Volume 12 Number 11 1984 Nucleic Acids Research

Hyperacetylation of histone H4 promotes chromatin decondensation prior to histone replacement by protamines during spermatogenesis in rainbow trout

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Received 18 October 1983; Revised and Accepted 11 May 1984

ABSTRACT

During the final stages of spermatogenesis in rainbow trout a dramatic increase in the level of histone H4 hyperacetylation is observed which is closely correlated with the replacement of histones by protamines. In order to understand further how H4 hyperacetylation might assist in protamine replacement of the histones, we have investigated the effect of H4 hyperacetylation on chromatin structure in trout testes actively undergoing the replacement process. Long chromatin fragments enriched in hyperacetylated H4 have been isolated and characterized. Evidence is presented that hyperacetylated H4 is clustered in certain regions (domains) of late stage testis chromatin and within these domains the chromatin exhibits an altered, highly relaxed structure which is believed to be the result of the extensive hyperacetylation. These domains, which are nearly devoid of protamine, are postulated to represent an initial structural transition which is necessary for the proper histone removal and protamine replacement process to take place.

INTRODUCTION

Spermatogenesis is the process whereby the male germline cells undergo differentiation to produce mature sperm cells. The final step involves the morphogenesis of haploid spermatid cells into sperm cells, during which time transcription ceases (1-3) and the DNA is condensed into a tightly-packaged, inert configuration (1,4). Condensation is often accompanied by the appearance of a new set of DNA-binding proteins (the protamines) which, in several forms including salmonid fishes and mammals, completely replace the histones (4,5).

It has recently been shown that during spermatogenesis in rainbow trout, spermatid cells show a significant rise in the steady state level of hyperacetylated histone H4 (3). This phenomenon has also been observed during spermatogenesis in the rat (6). In the case of the rainbow trout the proportion of histone H4 molecules which are hyperacetylated (that is, they contain three to four acetylated lysine residues per molecule) approaches half of the total H4 present in the spermatid chromatin and this level of acetylation

apparently persists until removal and replacement with protamines (3). The observed increase in hyperacetylated H4 is clearly not associated with transcription since the amount of transcription is rapidly declining at the time of the onset of hyperacetylation (if anything, a reverse correlation exists) (3). The rise in hyperacetylation is closely correlated with the time of protamine deposition and from this it is postulated that the hyperacetylation may be a necessary step in the transition from histone to protamine-bound DNA.

How might histone acetylation assist in this transition process? Histone hyperacetylation may simply reduce the affinity of the core histones for DNA and permit an easier displacement by protamine. Several previous investigations have tested the effect of random chemical acetylation of histones in the in vitro displacement of histones with protamine (7-9), and although acetylation was found to make displacement slightly more effective, the process was still incomplete and incorrect with regard to the extent and order of histone removal.

Alternatively, numerous investigations have associated histone acetylation with transcriptionally-active chromatin (10), and in this case the acetylation is believed to function by decondensing those regions of chromatin which are to become active, presumably in such a way that they are more accessible to the components of the transcription complex. Since the hyperacetylation in the case of the late stage trout testis is occurring at exactly the same four internal lysine residues in the histone H4 molecules as in the case of the transcription-associated acetylation (11,12), it is reasonable to predict that the effect that acetylation would have on chromatin structure would be the same or similar in both cases. This raises the possibility that the transition from condensed histone-bound DNA to condensed protamine-bound DNA might involve an intermediate state which is decondensed as a result of H4 hyperacetylation.

In this study, the effect that histone H4 hyperacetylation has on the structure of chromatin in late stage trout testis has been investigated. Using a protocol similar to one developed previously in our laboratory (13), but with much briefer nuclease digestion, large chromatin fragments have been generated and fractionated, permitting isolation and characterization of a subset of fragments enriched in hyperacetylated H4.

MATERIALS AND METHODS

Tissue: Naturally-maturing late stage testes were collected from rainbow trout in October, roughly 8-10 weeks after the start of spermatogenesis. The testes

were stored at -80°C until used. The testes were checked for stage of maturation both by phase contrast microscopic examination of the cell types present (the appropriate late stage testes are composed predominantly of spermatids at varying stages of morphogenesis into sperm) and by analysis of histone to protamine ratios. The testes used in this study routinely had a histone/protamine ratio of 40:60.

Nuclear Isolation: Nuclei were isolated essentially according to the procedure previously described in detail (14).

