx. 50% of the amount of linker histones found in unfractionated chromatin.

The distribution of the ubiquitinated and poly-ubiquitinated histone species among the immature erythroid chromatin fractions was determined. Similar amounts of histones from the various fractions were resolved by two-dimensional gel electrophoresis, transferred to nitrocellulose, and the ubiquitin–protein conjugates were detected by immunochemical staining with an anti-ubiquitin IgG and ^{125}I-protein A. Fig. 3 shows that uH2B and the polyubiquitinated species of H2A are enriched in the 0.15 M-NaCl-soluble oligo- and poly-nucleosomes of fractions F_I, F_{II} and F_{III}. The histones of fraction P_E are not enriched in uH2B, but histone H2A is enriched in polyubiquitinated species.

A range of nuclease digestions does not alter the enrichment of active DNA in the low-salt-insoluble nuclear material

Rose & Garrard (1984) observed that increasing the fragmentation of MPC-11 chromatin resulted in the release of the active κ immunoglobulin light chain gene sequences from the low-salt-insoluble nuclear material into a salt-soluble chromatin fraction. To test whether increasing the extent of nuclease digestion would result in liberation of the active DNA sequences from the low-salt-insoluble nuclear material into fraction S_{150}, mature and immature chicken erythrocyte nuclei were incubated for various times with micrococcal nuclease, and the chromatin was fractionated. Fig. 4 shows the distribution of the histone H5 gene sequences in chromatin fractions P_E and S_{150}. The results demonstrate that increasing the extent of nuclease digestion reduces the average size of the histone H5 DNA fragments, but not the magnitude of enrichment in the insoluble chromatin fraction P_E. Moreover, increasing the digestion time to 60 min had little effect on the selective partitioning of the active histone H5 gene sequences with the insoluble nuclear material (not shown).

Among the smear of histone H5 DNA fragments, two discrete bands of about 2.3 kb and 1.15 kb are visible for each fraction isolated from the mature erythroid nuclei. These bands are probably generated by digestion at the micrococcal hypersensitive sites (MHS) which are located at positions -111 ± 6 (MHS 5), $1016\pm12/1211\pm12$ (MHS 7) and 2182 ± 30 (MHS8) (Renaud & Ruiz-Carrillo, 1986). Preferential digestion at MHS 5 and MHS 8 would generate a band of approx. 2.3 kb, while preferential digestion at MHS 5 and 7 or MHS 7 and 8 would generate a band of approx. 1.15 kb. As the extent of digestion increases, the intensity of the 2.3 kb fragment decreases while that of the 1.15 kb fragment increases. In immature erythrocytes, the 2.3 kb band is less distinct in all fractions and is absent from the S_{150} fractions, where the majority of the H5 DNA fragments are shorter than

