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Selective association of the trout-specific H6 protein with chromatin regions susceptible to DNase I and DNase II: Possible location of HMG-T in the spacer region between core nucleosomes

(nonhistone proteins/micrococcal nuclease/trout testis)

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ABSTRACT **Nuclei and chromatin from trout testis cells were digested with three different nucleases (DNase I, DNase II, and micrococcal nuclease), and the acid-soluble proteins that were solubilized and those remaining bound to the nuclease-resistant DNA were compared electrophoretically. With the conditions described by H. Weintraub and M. Groudine [(1976) *Science*, 193, 848–856], which we previously found to be selective in digesting actively transcribed regions in trout testis chromatin, a single chromosomal protein, H6, was solubilized. The nucleosomal histones and H1 remained insoluble, bound to the resistant DNA. In contrast, digestion with micrococcal nuclease led to a preferential solubilization of a second protein, HMG-T, together with the release of some nucleosomal histones and H1 into the soluble fraction. DNase II also discriminated between "active" and "inactive" chromatins; when a DNase II-solubilized "active" chromatin fraction was prepared, it too was enriched in H6 and HMG-T. Thus, both H6 and HMG-T, the two major low-salt extractable chromosomal nonhistone proteins from trout testis, are associated with chromatin regions selectively sensitive to nucleases. The preferential solubilization of HMG-T by micrococcal nuclease action suggests that it might be located at the internucleosomal "spacer" region.**

A detailed knowledge of the structural organization of chromatin is a fundamental prerequisite for understanding the mechanisms that regulate the expression of specific genes in eukaryotes. The work of several investigators using micrococcal nuclease as a probe has led to the conclusion that the basic nucleosomal structure (consisting of a core particle of 140 base pairs of DNA and an octameric complex of histones made up of 2 molecules each of H2A, H2B, H3, and H4 and an internucleosomal region, containing about 60 base pairs of DNA and 1 molecule of H1) is a characteristic feature of both transcriptionally-active and inactive regions of chromatin (1–6). However, it is clear that "active" chromatin must possess some distinctive structural properties, as judged by its preferential susceptibility to digestion by DNase I (7–9) and DNase II (10, 11) and also by its preferential recognition by *Escherichia coli* RNA polymerase (12, 13). Therefore, it seems likely that the distinctive features characteristic of the structure of active chromatin might be due to the association of these regions with specific nonhistone proteins.

In recent years, our laboratory has been involved in the isolation and characterization of a restricted population of nonhistone proteins that can be extracted from purified trout testis chromatin by low concentrations of salt (14). Three major components were observed upon polyacrylamide gel electrophoresis and have been designed components R, S, and T. Component T (more recently termed H6) and component R (or HMG-T) have been extensively purified and their amino acid composition and partial amino acid sequence have been determined in our laboratory (15–17). Although these proteins are present in somewhat lower amounts than the histones (10–12% of the concentration of each nucleosomal histone), they still comprise above 10^6 molecules per nucleus, so that it is possible that they might also have a role in the structural organization of chromatin.

In the present report we present evidence that demonstrates that H6 is preferentially associated with chromatin regions particularly sensitive to digestion by DNases I and II. Experiments with micrococcal nuclease also suggest that one of the other major low-salt extractable proteins, HMG-T, is located in the internucleosomal region in trout testis chromatin.

Abbreviation: RSB buffer, 0.01 M Tris·HCl, pH 7.4/0.01 M NaCl/3 mM $MgCl_2$.

MATERIALS AND METHODS

Trout Testis. Trout testis were collected at a late stage of maturation (October 1974) from freshly killed trout (Dantrout, Brande, Denmark), immediately frozen on dry ice, and stored frozen at −70°.

Preparation of Trout Testis Nuclei. Trout testis nuclei were prepared as described (9, 18). The nuclear pellet was washed twice with RSB buffer (0.01 M Tris·HCl, pH 7.4/0.01 M NaCl/3 mM $MgCl_2$). Microscopic examination of the nuclear suspension revealed nuclei free of cytoplasmic tabs.