Nuclease Digestion and Chromatin Fractionation: Nuclei from one gram of starting tissue were routinely used in a given experiment. This amount of nuclei contained reproducibly from 900-1000 A_{260} units of DNA. The isolated nuclei were initially washed with 10 ml of RSB buffer plus calcium (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris, pH 7.4, 1 mM $CaCl_2$) and centrifuged at 1000 xG for 5 min. The supernatant was discarded and the pelleted nuclei were redispersed in 2.2 ml of RSB plus calcium giving a final volume of 2.5 ml. This nuclear suspension was preincubated for 2 min at 37°C at which time micrococcal nuclease (Worthington Biochemicals) was added to a final enzyme concentration of 100 units/ml. The incubation was continued for another 2 min, the incubation tube was then shaken in an ice water bath for an additional 2 min and centrifuged at 1000 xG for 5 min. The supernatant (S1) was retained for determination of A_{260} which represents a check of the extent of digestion. S1 never contains chromatin fragments or histones, but only acid-soluble DNA oligonucleotides, which, under the conditions of digestion used, constitute 0.4-0.5% of the total starting A_{260} units. The digested nuclei in the pellet were dispersed in 2 ml of 5 mM EDTA, pH 7.5 for a period of 30 min at 4°C and recentrifuged at 6000 xG for 10 min. The residual nuclear pellet was discarded while the supernatant contained the chromatin fragments generated by the nuclease digestion. This fraction (designated S2) contained 12-15% of the total starting chromatin, an amount equal to 100-150 A_{260} units in 2 ml with a DNA concentration of about 2-3 mg/ml. This concentration is important because the subsequent fractionation with 0.1 M NaCl is not reproducible if the concentration is too high or too low. At a concentration about 10 mg/ml virtually all histone H1-containing chromatin precipitates in 0.1 M NaCl, even that which is hyperacetylated.

Fraction S2 was gently vortexed and 1.0 M NaCl was pipetted in until a final concentration of 0.1 M was obtained. This resulted in the immediate formation of a precipitate, but the sample was incubated at 4°C for 30 min to allow equilibration. The sample was then centrifuged at 6000 xG for 10 min.

The supernatant was carefully removed and constituted the S2-sol subfraction, while the pellet represented the S2-insol subfraction. S2-sol normally contained 25-35% of the S2 chromatin fragments. The pelleted S2-insol fragments were resolubilized, if necessary in 1 mM EDTA, 10 mM Tris, pH 7.2.

Sucrose Gradient Centrifugation: Chromatin samples were loaded onto 15-35% sucrose gradients made in 1 mM EDTA, 10 mM Tris, pH 7.2. The gradients were centrifuged for 18-20 hours at 35,000 RPM in a Beckman SW27.1 rotor at 4°C. Gradients were fractionated using an Isco Gradient Fractionator and the chromatin fragment distribution was monitored by absorption at 260 nm.

Isolation of Protamine and DNA from Chromatin Fractions: Acid-soluble proteins were extracted by addition of 2.0 M H_2SO_4 to a final concentration of 0.2 M. After 30 min the samples were centrifuged and the proteins in the supernatant extract were precipitated by addition of 100% tricholoroacetic acid (TCA) to a final concentration of 20%. Precipitates formed after overnight incubation at 4°C were recovered by centrifugation and washed three times with acetone and dried.

DNA was isolated by initially digesting the chromatin sample with 100 µg/ml Proteinase K (Sigma) at 37°C overnight. Usually 0.1% sodium dodecyl sulfate was also added to the incubation mixture. DNA in the sample was then precipitated by addition of 2 volumes of ethanol, washed at least two times with 70% ethanol and dried.

Electrophoretic Analysis of Protein and DNA: Proteins were analyzed in 15% polyacrylamide acetic acid/urea gels according to the procedure of Panyim and Chalkley (15) as modifed by Kuehl (16). DNA was analyzed in 1% agarose (Bio-Rad) gels according to the procedure of Sharp et al. (17).

Electron Microscopy: A 200 mesh grid with a carbon coated parlodion film was floated on a drop of the sample for 30 seconds, washed in a dilute solution of Photoflo 200 and air dried. The grids were rotary shadowed with platinum: palladium 80:30 and examined in a Hitachi H500H electron microscope operated at 50 KV.

RESULTS AND CONCLUSIONS

Chromatin Containing Hyperactylated Histone H4 is Not Preferentially Digested: Late stage trout testis chromatin fragments were generated, released and fractionated according to the protocol detailed in the Materials and Methods. Histones of the starting nuclei and the subsequent fractions are shown in Figure 1. Quantitation of the relative levels of hyperacetylated H4 in the total starting chromatin, the released fraction S2, and the final