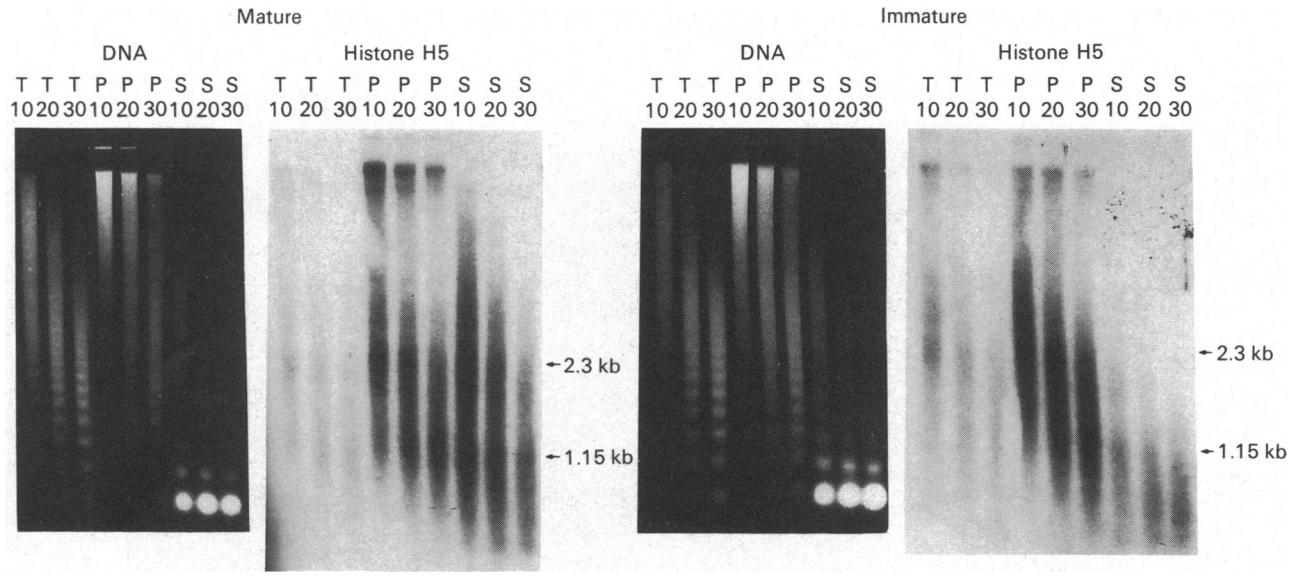

Fig. 4. Distribution of active histone H5 DNA in chromatin fractions P_E and S_{150} as a function of extent of micrococcal-nuclease digestion

Immature or mature erythrocyte nuclei were incubated for 10, 20 and 30 min with micrococcal nuclease. DNA (8 μg) isolated from nuclei (T), the insoluble nuclear P_E fractions (P) or the 0.15 M-NaCl-soluble S_{150} fractions (S) was run on 1% agarose gels, stained with ethidium bromide (DNA) or transferred onto nitrocellulose and hybridized to the ^{32}P-labelled histone H5 probe.

1.15 kb. The 1.15 kb band is quite apparent in the 10 min digestion S_{150} fraction. Note that, in contrast to mature erythroid chromatin, reducing the extent of fragmentation of immature erythroid chromatin does not result in the solubilization of long histone H5 gene chromatin fragments into fraction S_{150}.

The loss of RNA or acetylated histone species does not alter the enrichment of active genes with the low-salt-insoluble nuclear material of immature erythrocytes

The nature of the association of the active genes with the insoluble nuclear material in fraction P_E of immature erythrocytes was investigated. Fraction P_E was incubated with RNAase, centrifuged and DNA was isolated from the treated insoluble nuclear material. Fig. 1, lane P_{RE}, shows that RNAase digestion does not alter the enrichment of active DNA sequences in this fraction, suggesting that the active gene sequences are not attached to the residual nuclear material via RNA.

Alonso *et al.* (1987) demonstrated that the removal of acetyl groups from the hyperacetylated histones resulted in a loss of the low-salt solubility of the β-globin and histone H5 gene chromatin. Conversely, the amount of low-salt-soluble active gene chromatin was increased by augmentation of the amount of hyperacetylated histones (D. A. Nelson *et al.*, 1986). This was achieved by incubating cells in the presence of butyrate, an inhibitor of histone deacetylase (Candido *et al.*, 1978). To determine whether changing the amount of hyperacetylated histones would alter the partitioning of the active genes with the low-salt-insoluble nuclear material, immature erythroid cells were incubated in the presence or absence of sodium butyrate under conditions known to hyperacetylate or hypoacetylate histones of active chromatin (Alonso *et al.*, 1987). The enrichment of β-globin and histone H5 DNA sequences in fraction P_E was not altered (not shown).

The enrichment of active DNA with the low-salt-insoluble nuclear material is not dependent on on-going transcription

Immature erythrocytes were incubated with or without transcription inhibitors, actinomycin D and DRB. The presence of actinomycin D and DRB or DRB alone did not affect the distribution of active histone H5 sequences among the different fractions, and the enrichment in the insoluble nuclear fraction P_E was not altered (not shown).

