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The release of high mobility group protein H6 and protamine gene sequences upon selective DNase I degradation of trout testis chromatin

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ABSTRACT

Limited digestion of trout testis nuclei with DNase I selectively degrades the protamine genes. Concomitant with the degradation of transcribed DNA sequences a series of chromosomal proteins are released; among these, the major species corresponds to the high mobility group protein H6. The amounts of H6 released from chromatin by limited DNase I action and that in the residual nuclear pellet have been determined. A very high proportion of H6 is associated with DNase I sensitive chromatin regions.

INTRODUCTION

The HMG (high mobility group) proteins are a group of non-histone chromosomal proteins which can be extracted from chromatin with 0.35 M NaCl and which are soluble in 2%, but precipitated by 20% trichloroacetic acid (1). Their presence in high concentrations, 10^5 - 10^6 molecules, in the nucleus of a variety of eukaryotic cells, implies an important structural role in chromatin (2). Four major HMG proteins have been purified to homogeneity from calf thymus: HMGs 1, 2, 14 and 17 (2). In trout testis there are two major HMG proteins: HMG-T, similar in size and amino acid composition to calf thymus HMG's-1 and 2; and H6, similar to calf thymus HMG's-14 and -17 (3,4). A third non-histone protein, ubiquitin (5) also co-purifies with the HMG proteins in trout testis (6) and calf thymus (7).

Our earlier work with trout testis strongly suggests that these proteins as a group are a fundamental structural component of active chromatin (6,8). In particular, H6 has been shown to be a major stoichiometric structural component of a subset of nucleosome cores highly enriched in transcribed DNA sequences (9,10). Furthermore, HMG's 14 and 17 (analogous to trout testis H6) have been shown to be responsible for conferring DNase I sensitivity on the globin gene in avian erythrocyte chromatin (11).

Nevertheless, the HMG group comprises a complex group of proteins not all of which are equally abundant in every cell type (2). For this reason

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and due to technical considerations, different authors do not entirely agree as to which HMG proteins are preferentially localized in active chromatin and whether some individual species are exclusively associated with those regions. Although both Vidali and collaborators working with avian erythrocyte chromatin (12) and Levy W. and colleagues working with trout testis (8), calf thymus, and mouse brain chromatin (13) reported that HMG proteins 1 and 2 are selectively associated with DNase I sensitive chromatin regions, similar studies by Goodwin and Johns using rabbit liver and thymus yielded conflicting results (14).

Since, at least part of the disagreement derives from inadequate methodology for quantitation of these proteins, we have begun a reevaluation of these issues by applying a quantitative method to the study of the distribution of H6 among domains of trout testis chromatin differing in their susceptibility to DNase I.

We also asked whether a specific set of DNA sequences of trout testis, the protamine genes, coding for an abundant mRNA and a major group of polypeptides in this tissue, are selectively degraded by limited DNase I action.

MATERIALS AND METHODS

DNase I Digestions

In a typical preparation, 30 g of trout testis were used. Nuclei were prepared as previously described (15). The nuclear pellet was washed twice in RSB (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$) by gentle homogenization, followed by centrifugation at 5000 rpm for 5 min in the Sorvall HB-4 rotor. The nuclear pellet was then resuspended in RSB at a concentration of approximately 20 A_{260} units/ml and incubated for various time periods with 15 µg/ml of DNase I (Worthington, 2000 units/mg). The reaction was stopped by chilling the tubes on ice followed by centrifugation at 5000 rpm for 5 min. The supernatant was designated as S and the pellet P.

Alternatively, the nuclear pellet was washed once with RSB and once with RSB containing 1 mM $CaCl_2$ (RSB-Ca^{+2}). The pellet was then resuspended in RSB-Ca^{+2} at a concentration of 20 A_{260}/ml and DNase I (20 µg/ml) was added for 15 min at 37°C. The digestion was stopped by the addition of 0.2 M EGTA to a final concentration of 1 mM, followed by centrifugation for 5 min at 5000 rpm at 4°C. The first supernatant was designated S_0. The pellet was resuspended in about 10 volumes of RSB-1 mM EGTA containing 0.1 M NaCl, incubated at 0°C for 20 min and centrifuged to yield a second supernatant, $S_{0.1}$, and a pellet, which was successively extracted with RSB-1 mMEGTA

containing 0.2 M NaCl, to yield supernatant $S_{0.2}$ and finally with 1 mM EDTA, to yield supernatant S_{EDTA} and a pellet, P.