DNase I Digestion of Trout Testis Nuclei. Nuclei were resuspended in RSB buffer, at a DNA concentration of 1 mg/ml (20 A_{260} units/ml) and incubated for the desired periods at 37° with pancreatic DNase I (Worthington DPFF, 2070 units/mg) at a concentration of 20 μg/ml. The reaction was terminated by chilling the tubes on ice and immediately centrifuging them for 5 min at 1000 × *g* in a Sorvall HB-4 rotor. The supernatant and pellet were immediately used for the extraction of proteins.

Micrococcal Nuclease Digestion of Trout Testis Nuclei. Nuclei were resuspended, in RSB buffer containing 1 mM $CaCl_2$, at a DNA concentration of 10 mg/ml (200 A_{260} units/ml) and incubated for the desired periods at 37° with micrococcal nuclease (Worthington, 250 units/ml). The reaction was terminated as described for DNase I. This supernatant was designed S_1. The pellet was resuspended in a minimum volume of 0.2 mM EDTA, pH 7.5, and then centrifuged at 12,000 × *g* for 30 min. The supernatant obtained was designed S_2. The pellet and both S_1 and S_2 were immediately adjusted to 0.2 M in H_2SO_4, and proteins were extracted as described below.

Preparation of Trout Testis Chromatin. For the preparation of chromatin, the clean nuclei were washed once with 10 mM Tris·HCl, pH 7.5, and then resuspended in distilled water for 20 min at 4°. The chromatin gel was washed twice with twice-distilled water and recovered by centrifugation at 12,000 × *g* for 10 min in the Sorvall HB-4 rotor.

DNase II Digestion of Trout Testis Chromatin. The

chromatin gel was resuspended in 25 mM sodium acetate (pH 6.6) for 20 min at 4°. The volume was adjusted to give a concentration of 10 A_{260} units/ml (500 μg/ml). The solution was brought to 24° and DNase II (Worthington) was added to a final concentration of 100 units/ml. After incubation for the desired periods, the reaction was terminated as described for DNase I.

Acid Extraction of Proteins and Gel Electrophoresis. Chromosomal proteins were extracted from the various fractions obtained after DNase digestion of nuclei or chromatin by incubation of that fraction with 0.2 M H_2SO_4 for 1 hr at 4°. After incubation, the acidified solution was centrifuged at 10,000 × *g* for 10 min. The solubilized proteins contained in the supernatant fraction were precipitated by addition of 2 volumes of 95% ethanol at −40°. The proteins were recovered by centrifugation at 10,000 × *g* for 30 min at −5°, washed twice with cold ethanol, and dried under reduced pressure.

The proteins were analyzed by starch gel electrophoresis in aluminum lactate/4 M urea buffers as described by Sung and Smithies (19). After electrophoresis for 12–16 hr at 3–4 V/cm, the gels were bisected horizontally; the top slice was stained with arginine-sensitive stain (1.3 g of Amido Black 10 B, 17 ml of glacial acetic acid, and 3 mM cobalt nitrate per liter) and destained with 1 M H_2SO_4. The arginine-sensitive stain allows a rapid and highly sensitive visualization of the protein bands, but the color development is not proportional to the amount of protein present in each band. The bottom slice was stained conventionally with Amido Black 10 B and destained with 1% (vol/vol) acetic acid.

The proteins were also analyzed by electrophoresis on 15% (vol/vol) polyacrylamide slab gels containing 2.5 M urea for 6 hr at 20 V/cm at 4°. The gels were stained with Fast Green (20) and destained with 1% acetic acid.

RESULTS

DNase I action on trout testis nuclei

We have shown (9) that, upon incubation of trout testis nuclei with DNase I under conditions that allow digestion of 10–20% of the total DNA, there is a preferential degradation of those DNA sequences that give rise to testis cytoplasmic poly(A) (+) RNA as assayed by hybridization of the resistant DNA with labeled cDNA prepared from the *in vivo* population of cytoplasmic polyadenylated RNA. In order to examine the proteins that were solubilized upon degradation of this limited fraction of the DNA apparently enriched in active genes, we performed DNase I digestions of trout testis nuclei for various periods and analyzed the acid-soluble proteins that were present in the chromatin and were released into the supernatant and also those that remained bound to the DNA (resistant to DNase I). Fig. 1 shows a starch gel pattern of the acid-soluble proteins from the DNase I-labile and -resistant fractions. The supernatant fraction contained almost exclusively H6 and small amounts of HMG-T. The pellet fraction contained all of the other histones and was depleted in H6.