DISCUSSION

We demonstrate that the erythroid-specific β-globin and histone H5 genes are enriched in the low-salt-insoluble nuclear material of immature and mature chicken erythrocytes. Approx. 50% of the active β-globin and histone H5 gene chromatin of immature erythrocytes and approx. 25% of the chromatin of these potentially active genes of mature erythrocytes have an insoluble nature. The histone H2A.F gene (also known as H2A.Z), which is expressed in immature erythrocytes (Harvey *et al.*, 1983), has the same distribution among the various chromatin fractions of immature or mature erythrocytes as that of the β-globin and histone H5 genes (not shown). In contrast, the competent ϵ-globin gene, which is part of the DNAase I-sensitive β-globin domain and is located next to the β-globin gene, is not enriched in the DNA of the residual nuclear material. In addition, genes, such as thymidine kinase and c-*myc*, that are no longer expressed in immature erythrocytes (Conklin & Groudine, 1986) and repressed genes (vitellogenin and ovalbumin) are not enriched in the low-salt-insoluble nuclear material. These results suggest that only those genes that are expressed or have the potential for expression (for example, the β-globin gene of mature erythrocytes that have RNA polymerase molecules

located at the 5′ end of the gene) are enriched in the low-salt-insoluble nuclear material of immature or mature erythrocytes. Furthermore, gene sequences that are sensitive to DNAase I (e.g. ϵ-globin) are not necessarily enriched in the insoluble nuclear material.

Since immature and mature erythrocytes lack an internal nuclear matrix (Lafond & Woodcock, 1983; Lafond *et al.*, 1983), this suggests that the insolubility of active genes is not due to their association with the internal matrix. Several reports indicate that RNA is essential for the maintenance of the internal matrix structure (for review see Verheijen *et al.*, 1988; Nickerson *et al.*, 1989). Incubation of the residual nuclear material with RNAase did not solubilize active gene chromatin. This supports the conclusion that the insolubility of active gene chromatin is not a consequence of an interaction of active genes with the internal matrix. Moreover, our results suggest that the insolubility of active gene sequences is not due to the formation of intermolecular disulphide bonds, amount of histone acetylation or presence of RNA polymerase. Kaufmann *et al.* (1986) reported that disulphide bonds may mediate the association between the glucocorticoid receptor and nuclear matrix. It is conceivable that the presence of proteins that are capable of forming disulphide bonds with active genes may mediate the attachment of active genes with the nuclear matrix. However, the addition of iodoacetamide to the buffer used to isolate the chicken erythrocyte nuclei would prevent the formation of such bonds. The incubation of immature erythrocytes in the presence of DRB, which blocks transcription at or near the initiation site (Andersson *et al.*, 1982; Zandomeni *et al.*, 1986), does not alter the enrichment of active histone H5 DNA in fraction P_E. DRB does not intercalate or interfere with elongation of transcription and allows the release of RNA polymerase upon termination of transcription (Zandomeni *et al.*, 1983). This suggests that the association of active genes with the insoluble nuclear pellet of immature erythrocytes is not due to the presence of RNA polymerase. Our results are consistent with the idea that the insolubility of active gene chromatin is a result of the presence of preinitiation transcription complexes. Culotta *et al.* (1985) observed the quantitative sedimentation of stable RNA polymerase II preinitiation complexes formed by incubation of a naked DNA template and a whole-cell extract. These complexes lacked a factor necessary for transcription, so the insolubility was independent of transcription.

In mature and immature cells, the majority of the EDTA-solubilized active/potentially active β-globin and histone H5 gene chromatin fragments are soluble in 0.15 M-NaCl (the present paper; Ridsdale & Davie, 1987; Ridsdale *et al.*, 1988). The majority of the salt-soluble immature erythroid histone H5 gene chromatin fragments are of lengths 1.15 kb or shorter, while the 0.15 M-NaCl-soluble histone H5 gene chromatin fragments of mature erythrocytes can be considerably longer. It is interesting to note that 1.15 kb is the distance between micrococcal-nuclease-hypersensitive sites (MHS) 5 and 7, which are located at the 5′ and 3′ ends of the histone H5 gene respectively (Renaud & Ruiz-Carrillo, 1986). It is conceivable that only the coding region of the active histone H5 gene chromatin of immature cells is salt-soluble, while the coding and flanking regions of the potentially active histone H5 gene chromatin of mature cells are soluble in 0.15 M-NaCl.

