

Reconstitution of a deoxyribonuclease I-sensitive structure on active genes

(high-mobility group proteins/chromatin/gene expression)

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ABSTRACT Chicken erythrocyte nuclei have been labeled in the active regions of the chromosome by using the nick translation reaction. In this procedure, accessible areas of the genome are preferentially nicked by the action of pancreatic DNase I and subsequently labeled by using DNA polymerase I from *Escherichia coli*. These nuclei were employed as a substrate for studying the factors responsible for maintaining the special chromatin conformation of the overall population of active genes. Treatment of nuclei with 0.35 M NaCl resulted in the loss of DNase I sensitivity in the active genes, but this sensitivity could be restored when nuclei were reconstituted with the NaCl eluate. Further purification of the released factors revealed that the HMG (high-mobility group) proteins HMG-14 and HMG-17 are involved in maintaining the conformation of the active regions. These factors are not tissue specific and seem to be involved in the chromosomal structure of most of the active genes.

Over the past few years much progress has been made in our understanding of the chromatin organization of active genes. By using various nucleases it has been possible to probe the disposition of these genes within the chromosome and to show that active genes are in a different conformation than genes in an inactive state. As an example, most genes are sensitive to DNase I digestion of nuclei from tissues active in the expression of the gene, whereas these same genes are unaffected by DNase I digestion in tissues that do not actively synthesize that gene product (1-5). In order to fully understand the mechanisms involved in the control of gene expression one would like to know which factors or proteins are involved in organizing the chromosomal conformation of active genes. Several different lines of evidence now suggest that one of the factors involved in establishing the conformation of active genes may be the high-mobility group (HMG) proteins. These proteins are specifically released from nuclei when treated with DNase I (6). In addition, Levy-Wilson and Dixon (7) have shown that a semipurified preparation of active chromatin is enriched for the HMG proteins. Finally, Weisbrod and Weintraub (6) have demonstrated that HMG proteins HMG-14 and HMG-17 are involved in maintaining the DNase I-sensitive conformation of the hemoglobin gene in embryonic chicken erythrocytes. In this paper we have made use of a previously described technique for specifically labeling all of the active regions of chromatin from any tissue or cell preparation (8). These labeled active regions are, as expected, sensitive to digestion by DNase I. This sensitivity can be abolished by the removal of HMG proteins from the labeled nuclei and is reestablished by the addition of HMG proteins, indicating that these proteins are

involved in the organization of most of the active genes in the genome.

MATERIALS AND METHODS

Nick Translation of Nuclei. Erythrocytes were obtained from freshly killed chickens. Nuclei were prepared from frozen erythrocytes by lysis with 0.1% Nonidet P-40, homogenization, and washings in a polyamine-containing buffer (9), and they were resuspended at a DNA concentration of about 1 mg/ml in 50 mM Tris-HCl (pH 7.9)/5 mM MgCl₂/10 mM 2-mercaptoethanol/50 μg of bovine serum albumin per ml (nick translation buffer). These nuclei were incubated for 15 min at 37°C with DNase I (Sigma) at 0.1 μg/ml in order to nick the DNA. Polymerization was then carried out in the presence of 4 μM each of dATP, dGTP, dCTP, and [³H]dTTP (New England Nuclear, 42-50 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) for 5 min at 15°C after the addition of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) at 10 units (as defined by the supplier) per ml. The reaction was stopped by transferring nuclei to 0°C and immediate separation of nuclei by centrifugation. The nuclei were washed two or three times at 0°C with nick translation buffer to minimize further polymerization.

NaCl Chromatin Extraction. The nuclear pellet was extracted twice with 0.35 M NaCl in nick translation buffer by homogenization in a tight-fitting glass homogenizer and centrifuged at 8000 × g for 10 min to yield a pellet of stripped chromatin and a NaCl eluate. The combined supernatants from the two NaCl extractions were used for reconstitution experiments. In order to analyze the proteins involved in reconstitution, nuclei were prepared from 20 ml of chicken blood and extracted with 0.35 M NaCl as described above. This extract was treated with 10% trichloroacetic acid, and the soluble proteins were precipitated with 35% trichloroacetic acid, rinsed with acetone, and analyzed by sodium dodecyl sulfate/15% polyacrylamide gel electrophoresis using a modification of the procedure of Laemmli (10). The yield from 20 ml of whole blood was 140 μg of HMG proteins.

