

Isolation of a subclass of nuclear proteins responsible for conferring a DNase I-sensitive structure on globin chromatin

(erythrocyte differentiation/reconstitution/high mobility group proteins/recognition and propagation)

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ABSTRACT The globin gene is preferentially sensitive to digestion by DNase I in erythrocyte chromatin but not in brain, fibroblast, or oviduct chromatin. Elution of the erythrocyte chromatin with 0.35 M NaCl leads to no detectable change in the gross structure of individual nucleosomes; however, in this depleted chromatin the globin gene is no longer preferentially sensitive to DNase I. Reconstitution of the depleted chromatin with either the entire 0.35 M NaCl fraction or a subclass from this fraction greatly enriched in two high mobility group proteins (nos. 14 and 17) results in the successful reconstitution of DNase I sensitivity of the globin gene. For all of these preparations, the inactive ovalbumin gene exhibited no preferential sensitivity to DNase I. Reconstitution of the erythrocyte 0.35 M NaCl fraction with depleted brain chromatin resulted in no preferential sensitivity of the globin gene in brain chromatin; however, reconstitution of the brain 0.35 M NaCl fraction with depleted erythrocyte chromatin led to successful reconstitution of DNase I sensitivity of the globin gene. Thus, the eluted proteins responsible for conferring DNase I sensitivity are probably not tissue-specific and probably do not recognize specific DNA sequences.

The globin gene in chicken erythrocyte nuclei is preferentially sensitive to digestion by DNase I but not to digestion by staphylococcal nuclease (1). The resistance of the globin gene to staphylococcal nuclease suggests that the globin gene is packaged into nucleosome-like particles; however, its sensitivity to DNase I indicates that these particles are conformationally different from most nucleosomes. The sensitivity of the globin gene to DNase I is tissue specific in that globin chromatin is preferentially digested in erythrocytes, but not in chicken oviduct. Conversely, the ovalbumin gene is preferentially digested in the chicken oviduct but not in erythrocytes (1, 2). The sensitivity to DNase I of actively transcribed genes seems to be general, because sequences coding for nuclear RNA (1) and the least abundant class of mRNAs (3, 4) are also preferentially digested. Moreover, the latter genes are digested at about the same rate as the ovalbumin gene in chicken oviduct nuclei (3). This indicates that the sensitive chromatin structure may be fairly universal and independent of the frequency at which an active gene is transcribed. In addition to these genes, various actively transcribed, integrated viral genes (exogenous and endogenous) are sensitive to DNase I (5-7), and ribosomal DNA is also preferentially sensitive (8-10).

The sensitivity of actively transcribed genes to DNase I probably reflects an altered nucleosome structure that is required for RNA chain elongation. It is also likely that DNase I sensitivity reflects a *potential* for a gene to be copied, because the globin gene remains sensitive in mature erythrocytes that are no longer active in transcription (1, 11), because the ovalbumin gene remains sensitive in the hormone-withdrawn chicken oviduct (12), and because physarum ribosomal DNA

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remains sensitive during mitosis (9) when ribosomal RNA transcription is not detectable.

The boundaries of the sensitive chromatin structure are also very precise because, in the case of a hamster line transformed by adenovirus, integrated adenovirus sequences that are transcribed are sensitive to DNase I, whereas the adjacent adenovirus sequences that are not transcribed are not sensitive (6); thus, the active stretch of chromatin has well-defined stopping and starting points.

One of the puzzling features of the DNase I results is that the sensitivity can extend over as many as 30-40 nucleosomes. In the case of globin and the exogenous and endogenous avian RNA tumor viruses, individual nucleosomes retain this sensitivity; however, in the case of the ovalbumin gene, individual nucleosomes are resistant, suggesting that a loosely bound protein may be responsible for conferring DNase I sensitivity. We believe that the simplest way to explain the sensitivity of a contiguous string of 30-40 nucleosomes is to assume that somewhere along the transcription unit there is a "recognition event" in which a particular DNA sequence is read. This is then followed by a non-sequence-specific "propagation event" in which a DNase I sensitive structure is propagated along the transcription unit. Here we describe experiments that define a group of proteins that are easily eluted from chromatin and are responsible in part for conferring DNase I sensitivity. These proteins are not tissue specific and they cofractionate with a specific subset of high mobility group (HMG) proteins (13), which probably corresponds to HMG-14 and HMG-17. We believe that they take part in the proposed "propagation event" and not in the "recognition event."