In every experiment, a control was performed in which the reaction was stopped immediately after the addition of the DNase I. Controls in which samples were incubated in parallel at 37° but in the absence of DNase I were also included. In both types of controls, we detected the solubilization of traces of protein, as judged by the absorbance at 230 nm, but no band of H6 could be detected when these fractions were separated on gels. In another experiment using polyacrylamide gel electrophoresis, which allowed us to obtain a quantitative densitometer scan, H6 constituted 90% of the additional protein

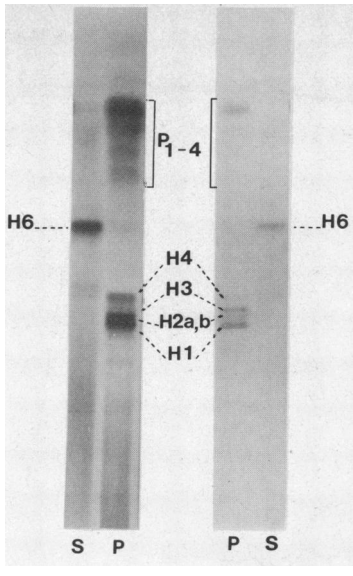

FIG. 1. DNase I action on trout testis nuclei. Nuclei were incubated for 10 min with DNase I (to the extent of 10% digestion of the input DNA) and the acid-soluble proteins from the solubilized fraction (*S*) and insoluble fraction (*P*) were analyzed by starch gel electrophoresis as described in *Materials and Methods*. (*Left*) Top portion of the gel, stained with arginine-sensitive stain. (*Right*) Bottom portion of the gel, stained with Amido Black. The protein bands were identified by comparison with the mobilities of purified protein standards run in the same slab gels. P_{1-4}, protamines; H6, component T, protein H6; H4, H3, H2, a, b, and H1, histones.

released into the supernatant fraction (data not shown). Individual protein bands from both starch and polyacrylamide gels were identified by reference to purified protein standards in parallel slots of the same slab gels. Several kinetic experiments were performed in which identical aliquots of nuclei and DNase I were incubated at 37° for different periods, and a progressive release of H6 into the supernatant fraction was consistently observed. None of the nucleosomal histones nor H1 was released. Identical results were also obtained when the same experiments were performed with purified chromatin rather than nuclei (Fig. 2).

DNase II action on trout testis chromatin

It has been established that, under defined experimental conditions (10), DNase II preferentially attacks a minor fraction of chromatin DNA; the amount of DNA solubilized is variable, depending on the source of the chromatin, but corresponds closely to the template activity of the particular chromatin for RNA synthesis as measured with *E. coli* RNA polymerase. If the structural feature recognized in chromatin by both DNase I and DNase II were the same and if the preferential association of H6 with DNase I-sensitive regions in chromatin were in any way related to transcriptional activity, we might also expect to find an enrichment in the content of H6 in the chromatin fraction solubilized by DNase II. Accordingly, we fractionated chromatin into DNase II-soluble (S_1) and insoluble (P_1) fractions, using a slight modification of the original procedure of Gottesfeld *et al.* (10). The acid-soluble proteins obtained from fractions S_1 and P_1 were analyzed by starch gel electrophoresis (Fig. 3). S_1 was clearly enriched in H6 (about 2-fold) and in HMG-T (about 1.5-fold) but the result was not as clear-cut as with DNase I because both S_1 and P_1 contained qualitatively the same protein bands—i.e., all of the histones plus protamines, H6, and HMG-T. Therefore, we conclude that DNase II can