The protein composition of the 0.15 M-NaCl-soluble oligo- and polynucleosomes, which are depleted in repressed DNA and enriched in active/competent DNA, of immature erythroid cells is similar to that of mature erythrocytes. However, the 0.15 M-NaCl-soluble polynucleosomes of immature erythroid cells have increased levels of uH2A and uH2B. It is possible that the increased amounts of the ubiquitinated histones may generate active/competent chromatin domains in immature erythrocytes that have a more 'open', disrupted, structure than the potentially active/competent chromatin domains of mature erythrocytes. The 0.15 M-NaCl-soluble polynucleosomes from both sources are enriched in hyperacetylated species of histones H4 and H2B. Recently, Hebbes *et al.* (1988) reported that acetylated histone species of the 0.15 M-NaCl-soluble oligonucleosomes are complexed with active genes. Interestingly, histone acetyltransferases are enriched in the linker DNA regions of the 0.15 M-NaCl soluble polynucleosomes (Chan *et al.*, 1988). It will be of considerable interest to determine whether other enzymes involved in modifying the histones are also preferentially localized with the active/competent gene chromatin domains.

Polyubiquitinated species of H2A are enriched in the 0.15 M-NaCl-soluble oligo- and poly-nucleosomes and in the insoluble chromatin fraction P_E. Recently, we determined that the major arrangement of ubiquitin in polyubiquitinated H2A is a chain of ubiquitin molecules joined to each other by isopeptide bonds to a ubiquitin molecule which is attached to the ϵ-amino group of Lys-119 of histone H2A (Nickel & Davie, 1989*b*). The role of histone polyubiquitination is not known. It is possible that polyubiquitination of the histones alters nucleosome structure and/or nucleosome packaging into higher order chromatin structures. Another possibility is that polyubiquitinated histones are tagged for turnover when displaced from the DNA. It is interesting to note that Chau *et al.* (1988) observed that cytoplasmic proteins tagged with a chain of ubiquitin molecules, but not those that were mono-ubiquitinated, were recognized in ubiquitin-dependent degradation of proteins.

We wish to thank Darcy Salo for excellent technical assistance, as well as Andy Ridsdale and Michael Hendzel for the erythrocyte cell preparation. This project was supported by a grant from the Medical Research Council of Canada and by the award of a Medical Research Council Scholarship to J.R.D.

REFERENCES

Affolter, M., Côté, J., Renaud, J. & Ruiz-Carrillo, A. (1987) Mol. Cell. Biol. **7**, 3663–3672

Alonso, W. R., Ferris, R. C., Zhang, D.-E. & Nelson, D. A. (1987) Nucleic Acids Res. **15**, 9325–9337

Andersson, K., Mahr, R., Bjorkroth, B. & Daneholt, B. (1982) Chromosoma **87**, 33–48

Burch, J. B. E. & Weintraub, H. (1983) Cell **33**, 65–76

Candido, E. P. M., Reeves, R. & Davie, J. R. (1978) Cell **14**, 105–113

Chan, S., Attisano, L. & Lewis, P. N. (1988) J. Biol. Chem. **263**, 15643–15651

Chau, V., Cousins, L. G., Marriott, D., Marsh, J. & Ecker, D. (1988) J. Cell Biol. **107**, 613a