Extraction of acid soluble proteins

To an aliquot of each supernatant and pellet fraction 5 M H_2SO_4 was added to a final concentration of 0.2 M. Samples were incubated for 1 hr at 4°C, then centrifuged for 15 min at 10,000 rpm in the Sorvall HB-4 rotor. The supernatants were either precipitated by the addition of two volumes of 95% ethanol at -40°C overnight, followed by repeated washes with 95% ethanol prior to lyophilization of the samples, or alternatively dialyzed extensively (spectropore membranes #3) against water. When the latter treatment was applied to the DNase I-generated samples, we observed a white precipitate inside the dialysis bag at the end of the dialysis step. This insoluble precipitate was separated from the soluble fractions by centrifugation for 5 min at 10,000 rpm.

Separation and quantitation of acid soluble proteins

Accurately weighed portions of the acid soluble proteins were dissolved in buffer and electrophoresed on polyacrylamide gels as described previously (16). The gels were stained for 30 min in 0.25% Coomassie Blue, 9.2% acetic acid, 45% methanol and then destained in 7.5% acetic acid, 22.5% methanol. The band of interest was cut from the gel and shaken overnight with a measured volume of 25% pyridine in water. The absorbance in 595 nm of the resulting solution was determined, and the amount of protein in the original band was calculated by reference to standard curves prepared by electrophoresis of known amounts of the appropriate homogeneous protein. The concentration of each one of the standard proteins was determined by the method of Lawry as described previously (16). Standard curves for the HMG proteins were linear and reproducible from gel to gel (16).

Extraction of nucleic acids from testis nuclei

The extraction of DNA from control and DNased nuclei was accomplished as described earlier (17). Both control and DNased DNA were sheared to a size of 300-500 nucleotides, as determined by gel electrophoresis. Protamine mRNA and ^{32}P-cDNA representing protamine sequences were prepared as previously described (18).

cDNA/DNA reassociation reactions

Reactions were performed in triplicate in 200 μl capillaries in a buffer containing 0.8 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA. 50 pg of cDNA (2500 cpm) and 1 mg of DNA were used per capillary. The extent of self annealing of the cDNA was determined as previously described (18) by including

in each experiment a zero time control. After incubation at 70°C for the desired amount of time, each sample was flushed into 4 ml of S_1 buffer and incubated with 15,000 units of S_1 nuclease for 90 min at 45°C (18).

RESULTS

DNase I sensitivity of the protamine genes

We have previously established that limited DNase I digestion of trout testis nuclei promotes the selective degradation of those DNA sequences which give rise to polyadenylated cytoplasmic RNA (17). We have now examined the DNase I sensitivity of a specific gene family that codes for protamines.

To this end, trout testis nuclei were incubated with DNase I for a period of time sufficient to degrade some 5-10% of the input DNA to acid solubility. The DNA resistant to DNase I treatment, obtained as described in Methods, was used as a driver in renaturation experiments with a radioactive protamine cDNA tracer. As a control, we examined the kinetics of the reaction of DNA extracted from nuclei which were incubated under identical conditions but without enzyme. The results from two such series of experiments (illustrated in Figure 1) clearly show that the reaction driven by the DNase resistant DNA is slower than that of the control reaction, as evidenced by an 8-fold shift to higher Co·t values. At a Co·t value of 2×10^4 M sec, at which the control reaction has achieved completion, the reaction of the resistant DNA has reached only 75%. Even though we were not able to achieve higher saturation values for the reaction driven by the resistant DNA when the reaction was carried out at higher Co·t values, we are not certain that this value of 75% represents a real plateau since we noticed that our cDNA probe was subject to some degradation when the period of incubation at 70°C was longer than that required for a Co·t of 20,000. We conclude that, with only 5-10% digestion of the trout testis genome by DNase I, there occurs a preferential rapid degradation of the protamine genes. This observation confirms our previous results showing that all DNA sequences that are transcribed into polyadenylated cytoplasmic RNA are selectively degraded by limited DNase I action on trout testis nuclei (17).