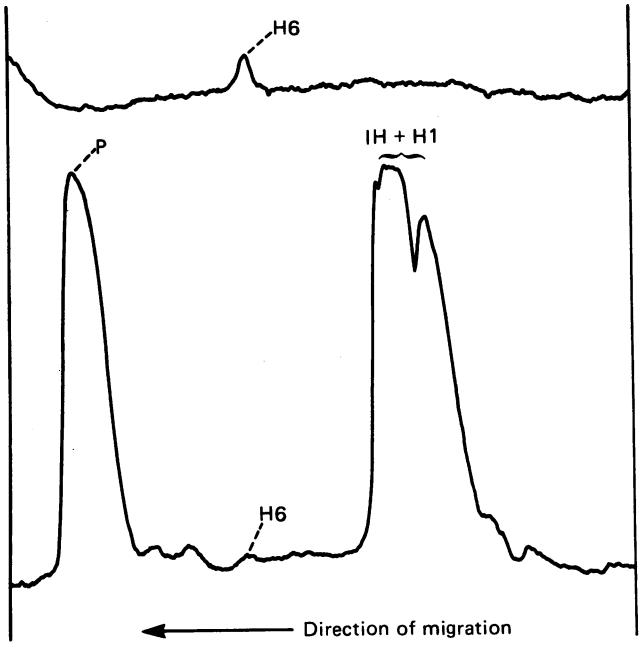

FIG. 2. DNase I action on trout testis chromatin. Chromatin was incubated for 30 min with DNase I, exactly as described for nuclei in the *Materials and Methods* section. Equal amounts (based on A_{230}) of acid-soluble proteins derived from the soluble fraction (*Upper*) and insoluble fraction (*Lower*) were analyzed on starch gels and stained with Amido Black. The gels were photographed and the negatives were scanned with a Joyce Loebl microdensitometer. P, protamines; IH + H1, inner histones plus H1.

also solubilize selectively a fraction of trout testis chromatin enriched in H6 and, to a lesser extent, HMG-T.

Micrococcal nuclease action on trout testis nuclei

To investigate whether the enrichment in H6 of chromatin fractions selectively sensitive to DNases I and II could also be observed in those regions of chromatin most accessible to micrococcal nuclease, intact trout testis nuclei were digested with this enzyme. Micrococcal nuclease does not show selective specificity toward chromatin regions enriched in template activity but digests the chromatin in a nonsequence-specific manner (7, 8) with the accessible spacer regions between nucleosomes being attacked first. The first supernatant fraction after digestion under the present conditions is equivalent to that of the DNase I experiments. The major protein released into this soluble fraction was HMG-T (Fig. 4). This nonhistone protein contains two cysteine residues and has previously been observed (17) to convert, upon standing, to two disulfide forms, an intramolecular disulfide, HMG-T′, that migrates faster in starch gels and a dimer that migrates more slowly. In the portion of the gel stained with the arginine-sensitive stain, we could also observe a small release of H6 at later times accompanied by the release of the nucleosomal histones (data not shown).

After the removal of the first supernatant (S_1), the nuclear pellet was disrupted by homogenization in 0.2 mM EDTA. This procedure has two effects: it breaks the nuclear membrane (7) and it allows the release into the supernatant of those proteins that were solubilized by the enzyme but remained insoluble probably because of nonspecific aggregation to the chromatin pellet promoted by the divalent cations present in the solution. After treatment of digested nuclei with EDTA, it was apparent that several other chromatin proteins had been released from the chromatin but had remained bound to the nuclear pellet. This group included approximately 60% of the H6, most of the

