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Nuclease sensitivity of active chromatin

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ABSTRACT

The active regions of chicken erythrocyte nuclei were labeled using the standard DNase I directed nick translation reaction. These nuclei were then used to study the characteristics and, in particular, the nuclease sensitivity of active genes. Although DNase I specifically attacks active genes, micrococcal nuclease solubilizes these regions to about the same degree as the total DNA. On the other hand micrococcal nuclease does selectively cut the internucleosomal regions of active genes resulting in the appearance of a mononucleosomal fraction which is enriched in active gene DNA. A small percentage of the active chromatin is also released from the nucleus by low speed centrifugation following micrococcal nuclease treatment. The factors which make active genes sensitive to DNase I were shown to reside on individual nucleosomes from these regions. This was established by showing that isolated active mononucleosomes were preferentially sensitive to DNase I digestion. Although the high mobility group proteins are essential for the maintenance of DNase I sensitivity in active regions, these proteins are not necessary for the formation of the conformation which makes these genes preferentially accessible to micrococcal nuclease. The techniques employed in this paper enable one to study the chromatin structure of the entire population of actively expressed genes. Previous studies have elucidated the structure of a few special highly prevalent genes such as ovalbumin and hemoglobin. The results of this paper show that this special conformation is a general feature of all active genes irregardless of the extent of expression.

INTRODUCTION

The use of nucleases to probe the structure of the chromosome has provided some valuable general information on the arrangement of proteins on the DNA as well as specific information on the conformation of the active regions of the genome. In particular the enzyme DNase I has been used extensively to probe the structure of active chromatin. There are numerous examples in the literature which support the claim that active genes are in a DNase I sensitive conformation, whereas these same genes are not preferentially digested in nuclei of cells in which these genes are phenotypically inactive (1,2,3,4). This special conformation seems to be dependent on the

presence of specific proteins. When high mobility group proteins HMG 14 and 17 are removed from nuclei, active regions lose their special sensitivity, but this conformation can be regained by reconstitution (5,6). These proteins seem to be associated with many active genes and as such are not gene specific.

Over 85% of the eukaryotic genome is packaged in the form of nucleosomes which are probably distributed over the entire genome, including the active regions (7). Although most active genes have been found to be in a nucleosomal structure, there are several indications that the arrangement of nucleosomes over active genes may be different than that of the rest of the DNA. The nucleosome monomers protecting the ovalbumin gene, for example, are preferentially released by micrococcal nuclease digestion (8,9). Furthermore, distribution of monosomes over the heat shock genes of drosophila is dependent on the activity state of these genes (10).

We have previously developed a convenient technique for studying the active regions of the chromosome, without resort to hybridization analysis. When DNase I is used to direct the nick translation of nuclei, the resulting label is found to be concentrated in the expressed genes of the nuclei (11). These labeled nuclei provide a convenient vehicle for studying the conformation of the active chromosomal regions. In this report we concentrate on the nucleosome conformation in the region of active genes. Our results clearly demonstrate that the nucleosomes in the region of active genes are a preferential target for micrococcal nuclease. In addition, the factors which are responsible for the DNase I sensitivity of active regions are local effects, since even isolated nucleosomes within the active regions are in a DNase I sensitive conformation.

MATERIALS AND METHODS

Nick Translation of Nuclei

Erythrocytes were obtained from freshly killed chickens. Nuclei were prepared from frozen erythrocytes by homogenization with 0.1% Nonidet P-40, and repeated washing in a polyamine-containing buffer (5). They were resuspended at a DNA concentration of about 1 mg/ml in 50 mM Tris-HCl (ph 7.9), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 µg of bovine serum albumin per ml (nick translation buffer). Nuclear DNA was nicked by incubation with 0.1 mg/ml DNase I (Sigma) for 15 min at 37°C. Polymerization was then carried out in the presence of 4 µM each of dATP, dGTP, dCTP and (^{3}H)dTTP (New

England Nuclear, 42-50 Ci/mmole) 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.