Reconstitution and Digestion. The 0.35 M NaCl extract was added to the stripped chromatin pellet and dialyzed against 10 mM Tris-HCl (pH 6.8)/0.25 mM EDTA for 1-2 hr at 4°C. Reconstitution was performed with a 2-fold molar excess of eluate to depleted chromatin. Reconstitution using purified HMG proteins was accomplished by treating the 0.35 M NaCl extract with 10% trichloroacetic acid. The acid-soluble fraction was neutralized and dialyzed against 10 mM Tris-HCl (pH 6.8)/0.25 mM EDTA and then added back to depleted nuclei as described above. After reconstitution, nick-translated control

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Abbreviations: DNase I, deoxyribonuclease I (EC 3.1.21.1); HMG, high-mobility group.

nuclei, NaCl-washed nuclei, or reconstituted chromatin was suspended in nick translation buffer to a 0.5 mg/ml DNA concentration and treated with DNase I at 350 ng/ml in order to examine the sensitivity of the labeled regions. Percent DNA digestion at 37°C was determined by measuring the release of radioactive nucleotides from labeled DNA as soluble material in 1 M HClO₄/1 M NaCl. In order to determine the degree of DNase I sensitivity, the digestion of labeled DNA was compared to the digestion of total DNA by measuring the release of 260-nm-absorbing material soluble in 1 M HClO₄/1 M NaCl (3).

RESULTS

We have previously shown that nuclei can be specifically labeled in the DNA of the active regions by using the nick translation reaction (8). This site-directed labeling procedure is based on the observation that the active genes of the nucleus are preferentially digested by DNase I. In the nick translation reaction, low concentrations of DNase I are employed to introduce nicks into the DNA of the active regions. These nicks then serve as a template for *E. coli* DNA polymerase I, allowing the labeling of these areas of the chromosomes. The specificity of this technique was unequivocally demonstrated by the fact that over 80% of these labeled sequences can be hybridized to cellular RNA. These nuclei provide an excellent substrate for studying the conformation of the overall population of active genes without the need for hybridization analysis. In order to

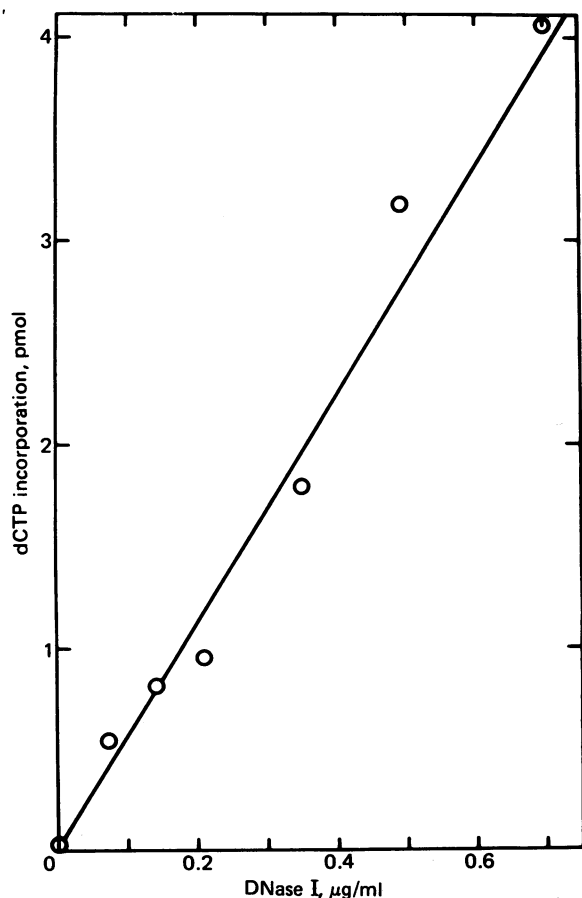


FIG. 1. Effect of DNase I on the nick translation reaction. Chicken erythrocyte nuclei were incubated for 15 min at 37°C with various concentrations of DNase I. The nuclei were then nick translated in a volume of 50 µl containing 20 µg of nuclear DNA and 0.5 unit of DNA polymerase. The extent of polymerization was determined by trichloroacetic acid precipitation.