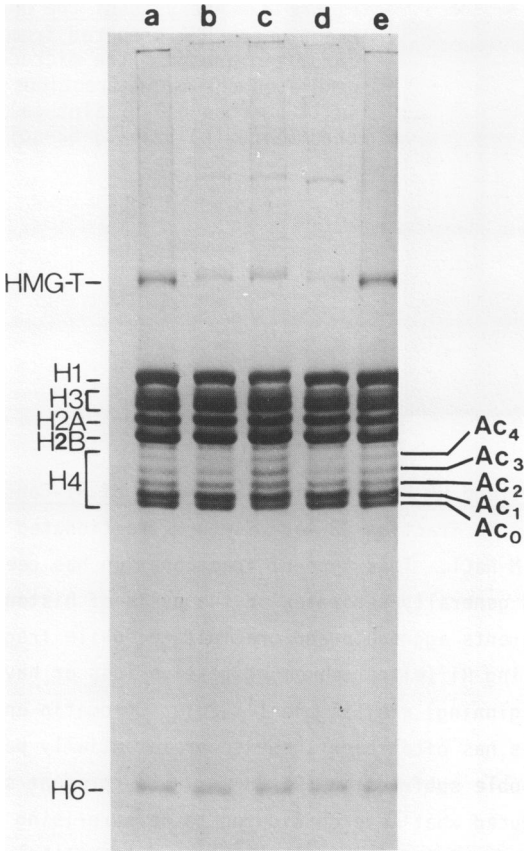

Figure 1. Histones in the Various Chromatin Fractions. Histones prepared as described under methods were electrophoresed in 15% polyacrylamide gels for five hours. Samples were adjusted such that equal amounts of core histones were present in each case, permitting direct comparison of the relative amount of hyperacetylated H4 in each case. The bands corresponding to the acetylated forms of H4 are denoted by the numbers on the right side of the figure and refer to the number of acetylated lysine residues present. Slot (a) total starting chromatin, (b) fraction S2, (c) subfraction S2-sol, (d) subfraction S2-insol, (e) final pellet. HMG-T and H6 are trout high mobility group proteins.

unreleased chromatin revealed no significant differences. Since only about one-sixth of the total starting chromatin is released into S2, it may be assumed that fragments derived from regions preferentially digestible with micrococcal nuclease would be enriched in S2. Such is apparently not the case with chromatin containing hyperacetylated H4 since it is released into S2 to an extent proportional to its overall content in undigested chromatin.

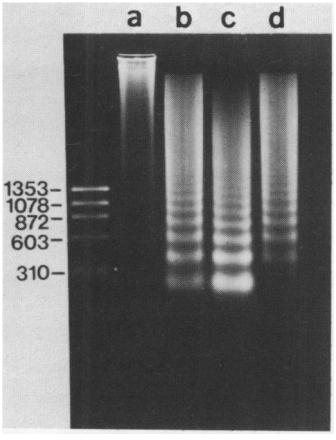

Figure 2. Analysis of the DNA in the Chromatin Fractions. DNA prepared from the total starting chromatin and from the microccal nuclease-generated chromatin fractions was electrophoresed in 1% agarose gels. Slot (a) total starting chromatin, (b) S2, (c) S2-sol, (d) S2-insol.

Hyperacetylated Histone H4 Enhances the Solubility of H1-Containing Chromatin: Chromatin fragments in fraction S2 were further fractionated on the basis of solubility in 0.1 M NaCl. This type of fractionation has been used in many investigations and generally separates on the basis of histone H1 content; H1-containing fragments aggregate and precipitate, while fragments reduced in or totally lacking H1 (either through digestive loss or having been absent from the beginning) are soluble (18-20). Chromatin enriched in acetylated histones has often been shown to preferentially partition into the 0.1 M NaCl-soluble subfraction (21-23). In the present study the fractionation produced what were considered to be surprising results. The subfraction soluble in 0.1 M NaCl (S2-sol), Figure 1C, although it does show a significant enrichment (3-6 fold) in hyperacetylated H4, also contains nearly the same amount of Histone H1 as is present in the S2-insol subfraction (Figure 1D). This finding is consistent however with the presence in S2-sol of a significant proportion of large fragments (greater than 10N in size; see Figure 2C) which might be expected to contain H1. The questions which arise are 1) what is the basis for the solubility difference in these chromatin fragments, if not H1 content, and 2) is the difference in solubility reflecting something meaningful about the regions of intact chromatin from which the fragments were derived?

In order to investigate this further, fragments of S2-sol and S2-insol were centrifuged in sucrose gradients such that the protein content could be analyzed as a function of fragment size. The results are given in Figure 3 and 4 for S2-sol and S2-insol, respectively. The somewhat reduced amount of H1 seen in S2-sol is accounted for by the complete absence of H1 in the