MATERIALS AND METHODS

Isolation of Nuclei. Embryonic erythrocytes were isolated from the circulating blood of 14- to 16-day-old White Leghorn chicken embryos by vein puncture. Brain cells were isolated from freshly extracted brain tissue derived from 16- to 20-day-old embryos. The brains were sheared with a loose-fitting Dounce homogenizer and washed twice in phosphate-buffered saline (GIBCO). Nuclei were isolated by suspension in reticulocyte standard buffer (RSB) (0.01 M Tris-HCl, pH 7.4/0.01 M NaCl/3 mM MgCl₂) containing 0.5% Nonidet P-40. Subsequent procedures were performed in the presence of 1 mM phenylmethylsulfonyl fluoride as a proteolysis inhibitor.

NaCl Chromatin Extraction. The nuclei were washed several times with RSB and then lysed and washed with EDTA wash solution (0.2 mM EDTA/75 mM NaCl/0.01 M Tris-HCl,

Abbreviations: RSB, reticulocyte standard buffer; NaDodSO₄, sodium dodecyl sulfate; C₀t_{1/2}, the value of initial concentration of DNA (moles of nucleotide/liter) × time (seconds) necessary for 50% hybridization; HMG, high mobility group.

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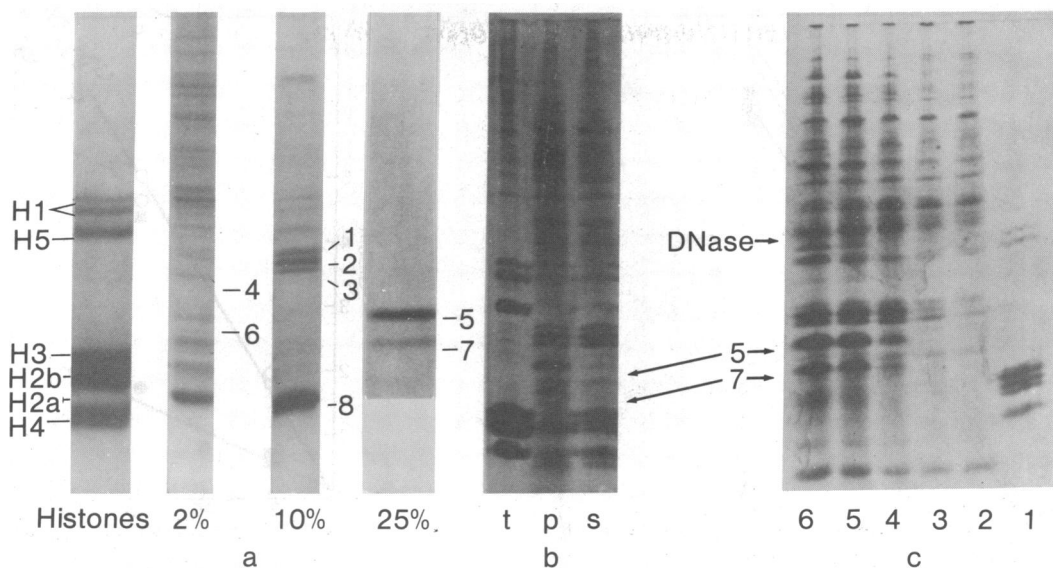