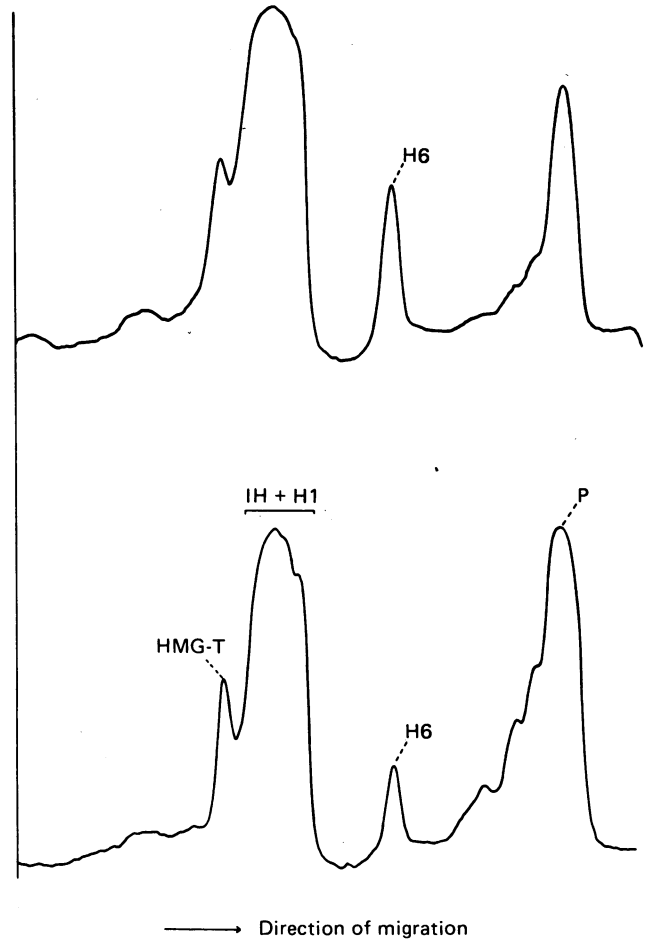

FIG. 3. DNase II action on trout testis chromatin. Chromatin was incubated for 5 min at 24° with DNase II, as described in *Materials and Methods*. Equal amounts of acid-soluble proteins obtained from the soluble fraction (S_1) (*Upper*) and the insoluble fraction (P_1) (*Lower*) were analyzed on starch gels and stained with Amido Black. The gels were photographed and the negatives were scanned on a Joyce Loebl microdensitometer. P, protamines; IH + H1, inner nucleosomal histones plus H1.

remainder of the HMG-T, and substantial quantities of the nucleosomal histones and traces of protamine. The insoluble pellet after EDTA treatment, representing chromatin resistant to micrococcal nuclease, contained nucleosomal histones, the bulk of the protamines, and only traces of H6 and HMG-T. Thus, in contrast with DNases I and II, the action of micrococcal nuclease leads to the preferential solubilization of HMG-T. However, it is clear that a substantial portion of the chromatin is also solubilized as previously demonstrated by Honda *et al.* (21, 22) but does not leave the nuclear pellet until after EDTA treatment.

DISCUSSION

We have examined the distribution of acid-soluble proteins in localized regions of chromatin from rainbow trout testis by selective digestion with the endonucleases DNase I, DNase II, and micrococcal nuclease. Each of these enzymes shows a characteristic pattern of digestion of chromatin. Micrococcal nuclease cleaves DNA at sites regularly spaced along the repeating chromatin subunit structure (2) in a nonspecific manner with regard to base sequence (5, 6). At early times of digestion the cleavage is restricted to the DNA located in the spacer regions between subunits (2). In contrast, with DNase I, the DNA