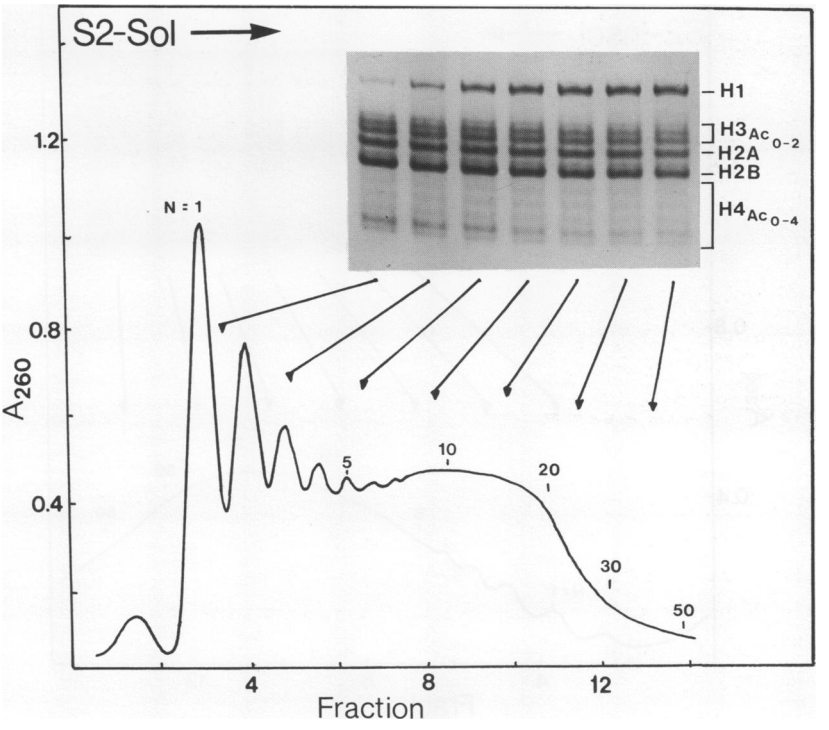

Figure 3. Analysis of Histones of S2-sol Chromatin Fragments Separated on the Basis of Size. S2-sol fragments were centrifuged in a sucrose gradient (see Methods) after which their distribution in the gradient was monitored by absorption at 260 nm. Sizes of the fragments through the gradient are expressed in units of the monomer nucleosome length (N=1) and were determined by analysis of the DNA length present in each fraction. Histones were extracted from selected fractions and are shown in the gel insert. The arrows indicate the region of the gradient from which the corresponding histones were derived.

monomer peak (N=1) and the presence of only half of the stoichiometric amount in the dimer, two thirds in the trimer, etc. Above N=10 the fragments exhibit roughly stoichiometric amounts of H1, the same amount as in S2-insol fragments of equivalent sizes. All the fragments of S2-sol were uniformly enriched in hyperacetylated H4, even those near the bottom of the sucrose gradient whose DNA length was equivalent to a 50N size fragment. The fragments of S2-insol, in contrast, were uniformly depleted in H4 hyperacetylation (Figure 4). These findings suggest that hyperacetylation of H4 is not only correlated with enhanced chromatin solubility, but may constitute the basis for the solubility difference. This is substantiated by the results of analogous chromatin fractionation experiments conducted with late stage

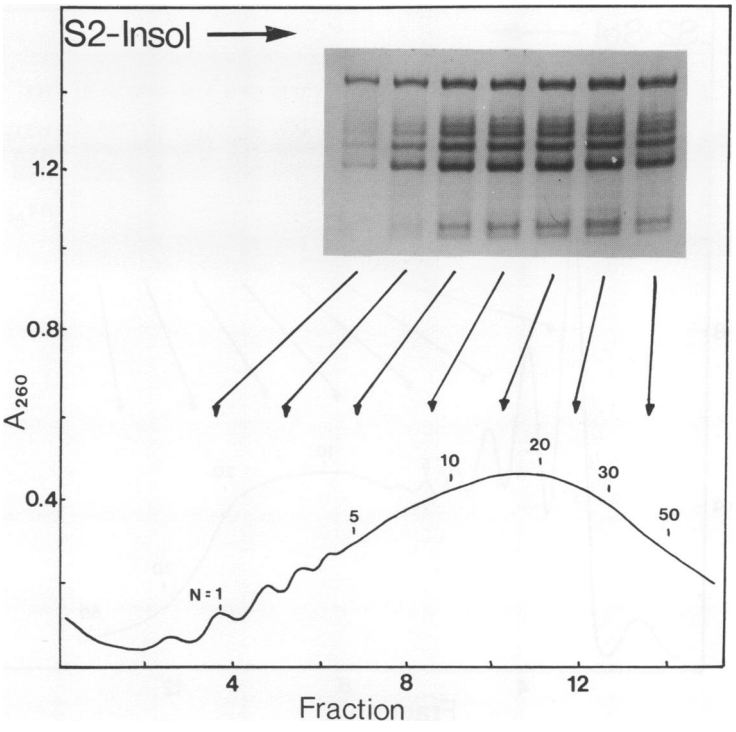

Figure 4. Analysis of the Histones of S2-insol Chromatin Fragments Separated on the Basis of Size. Fragments of the S2-insol subfraction were centrifuged under identical conditions to those used for the separation of S2-sol fragments shown in Figure 3. See the legend of Figure 3 for explanation.

testes from carp (Cyprinus carpio), a fish, in which, during spermatogenesis, the histones are not replaced by protamines, but rather the sperm cell retains somatic type histones (Figure 5A). We have found that the histone H4 in late stage carp testes does not become hyperacetylated as is observed in trout (Figure 5B), and when chromatin fractionation is conducted with carp using the identical protocol to that used with trout, the carp S2-sol subfraction fails to exhibit any fragments greater than 4N in size (Figure 6B) and generally all the S2-sol fragments are depleted in H1 content (Figure 6B). These observations with carp, acting as a negative control, support the conclusion that the enhanced solubility of long H1-containing fragments present in S2-sol derived from late stage trout testes is due to the presence of elevated levels of hyperacetylated H4.