FIG. 1. Preferential release from nuclei of 10% trichloroacetic acid-soluble HMG proteins by limited DNase I digestion. (a) The 0.35 M NaCl extract from embryonic 14-day erythrocyte nuclei was precipitated successively with 2, 10, and 25% trichloroacetic acid, and the pellets from each precipitate were washed with acetone and run on NaDodSO₄/18% acrylamide gels. Each lane represents a different gel; however, relative mobilities are comparable. (b) Erythrocyte nuclei (1 mg/ml in DNA) were treated with 1 μg of pancreatic DNase (p) per ml or 10 μg of staphylococcal nuclease(s) per ml for 30 min at 37°C in RSB. DNase I digestion was to 10% acid solubility; staphylococcal nuclease digestion was to 35% acid solubility. Nuclei were pelleted after addition of EDTA to a final concentration of 2 mM and cooling to 4°C, and the solubilized protein was analyzed on NaDodSO₄/15% acrylamide gels after concentration with 20% trichloroacetic acid and washing with acetone. t, Total nuclear protein. There are usually eight nonhistone proteins (numbered 1 through 8) that migrate below H5 in these gels. Most of these proteins display solubility properties of HMG proteins; however, separate experiments suggest that protein 8 is a proteolytic product from H1 or H5. (c) Time course of release of nuclear proteins by DNase I. Conditions are described in b except that DNase I was at 5 μg/ml. Lanes 2–6 show digestions of approximately 2, 5, 10, 15, and 20% acid solubility. Lane 1 shows acid-extracted histones.

pH 7.4). The chromatin pellet was extracted twice with 0.35 M NaCl/5 mM sodium phosphate, pH 7.0, by homogenization in a tight-fitting, glass Dounce homogenizer and centrifuged at 8000 × g for 10 min to yield a pellet of stripped chromatin and a 0.35 M NaCl eluate. The combined extracts were used for reconstitution or protein analysis. Proteins were separated on the basis of their solubility in trichloroacetic acid (see text) and assayed on sodium dodecyl sulfate (NaDodSO₄)/15 or 18% polyacrylamide gels by a modification (14) of the procedure of Laemmli (15).

Reconstitution and Digestion. The 0.35 M NaCl extract or the 10% trichloroacetic acid-soluble extract (neutralized and dialyzed into RSB), either alone or treated with trypsin and trypsin inhibitor, was added to the stripped chromatin pellet in an 8000-M_r-cutoff dialysis bag (Thomas) and dialyzed against RSB for 1–2 hr at 4°C. Trypsin treatment was at 10 μg/ml in 0.35 M NaCl at 37°C for 0.5 hr. The reaction was stopped with the addition of a 5-fold excess of trypsin inhibitor. NaDodSO₄/gel electrophoresis showed that all detectable proteins in the eluate were digested by this procedure and that the trypsin inhibitor was effective in preventing additional degradation of histones when the extract was reconstituted. All reconstitutions were performed with a 2-fold molar excess of eluate to depleted chromatin.

The reconstituted chromatin was digested at a DNA concentration of 2 mg/ml with pancreatic DNase I (Sigma) at 10 μg/ml for 5 min at 37°C. This corresponds to approximately a 10–15% decrease in acid-insoluble DNA. All preparations showed about the same distribution of DNase I fragments when analyzed on 10% acrylamide/8 M urea denaturing slab gels. An equal volume of 2-fold concentrated pH 5.5 extraction buffer (0.2 M NaAcO/0.02 M EDTA/0.3 M NaCl/0.03 M KCl/0.2% NaDodSO₄) was then added, and the mixture was incubated with 100 μg of proteinase K per ml (EM Biochemi-

cals) for 60 min at 37°C. The sample was extracted several times with equal volumes of phenol/chloroform (1:1) and several times with chloroform/isoamyl alcohol (24:1). The resultant aqueous phase was made 0.3 M in NaOH and incubated at 65°C for 0.5 hr. The sample was then neutralized and precipitated overnight with 2 vol of 95% ethanol. To assay the size distribution of DNA fragments after DNase digestion, approximately 20 μg of DNA was boiled in sample buffer (50% glycerol/0.01% bromphenol blue/0.01 M Tris-HCl, pH 7.4) and loaded onto a 10% polyacrylamide slab gel containing 8 M urea, 0.04 M Tris-HCl (pH 7.8), 2 mM EDTA, and 0.02 M sodium acetate. Electrophoresis was for 5 hr at 160 V in a running buffer containing 0.04 M Tris-HCl (pH 7.8), 2 mM EDTA, and 0.02 M sodium acetate. The gels were stained with ethidium bromide and visualized by illumination with an ultraviolet light.