Distribution of H6 among DNase I sensitive and resistant regions of trout testis chromatin

Having demonstrated the preferential DNase I sensitivity of a specific structural gene in trout testis, we devoted our attention to the study of the distribution of H6 among DNase I sensitive and resistant nuclear domains. We concentrated our efforts on H6 because it is the major acid-soluble protein

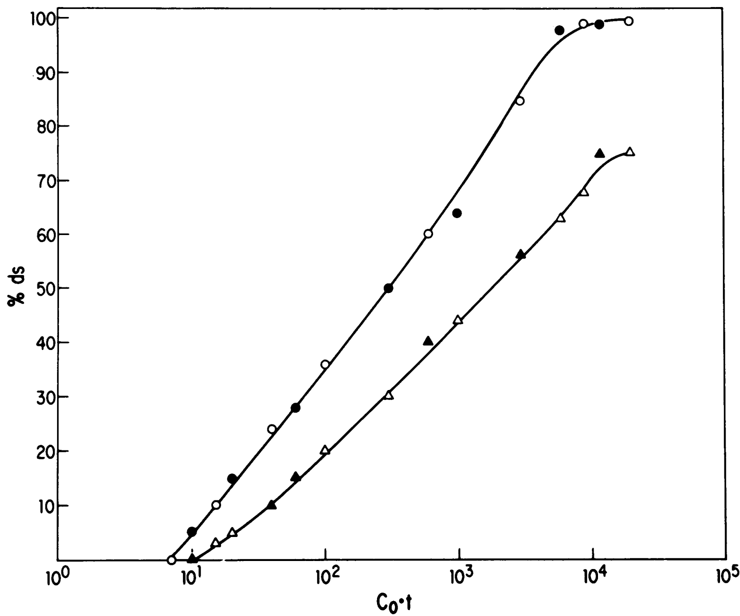

Figure 1. DNase I sensitivity of the protamine genes from trout testis. The reactions were performed as described in Methods. To facilitate comparison between the two sets of data, the curves were normalized assuming 100% hybridization between protamine cDNA and the control DNA. In these experiments, the actual percent reassociation betwen the ^{32}P-cDNA and control DNA was 59%. The extent of self-annealing of the ^{32}P-cDNA was 10%. In this figure we illustrate two sets of experiments with cDNA driven by a different batch of DNA. (o,●) Reassociation of protamine cDNA with DNA from control nuclei; (Δ,▲) reaction of the cDNA with DNase resistant DNA.

released by limited DNase I action (8) and it is the principal non-histone protein component of transcriptionally active nucleosomes (9,10). Trout testis nuclei were digested with DNase I for various time periods as described in Methods. The extent of acid solubility of the material in the supernatants are shown in Table I. Two kinds of controls were included in these experiments; in the first, an aliquot of nuclei was incubated at 37°C for 15 min without DNase to monitor endogenous nuclease action and in the second, nuclei were kept at 0°C for 1 hr in the absence of the nuclease. Less than 1% acid solubility was released into the supernatant fractions (S_0 and S_{37}) in both controls.

We then examined the patterns of acid soluble proteins obtained from each of these fractions. Fig. 2 illustrates the acid soluble proteins

TABLE I

KINETICS OF DIGESTION OF TROUT TESTIS NUCLEI BY DNase I

Nuclei from 30 g of testis were prepared and resuspended in RSB buffer (160 ml). 6-25 ml aliquots were separated and digested with DNase I for 5, 10, 30 or 60 min. The A_{260} released into a low speed supernatant was measured. The acid solubility was determined by measuring the A_{260} of a low-speed supernatant obtained after precipitation of an aliquot of each sample with 10% TCA S_0, S_5, etc. represent supernatant fractions after 0, 5, etc. minutes of digestion.

Fraction	% acid solubility	% A_{260}
S_0	0	6
S_5	1	12
S_{10}	5	33
S_{30}	11	57
S_{60}	15	65

released into the various supernatant fractions. Slots 2 and 3 show that only traces of HMG-T and H6 were released into the control supernatants S_0 and S_{37}. In the other fractions, S_5, S_{10}, S_{30}, and S_{60}, a complex pattern of proteins was observed (slots 4, 5, 6, 7 respectively).

H6 is released progressively as digestion proceeds although very little of this protein appears in the supernatant at very low digestion levels (Tables I and II). We have repeatedly observed a lag period in the release of H6. A plausible explanation of this lag is that a minimum number of nicks must be made on the DNA to allow the subsequent release of H6. After a certain number of cuts have been made, H6 is readily released from the nucleosomes (Fig. 2, and Table II). DNase I is known to cut the nucleosome at 10 base pair intervals (19), so that several single stranded cuts might be required to release the full length of the H6 molecule. In the experiments described in this report it would appear that even after digestion of 15% of the DNA to acid solubility, a significant fraction of the H6 remains bound to the nuclear pellet (P_{60}) (Tables I and II).