use this technique one must first ensure that the labeling procedure is indeed specific. One important but simple criterion is to demonstrate that the nick translation reaction is indeed dependent on the amount of DNase I and thus the number of inserted nicks. As shown in Fig. 1, incubation for 15 min at 37°C with low concentrations of DNase I resulted in a dramatic and linear increase in the polymerization reaction. A stimulation of 5- to 15-fold over the reaction with unnicked DNA was used in the studies presented in this paper. Too little stimulation was found to produce nuclei that were not preferentially labeled in the active regions (8), whereas too much DNase I resulted in extensive reduction in the size of the DNA (data not shown).

One of the properties that characterizes nick-translated nuclei is that the labeled regions are preferentially sensitive to digestion with DNase I. This sensitivity to DNase I can be used as a simple assay for determining the conformational state of the active regions. Thus, if nick-translated nuclei are manipulated in a way that changes the conformation of the active regions, the DNase I sensitivity of the labeled regions will be lost. When nuclei were washed with increasing concentrations of NaCl it was found that there was a striking loss of DNase I sensitivity in the active regions (Fig. 2), while the DNase I sensitivity of the total DNA was unaffected. Treatment of nuclei with up to 0.25 M NaCl had only a marginal effect on the DNase I sensitivity of the labeled regions. Thus at 0.25 M salt the accessibility was reduced to less than 1/2. When nuclei were washed with salt at a concentration greater than 0.3 M, a dramatic decrease in sensitivity was observed. After washing with 0.4 M NaCl, the active regions were less than 1/20th as sensitive to DNase I as the untreated nuclei were.

In order to investigate the nature of the change brought about by salt extraction, we asked whether this treatment perhaps resulted in the release of some factor that is necessary for maintaining the active conformation of chromatin. If this were the case, it might be possible to restore the original conformation

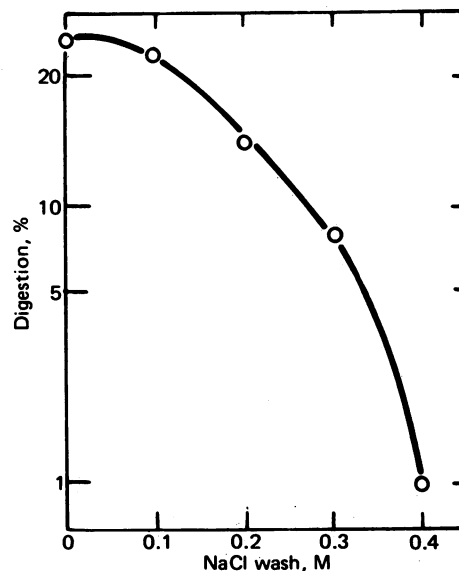


FIG. 2. Effect of NaCl concentration on the DNase I sensitivity of nick-translated nuclei. Nuclei were nick translated and washed. The nuclear suspension was separated into aliquots and each one was washed twice with nick translation buffer containing various concentrations of NaCl and subsequently resuspended in nick translation buffer at a DNA concentration of 350 µg/ml. The amount of DNase I digestion in each aliquot was determined and the results are expressed as percent digestion per 40-min incubation. In all cases total DNA digestion was approximately 2%.