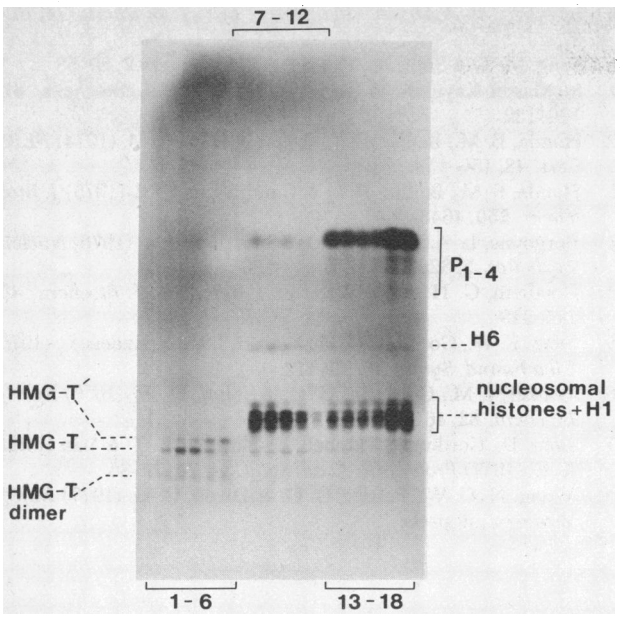

FIG. 4. Micrococcal nuclease action on trout testis nuclei. Nuclei were incubated at 37° with micrococcal nuclease for various periods as indicated in *Materials and Methods*. Acid-soluble proteins from fractions S_1, S_2, and P_1, were analyzed by starch gel electrophoresis. Slots 1 through 6 represent the acid-soluble proteins present in fraction S_1 after 0, 2, 5, 10, 30, and 65 min of incubation with the nuclease, respectively. Slots 7 through 12 show the acid-soluble proteins present in fraction S_2 after incubation with the enzyme for the same periods. Slots 13 through 18 show the acid-soluble proteins that remained bound to the insoluble fraction P_1 after micrococcal nuclease treatment for these periods. The individual protein bands were identified as described in the legend to Fig. 1.

within the nucleosomes is as accessible as the DNA in the spacer regions (23). Under our experimental conditions, DNase I preferentially digests the DNA of regions of the chromatin that are transcribed *in vivo* (7, 9). DNase II also shows selectivity toward transcribed regions and has been used to fractionate chromatin into active and inactive regions (10, 11).

The experiments with DNases I and II in which H6 is released selectively allow us to conclude that H6 is preferentially located in the domain of active chromatin. It is interesting to note that H6 is the only major acid-soluble protein solubilized by DNase I action that can be detected in our gel electrophoresis system. DNase II, on the other hand, solubilizes a substantial portion of the nucleosomal histones together with HMG-T and traces of protamine. However, H6 is enriched by about 2-fold and HMG-T is enriched by 1.5-fold in this fraction as compared to the insoluble fraction (Table 1). These findings suggest that the sites of cleavage within the chromatin may be different for DNase I and DNase II and also that DNase I may cleave the DNA preferentially at regions in intimate contact with H6, thus accounting for its rapid release.

The selective and rapid solubilization of HMG-T by micrococcal nuclease suggests that it may be located in the spacer region between nucleosomes. Partial amino acid sequence determinations of both HMG-T and H6 (17) have indicated that these proteins are related closely to the group of low-salt extractable proteins (HMG or high-mobility-group proteins) described by Johns and coworkers (24–26) in calf thymus chromatin. The major difference between these groups of proteins in the two tissues is that in trout testis the population is less heterogeneous and its two major member proteins, H6 and HMG-T, are easily detected on gels in the presence of the nucleosomal histones.

Table 1. Distribution of acid-soluble proteins in chromatin treated with DNase II

Protein fraction	Content in fraction S_1, %	Content in fraction P_1, %	Ratio, S_1/P_1
HMG-T	10.58	6.93	1.53
Nucleosomal histones + H1	48.63	36.27	1.34
H6	9.20	4.75	1.94
Protamines	31.6	52.04	0.60

The data are from Fig. 2. The protein content in each band was determined by measuring the area below each peak. The areas under the nucleosomal histones + H1 peak plus the protamine peak in S_1 and P_1 were 0.51 and 0.54, respectively, indicating that equal amounts of chromosomal proteins from each fraction were applied to the gel.

Recent experiments in our laboratory using calf thymus and mouse brain suggest a correlation between the complexity or heterogeneity of this group of HMG proteins and the genetic activity of the tissue. In addition, similar nuclease digestion studies with these two tissues confirm the presence of proteins of the HMG-group in regions on chromatin selectively sensitive to nucleases (B. Levy W., D. C. Watson, and G. H. Dixon, unpublished data). It has recently been established that the length of the spacer region between nucleosomes varies over a certain range in transcriptionally active chromatins (27) whereas inactive chromatins exhibit a relatively regular spacing of nucleosomes. It is not unlikely that the presence of HMG-type proteins, possibly in various combinations with H1 and other proteins, in spacer regions might determine the position and length of these spacer regions and contribute to the unique conformation of transcriptionally active regions of chromatin.

The selective solubilization of H6 by DNase I but not by micrococcal nuclease in turn suggests that it might be bound to the nucleosome core. If so, it is possible that this protein might be located on the outside of the nucleosome, as judged by its unusual amino acid composition, characterized by the lack of a hydrophobic core and by an overall high charge density. In addition, H6 is also highly modified by poly(ADP) ribosylation, a modification which so far has only been described for outer nucleosomal proteins such as H1 and protamine (28).

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