In addition, the carp S2-sol fragments were highly enriched for a

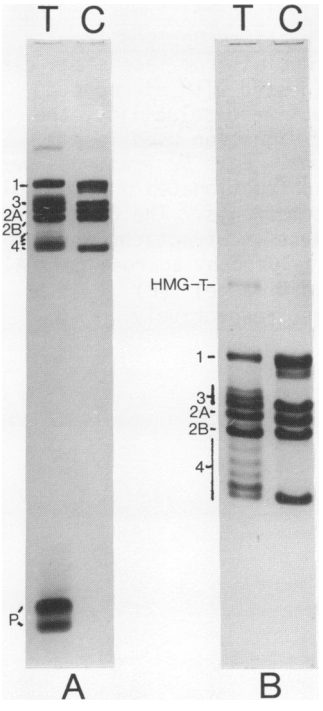

Figure 5. Analysis of the Histones from Late Stage Trout and Carp Testes. Histones were extracted from trout (T) and carp (C) testes at equivalent stages of maturation (middle spermatid stage) and compared electrophoretically in 15% polyacrylamide gels. A. Electrophoresis was conducted for two hours such that the protamines (P) were retained on the gel. B. Electrophoresis was for five hours in order to improve the resolution in the histone region.

protein with the same electrophoretic mobility as trout HMG-T (Figures 5B,6B) Assuming that this protein is in fact carp HMG-T, its association with short S2-sol fragments is consistent with our observations with trout that HMG-T is enriched in the S2-sol fraction as a whole (Figure 1), but upon size separation in sucrose gradients is only enriched in the 1-3 N S2-sol fragment size range (not shown). Our interpretation of these findings is that there is a subset of total chromatin in both trout and carp spermatids which contains HMG-T and is preferentially digested to small H1-depleted fragments during brief micrococcal nuclease digestion. This subset of chromatin is apparently distinct from the hyperacetylated chromatin domains in terms of its digestion and solubility properties since (1) the carp chromatin lacks histone acetylation altogether, and (2) the hyperacetylated chromatin of trout is not preferentially digested (see the Section above).

Hyperacetylated H4 is Clustered in Certain Regions (Domains) of Spermatid Chromatin: The content of hyperacetylated H4 among the various fragment size classes in S2-sol and S2-insol (Figures 3 and 4) provides information about the distribution of hyperacetylated H4 in the intact starting chromatin.

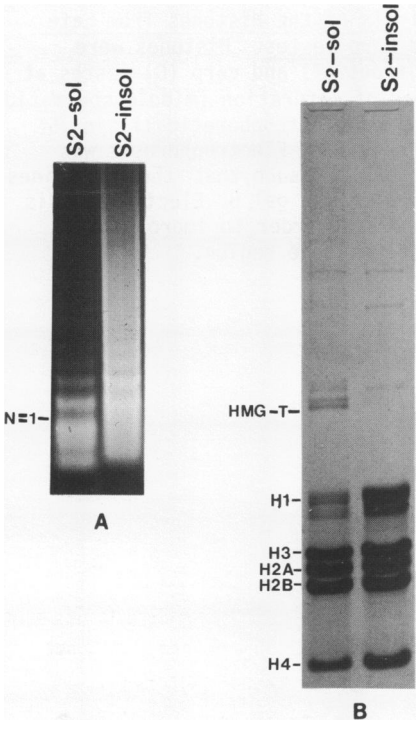

Figure 6. Analysis of histones and DNA in Chromatin Fractions Derived from Carp Testis. Nuclei from late stage carp testes were digested with micrococcal nuclease to 0.5% acid-solubility, the same degree of digestion used in the trout digestions. Carp S2 fragments were released and subfractionated into S2-sol and S2-insol components. The DNA and histones in these subfractions were isolated and analyzed by agarose gel (A) and acetic acid/urea gel (B) electrophoresis, respectively.

The fact that it has been possible to separate a class of long fragments (eq. 10-50N) which are uniformly enriched in hyperacetylated H4 from a second class of fragments of the same size range, but with reduced acetylation of H4, indicates that the distribution of hyperacetylated H4 in intact chromatin of the spermatids is neither uniform nor random. Rather, the hyperacetylation must to a large extent be clustered in certain regions (referred to as domains) of chromatin which are interspersed with other regions in which the level of hyperacetylation is significantly lower. Minimal cleavage with micrococcal nuclease then liberates fragments which are predominantly representative of either one domain or the other and permits subsequent fractionation of the fragments on the basis of differences in the properties of chromatin of the two domains.