Nucleases digestions

Washed nuclei were resuspended in nick translation buffer and incubated with DNase I (Sigma) at 15° or 37°C. For micrococcal nuclease digestions the washed nuclei were suspended in 10 mM Tris-HCl (pH 7.9), 0.25M sucrose, 0.5mM $CaCl_2$, 25 mM KCl, 5 mM $MgCl_2$ (Mnase buffer) and incubated with enzyme at 15°C. Percent DNA digestion was determined by measuring the release of radioactive nucleotides from labeled DNA as soluble material in 1 M $HClO_4$/1 M NaCl. In order to determine the degree of nuclease 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 $HClO_4$/1 M NaCl.

RESULTS

Nuclei can be conveniently labeled in their active regions using the standard nick translation reaction. When DNase I is used to introduce the nicks into nuclear DNA, the polymerase reaction preferentially incorporates radioactive nucleotides into the active parts of the chromosome. That this is indeed the case can be demonstrated by the fact that the labeled regions are subsequently digested by DNase I at a higher rate than the total DNA. As demonstrated in figure 1 the labeled regions are ten times more sensitive to DNase I than the rest of the genome. The degree of sensitivity of these labeled regions depends to a large extent upon the nick translation reaction (11), and varies between 5-15 fold. This sensitivity is not due to the extensive nicking in this area, since nick translated naked DNA does not show any preferential sensitivity in the labeled regions (11).

Nick translated nuclei are digested by DNase I in a characteristic manner, independent of the degree of sensitivity of these nuclei. Digestion of labeled regions always reaches a plateau after 5% of the total DNA has been digested. This is consistent with other published results which show that certain individual genes are 80-90% digested when only 5-10% of the genome has been solubilized (3,12). This suggests that the size of the active gene compartment is about 5-10% of the total genome. This represents about

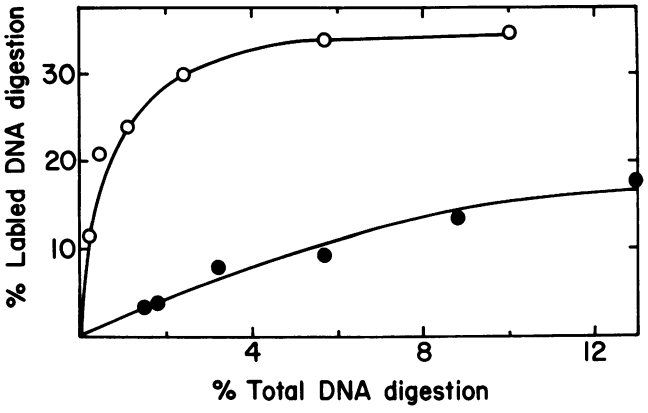

Fig. 1. Nuclease sensitivity of nick translated nuclei. Nick translated nuclei were incubated at 15°C with either 6.4 mg/ml DNase I (o) or 2 µg/ml micrococcal nuclease (•). At various times during digestion aliquots were removed and treated with 1 M $HC10_4$ / 1 M NaCl in order to determine the percent digestion of both total and labeled DNA. The results are expressed as percent digestion of labeled regions vs. the percent of total DNA digestion as determined from OD_{260}. At 15°C there was no detectable digestion of radioactively labeled nuclear DNA in the absence of added enzyme. Despite extensive washing of nick translated nuclei, about 5-10% of the total counts were soluble in $HC10_4$. This background has been subtracted to obtain the data shown in this figure.

50-100,000 genes, which corresponds to the number of different RNA species found in a given animal cell.

Using nick translated nuclei, we can now begin to ask questions about the structure and distribution of nucleosomes in the region of active genes. Micrococcal nuclease is a convenient enzyme for probing nucleosome conformation, since this enzyme preferentially digests internucleosomally. Only after 20% of the nuclear DNA has been digested does this enzyme attack intranucleosomal regions. The reaction comes to an end at 50% digestion leaving a series of discrete size chromatin pieces (13).