The next set of experiments was designed to elucidate whether amounts of H6 additional to those released into the low speed supernatant could be solubilized from the chromatin pellet remaining after DNase I digestion. To this end, a batch of testis nuclei was digested with DNase I by the second digestion procedure described in the Methods section. Following removal of the first supernatant (S) by centrifugation, the nuclear pellet was washed

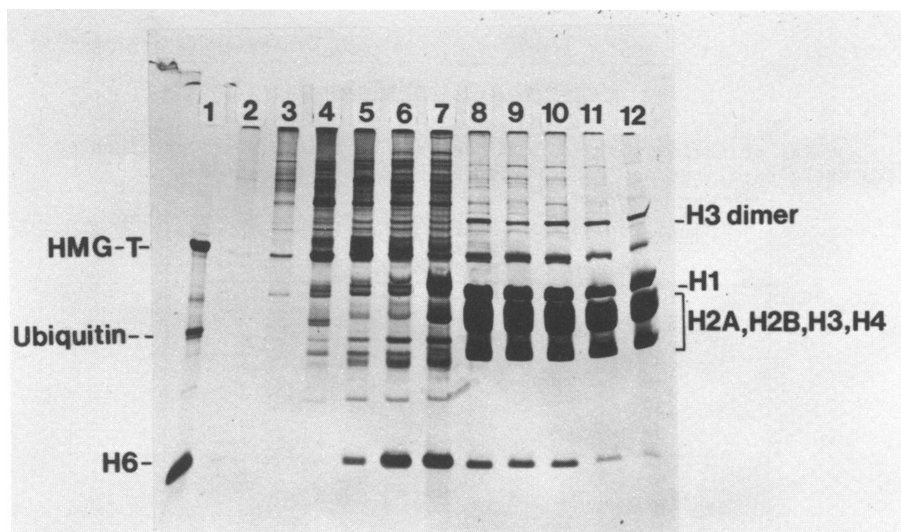

Figure 2. Electrophoretic analysis of the acid soluble proteins derived from DNase I sensitive and resistant fractions. Several identical aliquots of trout testis nuclei were digested with DNase I for different time periods. Acid soluble proteins were obtained and a measured quantity of each sample was electrophoresed as described in Methods. Slot 1 is a mixture of known amounts of purified trout testis HMG-T, ubiquitin and H6, slots 2 and 3 represent the proteins released into the control supernatants S_0 and S_{37} respectively. Slots 4, 5, 6 and 7 represent the proteins released into the supernatant fractions obtained after 5, 10, 30 and 60 minutes of incubation with DNase I. Slot 8 displays the proteins present in the control pellets incubated at 37°C (P_{37}). Slots 9, 10, 11 and 12 represent the proteins present in the pellets remaining after digestion with DNase I for 5, 10, 30 and 60 min., respectively.

successively with solutions of 0.1 M NaCl, 0.2 M NaCl and 1 mM EDTA. We reasoned that these solutions might solubilize additional H6 that might have been released by DNase I from nucleosomes but which failed to dissolve in RSB-Ca^{+2}. Acid soluble proteins extracted from all fractions were analyzed on polyacrylamide gels and the total content of H6 in the various fractions was determined (Table III). In these experiments, in which 7% of the acid soluble material was released into S, we found 19% of H6 associated with this fraction.

Of interest is the observation that H6 in fraction S (and to a lesser degree in $S_{0.1}$ and $S_{0.2}$) shows, in addition to the parental band, a slower running band whose amino acid composition is almost identical to that of the faster running band, except for a slightly higher glycine content (B.L.W.,

TABLE II

DISTRIBUTION OF HMG-T AND H6 AMONG FRACTIONS OBTAINED BY DNase I DIGESTION OF TROUT TESTIS NUCLEI

Samples of nuclei derived from one gram of testis were fractionated to yield these data. H6 was quantitated as described in Methods.

Fraction	μg H6 / g testis	% H6
S_0	0	0
P_0	102	100
S_{37}	0	0
P_{37}	96	100
S_5	1	1
P_5	109	99
S_{10}	7	5
P_{10}	112	95
S_{30}	52	49
P_{30}	54	51
S_{60}	48	59
P_{60}	33	41

unpublished observation). It is possible that the slower band may represent a fraction of the H6 molecules which are modified by poly ADP-ribosylation, since H6 is known to be poly-ADP-ribosylated and poly ADPR is degraded by acid hydrolysis to yield glycine (20). Turning our attention to the salt-eluted fractions it is apparent (Table III) that additional amounts of H6 can be solubilized under conditions in which the nucleosomal histones are not solubilized but remain bound to the residual pellet (not shown). A small fraction of the total protein (< 10%) that was acid extracted from fractions S, $S_{0.1}$, and $S_{0.2}$, precipitated during the dialysis step. These pellets were examined on gels and the amount of H6 in each fraction quantitated. The values obtained from each insoluble pellet were added to the values for H6 in the corresponding soluble fraction of each supernatant, to determine the total amount of H6 released onto each fraction. The cumulative values are listed in Table III. It is clear that substantial quantities of H6 were solubilized by the 0.1 and 0.2 M salt washes. In particular, fraction $S_{0.1}$ contained almost 50% of the H6 content of chromatin. The most straight-forward explanation is that a significant amount of H6, greater than that