Synthesis of cDNA. Embryonic globin mRNA was isolated as described (16). Ovalbumin mRNA was a kind gift of P. Thomas. cDNA was synthesized under conditions similar to those described by Friedman and Rosbash (17). A 100-μl mixture contained 50 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 10 μg of actinomycin-D, 5 mM dithiothreitol, 0.6 mg of (dT)₁₂₋₁₈, 0.5 mM dGTP, 0.5 mM TTP, 5 nM dCTP, 5 nM [³²P]dCTP (11.1 × 10¹² becquerels/mmol), 5 μg of RNA, and 40 μl of avian myeloblastosis virus polymerase. Incubation was for 4 hr at 37°C, and the reaction was stopped by addition of NaDodSO₄ to 0.1%. The reaction mixture was centrifuged through a 1-ml column of packed Sephadex G-50 layered over sterile sea sand. The excluded cDNA was adjusted to 0.3 M NaOH and incubated at 65°C for 0.5 hr. The mixture was then neutralized, and the cDNA was precipitated with 2.5 vol of ethanol overnight at -20°C. For most experiments, the globin cDNA had a specific activity of approximately 1–2 × 10⁸ cpm/μg; the ovalbumin cDNA was used at a specific activity of about 4–6 × 10⁷

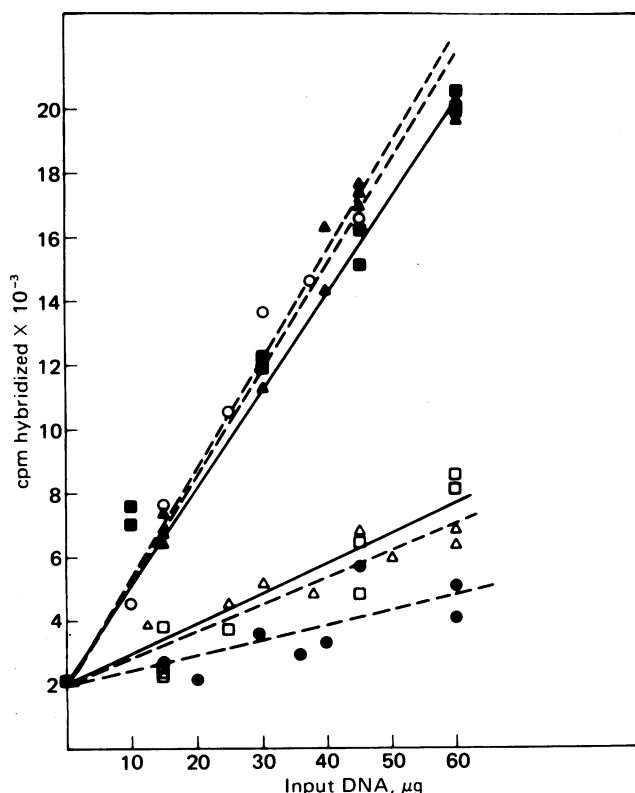


FIG. 2. Reconstitution of DNase I sensitivity of the globin gene with 10% trichloroacetic acid-soluble HMG proteins. Chromatin was eluted with 0.35 M NaCl and separated into several portions. One portion was reconstituted with the 0.35 M NaCl eluate directly (Δ); a second portion was reconstituted with the 10% trichloroacetic acid-soluble fraction from the 0.35 M NaCl eluate (\square); a third portion was reconstituted with the 10% trichloroacetic acid-soluble fraction from the 0.35 M NaCl eluate previously treated with trypsin and then trypsin inhibitor (\circ); and a fourth portion was digested directly (\blacktriangle). Each portion was then treated with DNase I so that 10–15% of the DNA was rendered acid soluble. The DNA was then purified and hybridized to an excess of globin cDNA. As controls, nuclei treated to 35% acid solubility with staphylococcal nuclease (\blacksquare) showed no preferential digestion, whereas nuclei treated to 10% acid solubility with DNase I (\bullet) showed a significant loss of globin sequences. Reconstitution with 0.35 M NaCl alone or with neutralized and dialyzed 10% trichloroacetic acid alone was unsuccessful in restoring DNase sensitivity (not shown). Input globin cDNA was 100,000 cpm.

cpm/ μ g. The globin cDNA probe used for the experiments shown in Fig. 2 was made with both [32 P]dCTP and [32 P]dTTP and had a specific activity of about $4\text{--}6 \times 10^8$ cpm/ μ g.