Active region DNA is digested by micrococcal nuclease at about the same rate as the rest of the chromosome (figure 1). Thus after 10% digestion of total DNA about 15% of the active regions were solubilized. This observation confirms the idea that active region DNA is protected by nucleosomes to about the same extent as other parts of the genome. When the micrococcal nuclease reaction was taken to completion (50%), however, as much as 80% of the active genes were digested, indicating that these areas have some preferential sensitivity to micrococcal nuclease.

Despite the apparent almost normal micrococcal nuclease sensitivity in active regions, careful analysis demonstrated that active regions are preferentially attacked by this enzyme resulting in the selective release of active DNA in the form of whole nucleosomes in the absence of extensive solubilization of DNA nucleotides. As shown in figure 2, upon 10% digestion of nuclear DNA with micrococcal nuclease the total DNA is distributed in the form of mononucleosomes, dinucleosomes and higher molecular weight fragments. On the other hand, active region DNA is almost entirely in the form of mononucleosomes. In order to study the kinetics of this process it was necessary to digest nuclei with minimal amounts of micrococcal nuclease and to observe the fate of total and active DNA. When these digested nuclei were extracted and the DNA run on agarose gels, even at early times in the digestion, labeled material appeared in the form of mononucleosome size fragments, despite the undectable digestion of total DNA (figure 3). These data strongly suggest that within active chromosome regions the nucleosomes are distributed in a

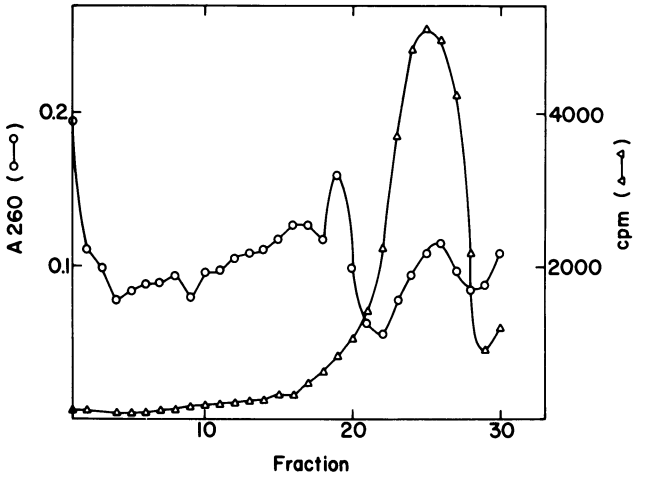

Fig. 2. Sucrose gradient analysis of nucleosomal particles from nick translated nuclei. Nick translated nuclei were suspended in micrococcal nuclease buffer and incubated with 80 µg/ml micrococcal nuclease for 1 min at 15°C. Digestion was stopped by the transfer of the reaction mixture to 0°C and the addition of EDTA to a concentration of 20 mM and sodium deoxycholate to 0.5%. This suspension was homogenized in a tight-fitting Dounce homogenizer and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was layered on a linear 5-20% sucrose gradient containing 10 mM Tris-HCl (pH 7.9), 0.1 mM NaCl, 5 mM EDTA, and centrifuged in a SW 27 rotor for 18 hr at 4°C at a speed of 24,000 rpm. Fractions (1 ml) were collected by puncturing the bottom of the tube and assayed for A_{260} and trichloracetic acid precipitable counts.