It is also possible to comment on the possible lengths of the hyperacetylated domains. If the domains are very long (eg. greater than 1000N) then cleavage to an average fragment length of 15N, as was generally done in this study, should yield fragments which are either uniformly hyperacetylated (derived entirely from within a hyperacetylated domain) or not hyperacetylated

at all (derived entirely from a nonhyperacetylated domain); few fragments would encompass a boundary between the two domains. Assuming that the hyperacetylation is in fact responsible for the solubility of fragments in 0.1 M NaCl (see the preceding section), then a very "clean" fractionation of hyperacetylation into S2-sol would be observed. This degree of separation was never observed in practice, but only an enrichment for hyperacetylated H4 in the S2-sol fragments. That is, the S2-insol fragments always exhibit some measurable level of hyperacetylated H4, a level which was observed to increase with increasing fragment length. We interpret this to mean that the hyperacetylated domains are, on the average, of short enough length that digestion to an average size of 15 N produces a significant proportion of fragments which overlap the two domains. Such fragments would be predicted to partition into the S2-insol subfraction since the nonhyperacetylated portion of the fragment is likely to interact and aggregate with other non-hyperacetylated fragments. The results of the electron microscopic examination of S2-sol and S2-insol fragments (see below) support this explanation.

Hyperacetylated H4-containing Fragments are Reduced in their Potential to Form Higher Order Structure: The results presented thus far indicate that the high content of hyperacetylated H4 found in late stage trout testis spermatid cells is clustered into domains dispersed throughout the intact chromatin. Long fragments derived from these domains, although possessing a normal content of histone H1, are capable of remaining soluble under conditions which normally result in the aggregation and precipitation of bulk nonhyperacetylated fragments. These observations indicate that the hyperacetylated domains may have altered structural features which are reflected in the observed solubility differences of isolated fragments.

In order to determine if major structural differences do exist, chromatin fragments in the total S2 fraction and the S2-sol and S2-insol subfractions were adsorbed onto grids and directly observed with the electron microscope (Figure 7). The fragments released into S2 show two distinct types of fiber morphologies (Figure 7a), one characteristic of nucleosomes in a higher order compact structure and the other characterized by decondensed or relaxed fibers often with reduced nucleosome density or in which there is an absence of nucleosomes altogether. Fractionation of S2 into S2-sol and S2-insol subfractions produced a reasonably clear separation of the two fiber morphologies seen originally in S2. The S2-sol fragments, enriched in H4 hyperacetylation, consist primarily of the relaxed fibers (Figure 7b) with normal nucleosome density, reduced nucleosome density

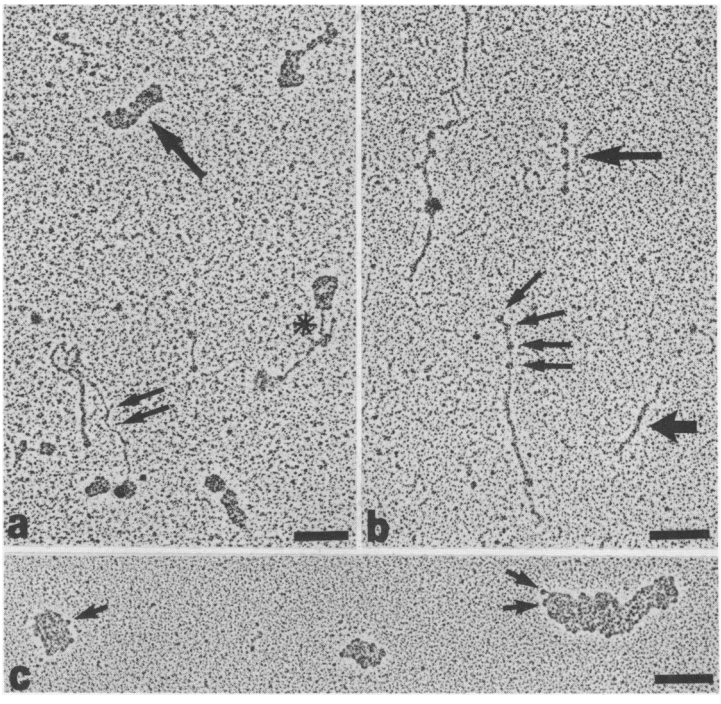

Figure 7. Electron Microscopic Visualization of Trout Chromatin Fragments. Fragments in S2, S2-sol, and S2-insol were usually diluted 30-fold prior to being adsorbed onto girds (see Methods). (a) Fragments of the S2 fraction showing two distinct fiber morphologies, compact higher order structures (single large arrow) and relaxed fibers often with reduced nucleosome density (double small arrows). (b) Fragments in the S2-sol subfraction exhibiting relaxed fibers with normal nucleosome density (single thin arrow), reduced nucleosome density (multiple arrows), or an entirely nucleosome-free appearance (single thick arrow). Dilution of S2-sol fragments was often into lower ionic strength conditions which might induce unfolding, but relaxed fiber morphologies as shown here were also observed at the boundaries of the structures (arrows). In all panels the bar represents a length of 0.1 μm.

(interspersed smooth regions present), or the absence of nucleosomes (entirely smooth fibers). Fragments in S2-insol were generally tightly condensed oligonucleosomes which, if left long enough under conditions of low ionic strength, would unravel into a beads-on-a-string configuration. Some of the tightly condensed structures observed in the S2-insol fraction had relaxed (often smooth) fibers associated with them (similar to the structure denoted by an asterisk in Figure 7a . These structures may represent fragments which contain both hyperacetylated and nonhyperacetylated regions within the same fragment.