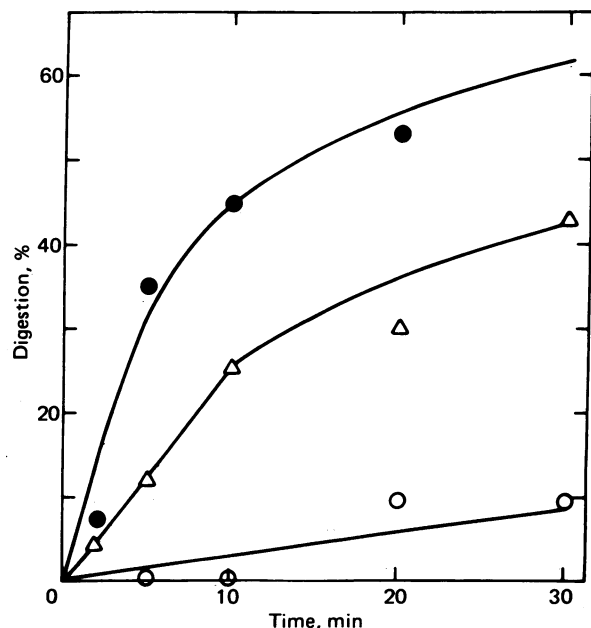


FIG. 3. Reconstitution of DNase I sensitivity. Nick-translated nuclei were suspended in buffer and washed twice with 0.35 M NaCl. The combined supernatants were added back to washed nuclei and the mixture was dialyzed against 10 mM Tris-HCl (pH 6.8)/2.5 mM EDTA for 2 hr at 4°C in order to obtain reconstituted nuclei (△). Unwashed nuclei (●) and salt-washed nuclei (○) were also dialyzed under the same conditions but without the addition of supernatant. After dialysis nuclei were digested in order to determine their sensitivity to DNase. In all cases the extent of total DNA digestion after 30 min of incubation was about 5%.

by reconstituting chromatin with the material released during the salt wash. To this end, salt-treated nuclei were dialyzed in the presence of the 0.35 M NaCl nuclear wash and the resulting chromatin was tested for sensitivity to DNase I. As shown in Fig. 3, about 80% reconstitution was obtained when dialysis was used to add back the extract. We repeatedly attempted to restore the DNase I sensitivity by reducing the salt concentration of the 0.35 M NaCl wash either by dilution or dialysis and directly adding this extract back to salt-treated nuclei resuspended in nick translation buffer. Reconstitution could not be obtained by this method, indicating that the mode of addition of the released factor is important for restoration of original conformation. The source of the salt extract did not seem to be important, because erythrocyte chromatin could be reconstituted at the same efficiency by using a liver NaCl extract (data not shown).

Because the above results indicated that some specific factor may be responsible for maintaining the conformation of the active regions of chromatin, we attempted to investigate the protein content of the 0.35 M NaCl extract. Weisbrod and Weintraub (6) have shown that HMG-14 and HMG-17 may be involved in the organization of the active conformation around the hemoglobin gene. It was thus of interest to see whether these same proteins are involved in maintaining the structure of other active genes. When the 0.35 M NaCl extract was precipitated with 10% trichloroacetic acid, only two major proteins that remained soluble in the acid solution were observed. These two proteins were determined to be HMG-14 and HMG-17 both by their acid solubility and by their mobility on sodium dodecyl sulfate/acrylamide gel electrophoresis (Fig. 4).

When the trichloroacetic acid-soluble material was neutralized, dialyzed, and then used to reconstitute salt-treated nuclei, 100% reconstitution of DNase I sensitivity was obtained, indicating that these proteins alone have the potential to restore

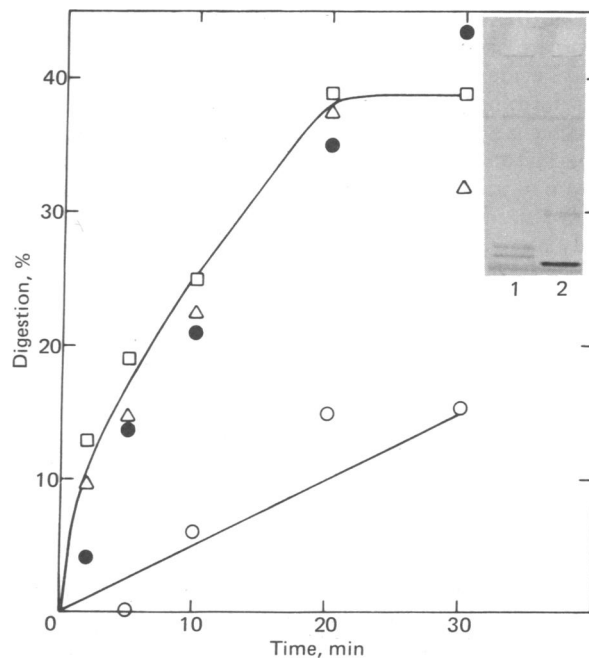


FIG. 4. Reconstitution with HMG proteins. HMG proteins were prepared from erythrocyte and liver nuclei by using differential trichloroacetic acid precipitation. The 10% trichloroacetic acid-soluble material was neutralized and dialyzed and used to reconstitute salt-treated nuclei as described in the legend to Fig. 3. Untreated nuclei (●), salt-washed nuclei (○), and nuclei reconstituted with HMG proteins from erythrocytes (△) or from liver (□) were tested for their sensitivity to DNase I digestion. The gel electrophoresis pattern of the HMG proteins from erythrocytes is shown in the *Inset* (lane 1). A standard containing histones H1 and H2B is shown in the same gel (lane 2).

the active conformation. HMG proteins extracted in the same manner from other tissues were also active in reconstitution (Fig. 4).