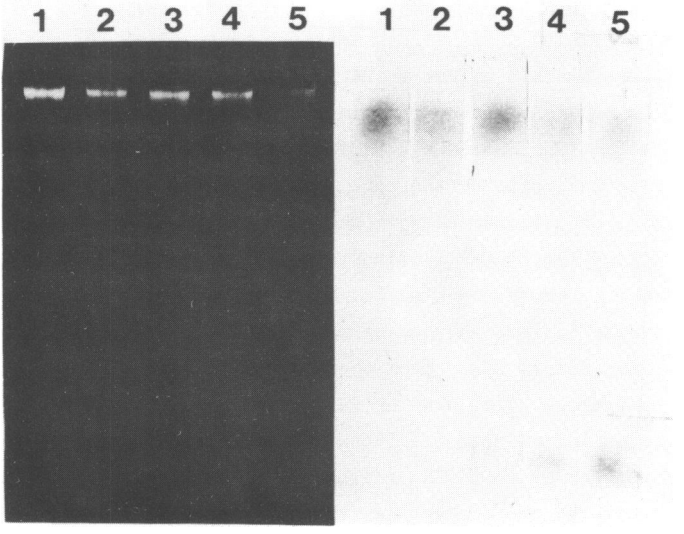

Fig. 3. Micrococcal nuclease digestion of nick translated nuclei. Nuclei were nick translated as described in Materials and Methods in the presence of $^{32}P\alpha$ dCTP and dATP (200 Ci/mmole, New England Nuclear Corp.). Labeled nuclei were suspended in Mnase buffer and digested for various times at 15°C with 80 µg/ml micrococcal nuclease. Aliquots were taken at 0 time (slot 1), 10 sec (slot 2), 20 sec (slot 3), 30 sec (slot 4) and 100 sec (slot 5). DNA was purified by phenol extraction and ethanol precipitation and 5-8 µg was applied to a 15% agarose slab gel and electrophoresed at 100 V for 2 hr in 0.4 M Tris (ph 7.9), 0.04 M Na Acetate, 0.01 M EDTA. The gel was stained with ethidium bromide and photographed under UV transillumination (A). After drying, the gel was autoradiographed using Agfa RP2 film (B).

way that makes them a selective target for micrococcal nuclease attack.

It is clear from these studies that the conformation of active regions is such that they permit preferential access to both DNase I and micrococcal nuclease. Since sensitivity to DNase I is dependent on the presence of the high mobility group proteins HMG14 and HMG17, it was of interest to determine whether the sensitivity to micrococcal nuclease is controlled by these same factors. To this end nuclei were washed with 0.4 M NaCl in order to remove these HMG proteins. These washed nuclei were resuspended in the standard buffer and treated with micrococcal nuclease to obtain the exact pattern of total DNA digestion as shown in figure 2 (this required the use of four times less micrococcal nuclease, since 0.4 M NaCl treated nuclei are in general more sensitive to micrococcal nuclease digestion). When labeled active regions were analyzed on the same gradient, results identical

to those shown in figure 2 were obtained indicating that active regions are preferentially sensitive to micrococcal nuclease even in the absence of HMG14 and HMG17. This suggests that additional factors are required for the maintenance of the conformation of active genes.

The demonstration that active region DNA is sensitive to digestion by several nucleases suggests that topographically these areas of the nucleus are more exposed to the extranuclear space. One possibility that could explain this phenomenon is that active regions are distributed on the periphery of the nucleus and thus are in an especially open conformation. It has been shown, in fact, that ovalbumin containing nucleosomes are preferentially released from nuclei following limited micrococcal nuclease digestion (9). In order to determine whether this is a general phenomenon applicable to all active genes, we digested nick translated nuclei with micrococcal nuclease and followed the release of DNA from the nucleus. As shown in figure 4, labeled active regions are selectively released from these nuclei. Despite the negligible release of total DNA, about 3% of the labeled DNA could be recovered in the supernatant after centrifugation of nuclease treated nuclei. The labeled material released from these nuclei was mainly in the form of mononucleosomes (data not shown). Despite further digestion of DNA, the release of active regions from the nucleus remained at a saturation level of 3-5%. This suggests that a small portion,