These observations support the conclusion, drawn earlier, that fractionation on the basis of 0.1 M NaCl solubility is separating distinct fragment classes derived from at least two distinct domains in the intact chromatin. Furthermore, the results of the electron microscopic examination indicate that fragments derived from the domains enriched in hyperacetylated H4 exhibit a relaxed configuration even under conditions which promote formation of higher order, more compact chromatin structures. It was interesting to find a significant amount of a smooth fiber morphology associated with the hyperacetylated fragments. Whether such a smooth fiber actually exists in vivo or simply is the result of the ready unfolding of certain regions containing unstable nucleosomes during the chromatin fractionation procedure is not known. In either event, however, since such unfolding is not normally observed in chromatin spreads, this would suggest that the smooth regions observed here do have intrinsic differences which favor a beaded to smooth fiber transition.

Electron microscopic observation of carp S2, S2-sol and S2-insol chromatin fractions are shown in Figure 8. The structures confirm what was previously found from analysis of the DNA in each of the fractions (Figure 6A); ie., that the carp S2-sol fraction is devoid of long fragments like those characteristic of the trout S2-sol fraction. It was interesting that the predominantly short S2-sol fragments (1-4N) from carp were often reduced in nucleosome density. This observation, taken together with the dominant smooth fiber configuration of the trout S2-sol fragments, suggest a correlation between reduced nucleosome density and enhanced solubility of chromatin under physiological salt conditions. This morphology may be due to unravelling of nucleosomes; promoted by histone H4 hyperacetylation in the case of trout and by histone H1 depletion or HMG-T enrichment in the case of carp. Unfolding of nucleosomes might lead to a masking of sites which promote higher order chromatin packing and usually give rise to the precipitation of chromatin fragments when exposed to physiological salt conditions.

Hyperacetylation of H4 Occurs Prior to the Binding of Protamine: Mature nucleoprotamine is totally inaccessible to digestion with micrococcal nuclease (24) and as a result the great majority of protamine in the late stage trout testis is found in the residual nuclear pellet after preparation of the S2 chromatin fraction. Chromatin in a transition state from a nucleohistone to a nucleoprotamine organization might be expected to contain some protamine, but still be digestible with nucleases. This is indeed the case as a small

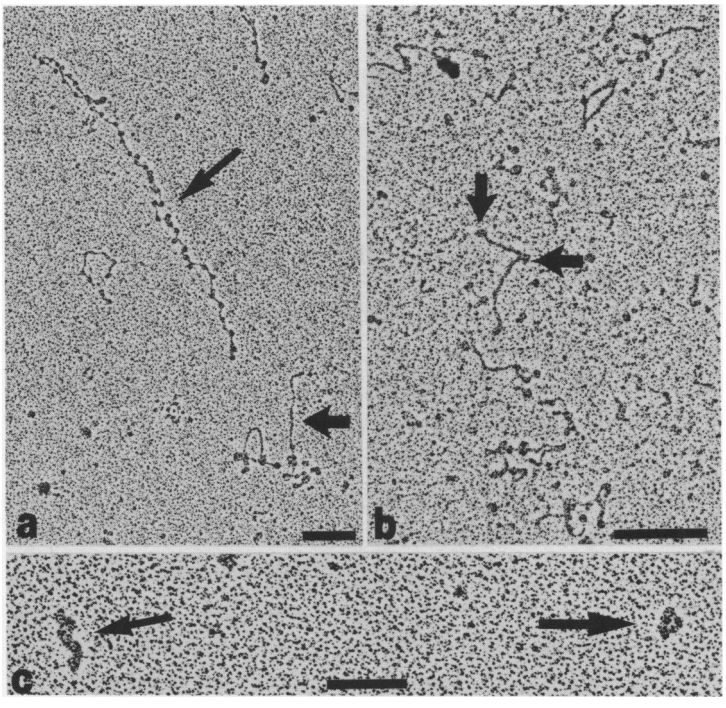

Figure 8. Electron Micrograph Illustrating the Chromatin Morphology Obtained from Carp Chromatin Fractions. (a) S2 fraction containing fragments which display a regular beaded morphology (large arrows) and fragments with a reduced nucleosomal density (small arrows), (b) S2-sol fraction containing a large population of small fragments with a reduced nucleosome density (arrows indicate nucleosomes), and (c) S2-insol fraction containing nucleosome clusters (arrows). Bar = 0.1 μm.

amount of protamine is always observed in the S2 fraction. Upon fractionation in 0.1 M NaCl the protamine is found in S2-insol, while the S2-sol fragments contain no detectable protamine unless the gels are overloaded in which case a trace amount is observed. This indicates that the hyperacetylation of H4 appears in a given region of chromatin prior to any significant binding of protamine. Assuming that the hyperacetylation of H4 has a function in the process of histone removal and protamine deposition, this would suggest that chromatin decondensation, brought about by H4 hyperacetylation, is a prerequisite for protamine deposition.