Hybridization Conditions. Hybridizations in excess DNA were performed as described (1). Hybridizations in excess cDNA were usually set up with 20,000 cpm of globin cDNA (100,000 cpm was used for the experiment shown in Fig. 2) or 25,000 cpm of ovalbumin cDNA in increasing volumes of 0.3 M NaCl/50 mM Tris-HCl, pH 7.4/0.1% NaDodSO₄ containing 5 mg of DNA per ml. Samples were heat denatured and annealed for 72 hr. The entire reaction mixture was assayed by resistance to S1 nuclease as described (1). Both the globin cDNA and the ovalbumin cDNA were in excess of from 3- to 30-fold. Under these conditions, the value of initial concentration of DNA (moles of nucleotide/liter) \times time (seconds) necessary for 50% hybridization ($C_{0t_{1/2}}$) for each reaction varied, so that samples with less DNA were approximately 40-fold beyond their $C_{0t_{1/2}}$ whereas samples with more DNA were about 6- or 7-fold beyond their $C_{0t_{1/2}}$. Thus, all points were taken well beyond saturation.

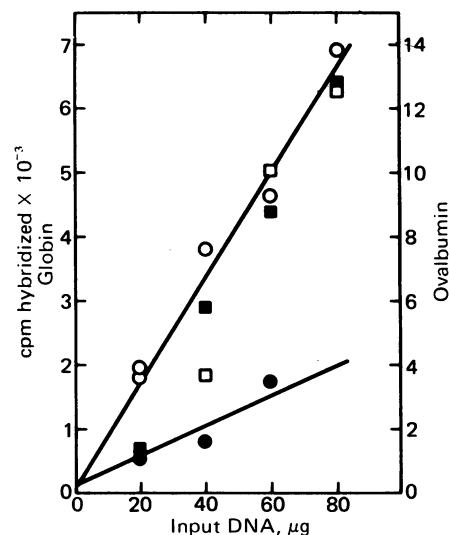


FIG. 3. Reconstitution of DNase I sensitivity in erythrocytes with the 0.35 M NaCl fraction from brain cells. The 0.35 M NaCl fraction from brain chromatin was reconstituted with the 0.35 M NaCl depleted erythrocyte chromatin (solid symbols) and the 0.35 M NaCl fraction from erythrocyte chromatin was reconstituted with the depleted brain chromatin (open symbols). Circles represent hybridization to globin cDNA; squares, to ovalbumin cDNA.

RESULTS

Proteins Released from Nuclei by DNase I. Fig. 1b shows a NaDodSO₄/acrylamide gel of the proteins released from embryonic chicken erythrocyte nuclei after digestion with DNase I or staphylococcal nuclease. Most of the proteins released by the two nucleases were the same; however, two very prominent nonhistone proteins (labeled 5 and 7) were greatly enriched in the supernatant from the DNase I digest (p) but not in the supernatant from the staphylococcal nuclease digest (s). These proteins are likely to be HMG proteins because they were