The fact that these proteins are involved in active region conformation could also be seen from the pattern of DNase I sensitivity as a function of salt concentration (Fig. 2). It is clear from this experiment that the major factor is removed at a salt concentration of 0.35 M. The proteins HMG-14 and HMG-17 remain bound to nuclei up to concentrations of 0.3 M NaCl. As demonstrated by gel electrophoresis analysis, they are then released as a sharp peak at about 0.35 M NaCl (data not shown). Some HMG-14 and HMG-17 are released only at 0.4 M, together with other 10% trichloroacetic acid-soluble proteins.

DISCUSSION

In order to understand the basis of differential gene expression in eukaryotes many investigators have turned their attention to the chromosomal structure of active genes. In general, the genes active in a particular tissue are characterized by increased accessibility to a variety of probes. Several nucleases, including DNase I (1), DNase II (11), and micrococcal nuclease (3, 5, 12) preferentially attack active regions of the chromosome, and both bacterial (13) and eukaryotic (14) RNA polymerases preferentially transcribe *in vitro* in these same regions. All of these studies suggest that the active regions of chromatin differ in their conformation from the rest of the genome, but none of these studies tells us anything about the nature of this structure.

By using a different approach, it has been shown that there are certain proteins that seem to be associated with the active regions of chromatin (15). Some of these proteins, including HMG-14 and HMG-17, are released after DNase I treatment

of nuclei (6). Although these experiments suggest that these proteins are present in the regions of the active genes, there is no direct evidence that these same proteins are involved in any way in forming or maintaining this unique conformation. In this paper we have demonstrated that trichloroacetic acid-soluble factors are directly responsible for maintaining the structure of most of the active regions of the chromosome. It is likely that these factors are HMG-14 and HMG-17. It has already been demonstrated that these proteins are responsible for the special conformation in the region of the active hemoglobin gene (6).

Earlier studies using probes to identify the active regions of the chromosome indicated that the conformations of all of the active genes are similar. Thus, many specific genes are sensitive to DNase I and the degree of sensitivity is the same for all active genes regardless of the extent to which these genes are expressed (16). In addition, many active genes are accessible to RNA polymerase to the same degree. These data suggest that the mechanisms for forming these special conformations may be similar for all active genes. Indeed, our studies show that the same proteins, probably HMG-14 and HMG-17, are involved in the structure of all of the genes labeled by the nick translation reaction. Because the labeled DNA produced by nick translation of nuclei represents 80% of the transcribed cellular sequences (8), it can be assumed that these results apply to most of the active genes in the genome. The addition of these two proteins to salt-extracted chromatin caused a 100% reconstitution of the active genes of the erythrocyte genome. Although these proteins are involved in the formation of DNase I sensitivity, it is clear that they may not be the primary factors responsible for determining the activity state of a gene. The reconstitution studies indicate that some factor or structure remains on the chromosome after treatment with 0.35 M NaCl and that these factors may be the primary element that determines the activity state of the genes and directs the reconstitution of the HMG proteins *in vitro*.

Although all active genes are characterized by their similarity, differences have been observed in many cases. Thus, the hemoglobin gene is sensitive to DNase I even when in a monomer form (1), although the ovalbumin gene is not (2). The response of various genes to treatment with micrococcal nuclease is not uniform, and although DNase II is site directed to

the region of the hemoglobin gene (17) it has not been found to be specific for other active regions. Finally, electron microscope studies seem to indicate the presence of nucleosomes for some genes but not for others (18). These studies suggest that other factors in addition to the HMG proteins may be involved in the control of gene expression at the structural level. The use of nick-translated nuclei might help in revealing these factors.

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