Cohen, R. B. & Sheffery, M. (1985) J. Mol. Biol. **182**, 109–129

Conklin, K. F. & Groudine, M. (1986) Mol. Cell. Biol. **6**, 3999–4007
Culotta, V. C., Wides, R. J. & Solner-Webb, B. (1985) Mol. Cell. Biol. **5**, 1582–1590
Davie, J. R. (1982) Anal. Biochem. **120**, 276–281
Ericsson, C., Goldknopf, I. L. & Daneholt, B. (1986) Exp. Cell Res. **167**, 127–134
Ferenz, C. R. & Nelson, D. A. (1985) Nucleic Acids Res. **13**, 1977–1995
Gariglio, P., Bellard, M. & Chambon, P. (1981) Nucleic Acids Res. **9**, 2589–2598
Giles, K. W. & Myers, A. (1965) Nature (London) **206**, 93
Goldknopf, I. L., Wilson, G., Ballal, N. R. & Busch, H. (1980) J. Biol. Chem. **255**, 10555–10558
Gross, D. S. & Garrard, W. T. (1987) Trends Biochem. Sci. **12**, 293–297
Harvey, R. P., Whiting, J. A., Coles, L. S., Krieg, P. A. & Wells, J. R. E. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2819–2823
Hebbes, T. R., Thorne, A. W. & Crane-Robinson, C. (1988) EMBO J. **7**, 1395–1402
Kaufmann, S. C., Okret, S., Wikstrom, A.-C., Gustafsson, J.-A. & Shaper, J. H. (1986) J. Biol. Chem. **261**, 11962–11967
Lafond, R. E. & Woodcock, C. L. F. (1983) Exp. Cell Res. **147**, 31–39
Lafond, R. E., Woodcock, C. L. F., Kundahl, E. R. & Lucas, J. J. (1983) J. Cell Biol. **96**, 1815–1819
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, pp. 387–389, Cold Spring Harbor Laboratory, New York
Nelson, D. A., Ferris, R. C., Zhang, D.-E. & Ferenz, C. R. (1986) Nucleic Acids Res. **14**, 1667–1682
Nelson, W. G., Pienta, K. J., Barrack, E. R. & Coffey, D. S. (1986) Annu. Rev. Biophys. Biophys. Chem. **15**, 457–475
Nickel, B. E. & Davie, J. R. (1989*a*) Biochim. Biophys. Acta **1007**, 23–29
Nickel, B. E. & Davie, J. R. (1989*b*) Biochemistry **28**, 964–968
Nickel, B. E., Roth, S. Y., Cook, R. G., Allis, C. D. & Davie, J. R. (1987) Biochemistry **26**, 4417–4421
Nickerson, J. A., Krochmalnic, G., Wan, K. M. & Penman, S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 177–181
Reeves, R. (1984) Biochim. Biophys. Acta **782**, 343–393
Renaud, J. & Ruiz-Carrillo, A. (1986) J. Mol. Biol. **189**, 217–226
Ridsdale, J. A. & Davie, J. R. (1987) Nucleic Acids Res. **15**, 1081–1096
Ridsdale, J. A., Rattner, J.-B. & Davie, J. R. (1988) Nucleic Acids Res. **16**, 5915–5926
Roberge, M., Dahmus, M. E. & Bradbury, E. M. (1988) J. Mol. Biol. **201**, 545–555
Rose, S. M. & Garrard, W. T. (1984) J. Biol. Chem. **259**, 8534–8544
Ruiz-Carrillo, A., Affolter, M. & Renaud, J. (1983) J. Mol. Biol. **170**, 843–859
Stalder, J., Larsen, A., Engel, J. D., Dolan, M., Groudine, M. & Weintraub, H. (1980) Cell **20**, 451–460
Strätling, W. H. (1987) Biochemistry **26**, 7893–7899
Strätling, W. H., Dolle, A. & Sippel, A. E. (1986) Biochemistry **25**, 495–502
Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
Verheijen, R., Van Venrooij, W. & Ramaekers, F. (1988) J. Cell Sci. **90**, 11–36
Villeponteau, B., Landes, G. M., Pankratz, M. J. & Martinson, H. G. (1982) J. Biol. Chem. **257**, 11015–11023
Williams, A. F. (1972) J. Cell. Sci. **10**, 27–46
Wood, W. I. & Felsenfeld, G. (1982) J. Biol. Chem. **257**, 7730–7736
Zandomeni, R., Bunick, D., Ackerman, S., Mittleman, B. & Weinmann, R. (1983) J. Mol. Biol. **167**, 561–574
Zandomeni, R., Zandomeni, M. C., Shugar, D. & Weinmann, R. (1986) J. Biol. Chem. **261**, 3414–3419
Zhang, D.-E. & Nelson, D. A. (1988) Biochem. J. **250**, 233–240

Received 15 February 1989/26 April 1989; accepted 5 May 1989