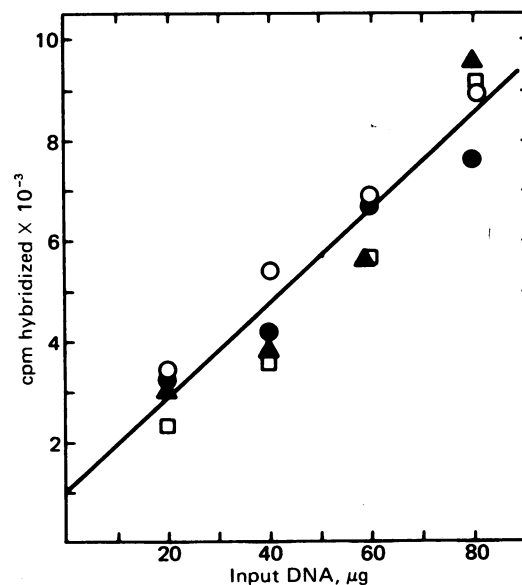


FIG. 4. Normal levels of the ovalbumin gene in depleted and reconstituted erythrocyte chromatin treated with DNase I. The various pancreatic DNase I-digested DNA fractions described in Fig. 2 were hybridized to an excess of an ovalbumin cDNA probe. \square , Depleted chromatin reconstituted with the 0.35 M NaCl fraction; \circ , chromatin depleted of the 0.35 M NaCl fraction; \blacktriangle , control chromatin.

eluted from chromatin in 0.35 M NaCl and were soluble in 10% trichloroacetic acid, but precipitable by 20% or 25% trichloroacetic acid (Fig. 1a). On the basis of their solubility properties and their migration in NaDodSO₄/acrylamide gels (18, 19), these proteins probably correspond to HMG-14 and HMG-17 and are probably different from those HMG proteins previously identified (20) as being preferentially released by DNase I in a different erythroid cell type. In addition, they are analogous to a related group of HMG proteins that have been described by Levy *et al.* (21, 22) as being associated with nucleosomes that are enriched in sequences coding for actively transcribed genes.

Reconstitution of DNase I Sensitivity. The globin gene was preferentially sensitive to DNase I in either mildly sheared erythrocyte chromatin or chromatin isolated by mild nuclease digestion (Fig. 2). When these preparations were eluted with 0.35 M NaCl, a large number of nonhistone proteins were released (Fig. 1a); however, the basic nucleosome conformation of the chromatin was intact, as shown by the following assays: The 10-base repeat produced by DNase I was unaltered; staphylococcal nuclease produced normal multimers of nucleosome particles as assayed on nucleoprotein gels; trypsin digested only the histone tails, leaving the histone core intact; Lomant's reagent crosslinked the histone core into an octamer; and electron micrographs exhibited a beaded chromatin fiber (data not shown).

In contrast to those assays that monitor the structure of bulk chromatin, chromatin eluted with 0.35 M NaCl displayed no preferential sensitivity for digestion of the globin gene by DNase I (Fig. 2) as assayed by hybridization in excess globin cDNA. That some constituent(s) of the 0.35 M NaCl eluate was responsible for conferring DNase I sensitivity to the globin gene was confirmed by the following experiment. When the 0.35 M NaCl eluate was reconstituted to half an equivalent of the depleted chromatin by dialysis into RSB, the globin genes regained their sensitivity to DNase I (Fig. 2). These general results are very reproducible and have been obtained in each of a total of 12 independent experiments performed to date.

Because the active component in the 0.35 M NaCl eluate was lost after treatment of the extract with trypsin (Fig. 2), we presume that at least one component was a protein. One clue to the identification of this protein comes from the data shown in Fig. 1 indicating that HMG-14 and HMG-17 were preferentially released from nuclei by low DNase I treatment. Thus, HMG-14 and HMG-17 were purified from the 0.35 M NaCl eluate by differential precipitation with trichloroacetic acid. These proteins were then neutralized and dialyzed into 10 mM Tris-HCl, pH 7.4, and then RSB and tested for their ability to restore DNase I sensitivity to the globin genes by dialysis (against RSB) onto the 0.35 M NaCl depleted chromatin. The data in Fig. 2 show that these proteins were also effective in restoring DNase I sensitivity to the globin gene.

In order to test whether the active component in the 0.35 M NaCl extract was tissue-specific, a similar 0.35 M NaCl wash from brain nuclei (in which the globin gene was not preferentially digested by DNase I) was isolated. Fig. 3 shows that this fraction could also confer DNase I sensitivity on the globin gene in depleted erythrocyte chromatin. These results clearly show that the active component responsible for reconstituting DNase I sensitivity in depleted erythrocyte chromatin was *not* tissue specific. In contrast, when the 0.35 M NaCl fraction from erythrocytes was reconstituted to the 0.35 M NaCl depleted brain chromatin, the globin gene in brain remained DNase I resistant. Thus, the specificity for the interaction between the eluted proteins and the residual chromatin still remained with the residual chromatin.