present in S was released by DNase I but failed to be solubilized in the RSB-CA^{+2} buffer and was recovered only after the low salt treatment. A cautionary note is pertinent here, since we have shown in control experiments (16) that some H6 can be solubilized by washes of intact nuclei with low salt solutions. Nevertheless, the fraction of total nuclear H6 which is soluble in 0.1 M NaCl and 0.2 M NaCl in controls corresponds to 10% and 20% of the total, respectively (16) while in our DNase I experiments, we observed almost 50% of the H6 solubilized by 0.1 M NaCl, and 16% by 0.2 M salt (Table III). Thus, we conclude that, at least 40% (of the 47.5%) of the H6 solubilized by 0.1 M NaCl, must be derived from DNase I sensitive regions of chromatin. The sum of the amounts of H6 present in S and $S_{0.1}$, is 60%, a value 3 times larger than that obtained when only the first supernatant, S is considered. The real value might be even higher, perhaps up to 100% since free H6 released by nuclease can bind tightly to other chromatin sites and thus appear in the pellet fraction (Kuehl, unpublished data).

DISCUSSION

We have examined the DNase I sensitivity of the protamine genes from trout testis. When trout testis nuclei were incubated with DNase I, to the extent of 5-10% digestion of the input DNA we observed a rapid and preferential degradation of the protamine genes, as assayed by cDNA/DNA reassociation kinetics. The protamine genes code for an abundant set of nuclear proteins.

TABLE III

DISTRIBUTION OF H6 AMONG CHROMATIN FRACTIONS DERIVED BY DNase I ACTION ON TROUT TESTIS NUCLEI

Nuclei from 24 g of testis were digested with DNase I by the second procedure described in Methods. Acid soluble proteins were obtained from each fraction and electrophoresed as illustrated in Figure 2. The amount of H6 associated with each fraction was quantitated as described in Methods.

Fraction	μg H6 / g testis	% H6
S	33.2	19.0
$S_{0.1}$	83.2	47.5
$S_{0.2}$	27.9	15.9
S_{EDTA}	0.5	0.3
P	30.3	17.3

These conclusions extend our earlier studies showing that DNA sequences which are transcribed into cytoplasmic polyadenylated RNA in trout testis, are selectively sensitive to DNase I action (17). The preferential DNase I sensitivity of active genes has been established in several other systems (21,22).

We then applied new methodology to the study of the distribution of the high mobility group protein H6 among DNase I sensitive and resistant chromatin domains. A major fraction of H6 is preferentially released by the limited DNase I action upon trout testis nuclei under conditions where transcribed DNA sequences are selectively degraded.

We previously reported the release of H6 by limited action of DNase I on nuclei from trout testis collected at a later stage of development than those employed in our present studies (8). Release of H6 paralleled the degradation of transcribed DNA sequences (17). These earlier studies were largely qualitative in nature because estimates of the amounts of H6 released were based on visual examination of the intensity of stained bands in starch gels (8). In recent more quantitative work, we have determined that some 75% of the H6 content of chromatin can be found in nucleosomal particles generated by selective micrococcal nuclease action on trout testis nuclei (16). Furthermore, H6 is a fundamental component of a subset of transcriptionally-active nucleosome cores, being present at a concentration of two molecules of H6 per octamer of inner histones in a subcomponent of the 0.1 M salt-soluble mononucleosome fraction released by micrococcal nuclease (10). Thus, the observation that H6 is selectively associated with transcriptionally-active nucleosomes is well substantiated.

Our data supports the conclusions of Weisbrod and Weintraub, who demonstrated that HMG's 14 and 17, proteins analogous to trout H6, are responsible for conferring selective DNase I sensitivity on the globin genes in chicken erythrocyte chromatin (11).

These combined observations suggest that the smaller HMG proteins serve a role in organizing the configuration of active chromatin.

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FOOTNOTES

The abbreviations used are: HMG, high mobility group; RSB, 10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$; EGTA, Ethyleneglycol-bis(β-amino-methylester)-N,N'-tetraacetic acid.

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