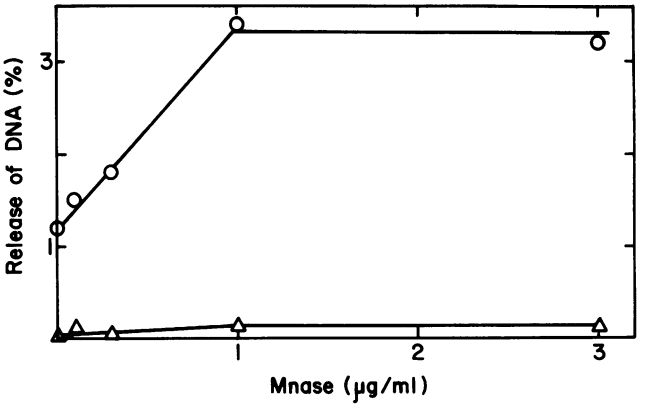

Fig. 4. Release of DNA from nuclei following micrococcal nuclease digestion. Nick translated nuclei were suspended in Mnase buffer and incubated for 2 min at 15°C with various amounts of micrococcal nuclease. The reaction was stopped by sedimenting nuclei at 4°C and the supernatant was assayed for A_{260} (Δ) and for TCA precipitable radioactive material (o). All of the A_{260} material released into the supernatant was found to be PCA precipitable.

but not all, of the active regions are indeed in some special topographical conformation which not only imparts sensitivity to nucleases but also allows the regions to be released from their place in the nucleus. It should be noted that even after 20% of the nuclear DNA has been solubilized and most of the DNA has been converted to mononucleosomes, only a small fraction of these nucleosomes are released by centrifugation.

As has been demonstrated in various systems, active region chromatin has a conformation which renders it sensitive to DNase I. It is interesting to ask whether this special conformation is formed by factors which modify every nucleosome within the active region or by factors which may reside a distance from the actual active areas, but can cause a conformational change over a large domain. One way to answer this question is to isolate monosomes and determine whether monosomes covering active regions retain their sensitivity to DNase I. Several different types of experiments suggest that mononucleosomes do indeed retain nuclease sensitivity.

A first indication that this is indeed the case comes from experiments in which nick translated nuclei were digested with micrococcal nuclease to various degrees before digestion with DNase I (figure 5). Despite the conversion of nuclear DNA to nucleosomal particles, the active regions retained their sensitivity to DNase I. In order to validate this observation, monosomal particles were isolated from nick translated nuclei by two independent techniques. Whether purified by sucrose gradient centrifugation (figure 6) or differential centrifugation (figure 7) the active nucleosomes retain their sensitivity to DNase I.

DISCUSSION

Although active genes are known to be in a conformation which differs from that of non-expressed genes, there is little information describing this structure in detail and there are still many questions which have been left unanswered. One of the difficulties in approaching these problems is that in most cases individual genes were used to study active gene conformation. Due to the large amount of hybridization analysis required, these studies tend to be tedious and the results only reflect the situation for one gene out of the entire population of expressed genes. The use of nuclei labeled in their active regions by nick translation provides a system for analyzing a large battery of expressed genes without the need to use hybridization analysis.

There is considerable disagreement in the literature concerning the

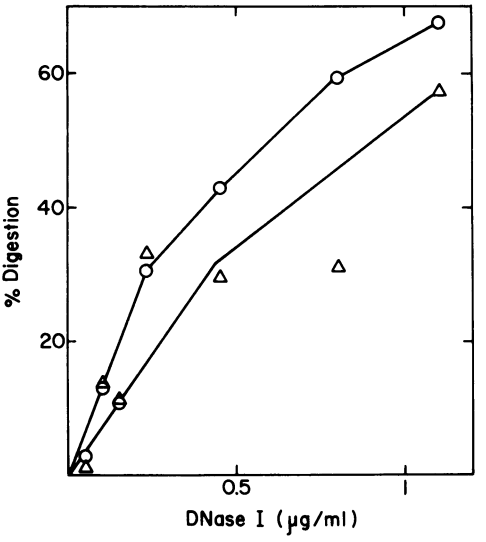

Fig. 5. DNase I sensitivity of nick translated nuclei following micrococcal nuclease digestion. Nick translated nuclei were suspended in Mnase buffer and incubated at 15°C either with (Δ) or without (o) 1 µg/ml micrococcal nuclease. The reaction was stopped after 5 min by dilution into the same buffer at 0°C. The nuclei were recovered by centrifugation and resuspended in 50 mM Tris (pH 7.4), 5 mM $MgCl_2$ and incubated for 2 min at 37°C with various amounts of DNase I. Percent digestion of labeled DNA was determined as described in Materials and Methods. Treatment with micrococcal nuclease resulted in the digestion of about 10% of the total DNA and about the same degree of digestion of radioactively labeled DNA. As determined by gel electrophoresis about 50% of the DNA was in the form of mono and dinucleosomes. Treatment with 1 µg/ml DNase I caused an additional 5% digestion of the total DNA.