As an internal control for these experiments, both the depleted and the reconstituted DNase I-treated erythrocyte DNA preparations displayed no preferential loss of ovalbumin DNA sequences (Fig. 4). In addition, the same general conclusions were also obtained when the depleted and reconstituted DNase I-treated DNA was analyzed by hybridization in excess DNA (data not shown). Both the depleted and the reconstituted DNA preparations exhibited the same pattern of DNA fragments on denaturing gels and both displayed approximately the same level of digestion as assayed by release of acid-soluble DNA. In addition, both types of preparations hybridized with the same kinetics to tracer-labeled DNA from the entire genome (data not shown). On the basis of these results, we believe that the active component responsible for conferring DNase I sensitivity is probably not recognizing a specific DNA sequence or a unique feature of the depleted globin chromatin, but rather is recognizing a more general feature that the depleted globin chromatin in erythrocytes shares with other actively transcribed genes both in erythrocytes and in many other cell types.

DISCUSSION

Our experiments show that the DNase I sensitivity displayed by the actively transcribed globin gene in embryonic chicken erythrocyte nuclei is conferred, in part, by a protein(s) that is easily eluted from chromatin in 0.35 M NaCl. Subsequent purification of this eluate indicates that at least one component in a fraction that is greatly enriched in HMG-14 and HMG-17 confers this sensitivity. Because of the harsh isolation procedures employed, we cannot determine the stoichiometry required for successful reconstitution, since we do not yet know the effectiveness of our renaturation conditions, nor do we know the effectiveness with which other active genes may be reconstituted. Furthermore, we cannot yet rule out the possibility that an undetected protein other than HMG-14 or HMG-17 is responsible for conferring DNase I sensitivity on the globin gene. However, preliminary experiments show that a purified, "native" complex of these HMG proteins is also capable of restoring DNase I sensitivity to the depleted globin gene; thus, it may soon be possible to titrate the binding of this complex to several different active genes in the same nucleus and show unambiguously that HMG-14 or HMG-17 or both are responsible, at least in part, for DNase I sensitivity.

HMG-14 and HMG-17 are also those proteins that are specifically released from undepleted nuclei by DNase I (and not by staphylococcal nuclease). This suggests that they are physically present on actively transcribed regions and that they probably do not work enzymatically. These results are also compatible with recent findings (23) showing that fluorescence-labeled antibodies to proteins released by DNase I specifically stain regions of the *Drosophila* salivary gland chromosome that are puffed or have puffed at some time during development. These experiments provide a clear demonstration that proteins of this type are in fact localized in regions of the chromosome that have been or are potentially active.

The 0.35 M NaCl fraction from erythrocytes can induce DNase I sensitivity in depleted erythrocyte chromatin, but not in depleted brain chromatin. In contrast, the 0.35 M NaCl fraction from either brain or erythrocytes can induce DNase I sensitivity only in depleted erythrocyte chromatin. Thus, there are at least two factors responsible for conferring a DNase I-sensitive structure on active genes—one that is elutable in 0.35 M NaCl and is not tissue specific and another that is not elutable, but remains tightly bound to the globin gene in erythrocyte chromatin and is not present or functional on the globin gene in brain chromatin. For convenience, we call such a component a "receptor" factor and the component that is eluted, an

“elutable” factor. The experiments reported here strongly suggest that the “elutable” factor is either HMG-14 or HMG-17 or both. In addition to the elutable and receptor factors, our previous work suggested the existence of at least two other functional elements important for globin gene activation—a “recognition” factor that presumably recognizes the globin gene during development and a “propagation” factor that is responsible for propagating a DNase I-sensitive structure to each nucleosome in the transcription unit. By use of the DNase I assay, the relationship among these four *functional* elements can now be approached experimentally.

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