DISCUSSION

The exchange of protamine for histones during the final stages of spermatogenesis involves a mechanism which is not understood at this time. Attempts to mimic the exchange *in vitro* by adding protamines to histone-bound chromatin under various conditions have generally failed (7-9, 25). Histone removal is incomplete and the order of removal of each class of histone is not the same as that observed *in vivo* (3,4). Furthermore, no one has ever demonstrated the formation *in vitro* of a condensed nucleoprotamine structure like that found in the head of a sperm. The *in vitro* studies have been conducted under the assumption that the mechanism of exchange might be a simple electrostatic competition process in which the protamines, by virtue of their small size and high basicity (they have stronger affinity for DNA than do the histones) displace the histones competitively. The apparent order of histone removal *in vivo*, however, suggests that the mechanism is more complex (eg. histone H1, the least tightly bound, is the last to be removed while H4, the most tightly bound core histone, appears to be the first removed).

We favor a sequential, regulated mechanism involving several steps which are necessary for histones to be adequately removed under conditions of physiological ionic strength and for the DNA to be repackaged into the correct nucleoprotamine configuration. Evidence from our own as well as other laboratories indicate that histone acetylation, especially the hyper-acetylation of H4 (and possibly also H3), constitutes one of the steps (3,6,26-28) in this mechanism. The data presented in this paper support a model in which elevated levels of hyperacetylated H4 induce a localized decondensation of chromatin which occurs prior to and may be required for the binding of protamine. Binding of protamine would be followed very shortly by complete histone displacement (possibly of hyperacetylated histone H4 first) as additional protamine molecules bind. At some time during or subsequent to the actual exchange process the chromatin would begin recon-densation into the nucleoprotamine configuration. This final step may also be accompanied by the dephosphorylation of the protamines which are phosphory-lated at the time of initial binding to DNA (29).

The elevated level of H4 acetylation observed during trout spermiogenesis could be controlled in a number of different ways. One way would be through an increase in the level of a specific histone acetylase or the activity of a pre-existing acetylase. A second possibility would be that the chromatin is initially altered in its conformation (by some other process besides acetylation) such that

the histone H4 molecules become more accessible for acetylation. Hyperacetylation of H4 might then lead to further deconsendation of the chromatin. A third possibility would be a decrease in the rate of deacetylation through a decrease in deacetylase activity. This latter possibility is supported by the previous demonstration by Candido and Dixon (30) that the deacetylase activity of late stage testes (90% spermatid cells) is very low, evidenced by the lack of turnover of ^{14}C -acetate incorporated *in vivo* in acetylated histone molecules. Consistent also with this is the observation that artificial blockage of deacetylase activity using sodium butyrate induces the formation of hyperacetylated stretches of chromatin in HeLa cells (31,32) which exhibit solubility properties quite similar to those described here from trout testis spermatids without the use of sodium butyrate treatment. In those studies (31,32) the hyperacetylated chromatin was found to be no more sensitive to micrococcal nuclease than the bulk chromatin. Also, long fragments enriched in hyperacetylated H4 were shown to be soluble at physiological ionic strength even in the presence of normal amounts of histone H1. Perry and Chalkley (32) have proposed that histone acetylation, followed by rapid deacetylation, occurs in a sequential manner throughout all the chromatin bringing about a transient decondensation which may function to provide for continuous repair and/or an opportunity for binding of regulatory proteins. A natural decline in deacetylase activity during spermiogenesis in trout could permit such a sequential acetylation to procede to an even greater degree, possibly leading to more extensive decondensation, as a prerequisite for protamine binding and eventual histone displacement.

Recently, Bode *et al.* (34) have examined the effect of increasing levels of histone acetylation on the stability of isolated nucleosomes. They propose that there is a critical level of histone acetylation within a given nucleosome above which the nucleosomes can potentially unravel or open up. They suggest, as this critical level, 10 acetylated residues per nucleosome. We have estimated a total number of 8-10 acetylated lysine residues per nucleosome in the hyperacetylated chromatin from spermatid cells. This must be considered a lower limit since the "hyperacetylated" chromatin is contaminated to some degree with non-hyperacetylated chromatin. Since the level of H4 acetylation in the hyperacetylated chromatin represents an increase equivalent to 3-4 acetylated lysine residues/nucleosome relative to non-hyperacetylated chromatin, this would suggest that hyperacetylation of H4 alone could shift the average number of acetylated lysine residues/nucleosome from a value well below 10 to a value near 10. Although these figures must be taken with caution since they only represent estimates, the idea of such an acetylation-related structural transition in chromatin is consistent with our results and our proposal that histone H4 hyperacetylation is a key early step in the replacement of histones with protamines.

ACKNOWLEDGEMENTS

The research presented in this paper was supported by a National Research Service Award (#GM07147) from the National Institutes of Health and more recently an Alberta Heritage Foundation for Medical Research Fellowship to M.E. Christensen, by a grant from the Alberta Heritage Foundation for Medical Research to J.B. Rattner and a grant from the Medical Research Council of Canada to G.H. Dixon.

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