distribution of nucleosomes in active regions. Analysis of the DNA material associated with mononucleosomal particles demonstrates that this represents a random sample of the genome (14). In addition, the entire ovalbumin and hemoglobin gene sequences in actively synthesizing cells are present in mononucleosomes and at about the same concentration as non-active genes, indicating that the distribution of nucleosomes around these genes is random and similar to non-active chromosomal regions (7). That this is not generally the case can be deduced from electron microscope studies which show that while many genes do have a normal distribution of nucleosomal particles (15), there are other active genes which show a relatively small number of particles. In a few instances it has been possible to show that the sensitivity

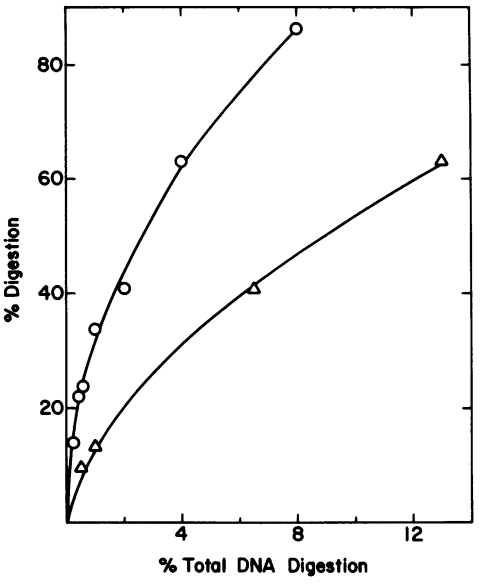

Fig. 6. DNase I sensitivity of nick translated mononucleosomes prepared by sucrose gradient ultracentrifugation. Nick translated nuclei were digested with micrococcal nuclease and subjected to sucrose gradient centrifugation as described in the legend to figure 2. Over 90% of this material was found to be mononucleosome particles as determined by gel electrophoresis. Mononucleosome containing fractions were diluted 1:10 into 50 mM Tris (pH 7.4), 5 mM $MgCl_2$ and incubated at 37°C with 0.4 µg/ml DNase I (Δ). Nick translated nuclei were suspended in this same buffer and incubated with 0.4 µg/ml DNase I at 37°C (o). At various times aliquots were assayed for percent digestion of total and radioactively labeled DNA as described in Materials and Methods.

of active regions to micrococcal nuclease is altered. The ovalbumin gene is preferentially released as nucleosomes in actively producing oviduct cells (8,9). Furthermore, Wu at al. have studied the sensitivity of the heat shock genes of Drosophila to treatment with low concentrations of micrococcal nuclease using blotting and analysis (10). This pattern of nucleosome sensitivity is changed by induction of these genes (16).

Our results help to clarify the picture of nucleosomes in the regions of active genes. Micrococcal nuclease solubilizes active genes at a slightly higher rate than the rest of the genome (figure 1), suggesting that the concentration of nucleosomes in these areas is near normal, but that some genes are digested more rapidly than others. In fact, other results from the literature support the notion that some specific genes are more sensitive to Mnase than others (3). The majority of the active genes show increased sen-

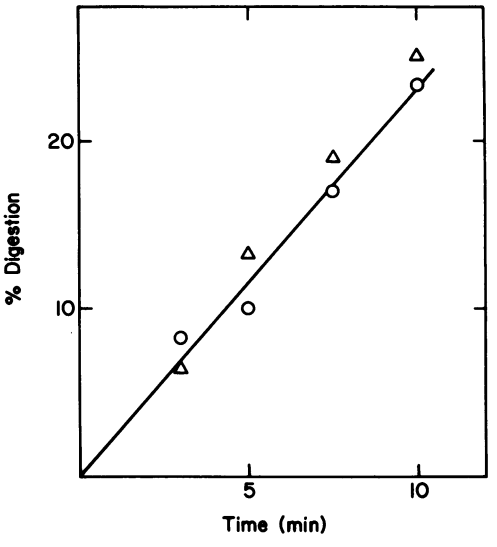

Fig. 7. DNase I sensitivity of mononucleosomes prepared by differential centrifugation. Nick translated nuclei were digested by micrococcal nuclease (7%), centrifuged and washed in 0.075 M NaCl, 0.02 M EDTA, 0.01 M Tris-HCl (pH 7.4). The resulting pellet was suspended in 5 mM sodium phosphate (pH 6.8) and the insoluble material was removed by low speed centrifugation (1). Over 90% of the soluble material was found to be mononucleosomal as determined by gel electrophoresis. These monosomes were diluted 1:10 into 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$. Mononucleosomes (Δ) and total nuclei (o) were incubated at 37°C with 2 µg/ml DNase I and aliquots were assayed from digestion at various times. The percent digestion of radioactively labeled DNA is presented in this figure. Total DNA digestion of both preparations was about 3% after 10 min.

sitivity to micrococcal nuclease between nucleosomes, causing the preferential release of active genes in the form of mononucleosome particles (18). Thus, although nucleosomes cover the entrie active gene region, there is a special conformation which renders them more exposed to clipping by micrococcal nuclease.

It should be noted that the mechanisms of micrococcal nuclease and DNase I are different. Whereas DNase I digests and solubilizes both inter and intra nucleosomal regions at the same rate, micrococcal nuclease preferentially attacks inter nucleosomally. Even after complete digestion some parts of the nucleosomal core remain resistant to micrococcal nuclease. Our results show that active regions are preferentially exposed to both of these enzymes, each digesting with its own mechanism.

Bloom and Anderson have reported that the ovalbumin gene is not only

rapidly converted to nucleosomes, but that the nucleosomes are preferentially released from the nucleus (9). We indeed find that more than 3% of the active genes are rapidly released from the nucleus after nuclease digestion. It is striking, however, that only a small subpopulation of the active regions are released. This special class of genes may be concentrated on the nuclear membrane thus making them not only sensitive to micrococcal nuclease, but in a position which places them in juxtaposition to the extranuclear space.

The factors which form and preserve the DNase I sensitive conformation of active genes are not completely known. Although the ovalbumin gene loses its sensitivity to DNase I after digestion of nuclei with micrococcal nuclease (2), the hemoglobin gene seems to retain its special conformation even after this gene has been converted to nucleosomal particles.[1] Our results suggest that most genes are indeed sensitive to DNase I at the level of the individual nucleosome. Although the mechanism of formation of this structure is not understood, it is clear that HMG proteins play some role in preserving this DNase I sensitivity. These HMG proteins also act at the level of nucleosomes, since monosomes containing the hemaglobin gene can be successfully reconstituted to active form by the addition of HMG proteins (5,17).

It should be noted that while HMG proteins are involved in the preservation of DNase I sensitivity in active regions, these proteins do not play a role in the sensitivity of these regions to micrococcal nuclease attack. This was demonstrated by the observation that active genes remain sensitive to micrococcal nuclease even in nuclei lacking HMG14 and HMG17. Furthermore, it is suggested by the fact that these proteins act at the level of individual nucleosome and probably do not affect the overall distribution of nucleosomes over the active genes. These results indicate that other factors must be involved in forming the special conformation typical of active chromatin.

In summary, most studies concerned with chromosome structure have concentrated on a few highly active and easily studied genes, such as ovalbumin and hemoglobin. The nick-translation technique enables one to study the conformational state of all active genes. Our results extend the observations of others by demonstrating that the nuclease sensitivity pattern is an inherent property of all actively expressed genes.

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