Chromatin Reorganization during Spermatogenesis in the Winter Flounder*

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During spermatogenesis in the winter flounder, the average repeat length of nucleosomal DNA in the testis increases from 195 ± 2 base pairs in prespermatid nuclei to 222 ± 3 base pairs in sperm. This increase in repeat length apparently occurs in the linker region since there is no change in the pattern of DNA fragments produced during micrococcal nuclease digestion of the nucleosome core. The timing of the increase coincides with the loss of phosphate from the high molecular weight basic nuclear proteins and histones H2A and H4.

When prespermatid nuclei are digested with micrococcal nuclease to the point where 10% of the DNA is acid-soluble, mononucleosomes and higher oligomers are readily released. However, when sperm chromatin is digested to the same extent, these products are no longer soluble and only traces of H1 and small DNA fragments are released. This situation is not changed in sperm chromatin that has been depleted of H1 by extraction with 0.4 M NaCl. However, if nuclease-treated sperm chromatin is lightly digested with trypsin, mono- and oligonucleosomes are released. At this level of proteolysis, the high molecular weight basic nuclear proteins are completely broken down, but the core histones are largely intact. These data are consistent with a model in which the unphosphorylated high molecular weight basic nuclear proteins function in cross-linking nucleosomes together within the sperm nucleus.

Chromatin condensation in terminally differentiating tissues is usually associated with changes in basic nuclear proteins (1). Perhaps the most radical change involves the complete replacement of histones by protamines that takes place during spermatogenesis in many organisms including mammals, birds, and various species of fish. During this replacement, the nucleosome structure (2, 3) common to somatic cells is abolished (4). A less radical transition in chromatin structure occurs during spermatogenesis in the sea urchin. Their sperm have a nucleosomal DNA repeat length of 250 bp compared to 222 bp for somatic cells (5), and they contain, as histone variants, a more basic H1 (6) and an H2B which is both longer and more basic than its somatic counterpart (6, 7). It has been suggested that these sperm-specific histones might be responsible for the increase in nucleosomal repeat length (8). An increase in nucleosome DNA repeat length was also observed during erythropoiesis in the chicken. In this system, the increase (from 190–212 bp) is gradual and coincides with the deposition of histone H5 (9).

Recently, we described a new group of proteins that might be associated with chromatin condensation. They are the HM, BNPs that appear late in spermatogenesis in the testes of the winter flounder (10). These proteins have an average molecular weight of 110,000 and an amino acid composition in which arginine, lysine, serine, and proline together make up three-quarters of the total residues. Approximately 80% of their serine residues (22 mol%) are phosphorylated when these proteins are first detected in the nucleus (11). The HM, BNPs are later dephosphorylated and eventually may make up 25% of the acid-soluble protein in mature sperm nuclei.

In this paper, we report on the reorganization of chromatin that accompanies the deposition of HM, BNPs during development of winter flounder sperm.

EXPERIMENTAL PROCEDURES

Tissue—Winter flounder testes were collected at the Marine Sciences Research Laboratory, St. John’s, Newfoundland, as previously described (10).

Preparation of Nuclei—Nuclei prepared from flounder testis and calf thymus (10) were washed three times by suspension and centrifugation at 9000 × g for 10 min in 0.22 M sucrose containing 3 mM MgCl₂, 10 mM Tris/HCl (pH 8.0), and 0.5 mM PMSF. The DNA concentration of this nuclear suspension was determined from the absorbance at 260 nm of an aliquot (0.5 ml) homogenized in 4.5 ml of 1 mM EDTA (pH 8.0) by addition of 1/10 volume of 0.2 M EDTA (pH 8.0). The degree of digestion was determined by measuring the amount of DNA rendered acid-soluble (13).

Extraction of DNA—Micrococcal nuclease-treated nuclei were centrifuged at 10,000 × g for 10 min and re-extracted with 1 volume of 1 mM EDTA (pH 8.0), 0.5 mM PMSF. DNA was extracted from the combined supernatants or from unfractionated nuclei by digestion with micrococcal nuclease (100 units/ml, Millipore) at 24 or 37 °C. At various times, aliquots were removed and the reaction stopped at 0 °C by adding 1/10 volume of 0.2 M EDTA (pH 8.0). The degree of digestion was determined by measuring the amount of DNA rendered acid-soluble (13).

Extraction of Proteins—Acid-soluble proteins were extracted from nuclear preparations with 0.2 M H₃PO₄, for at least 1 h at 4 °C with intermittent stirring. DNA was removed by centrifugation at 17,000 × g for 10 min. Proteins were precipitated from the supernatant at 0 °C by the addition of 100% trichloroacetic acid to give a final concentration of 25% trichloroacetic acid and were recovered after 2-3 h by centrifugation at 17,000 × g for 10 min. The pellet was washed with 95% ethanol containing 2% H₂SO₄, dried under nitrogen,
and dissolved in H₂O. The protein concentration of this solution was determined by the method of Lowry et al. (14) using calf thymus histones as a standard.

Digestion of Nuclei with Trypsin—January testis nuclei were suspended in 5 mM Tris/HCl (pH 8.0) containing 1 mM EDTA at a concentration of 20 2A₀₀₅ units/ml and were digested with trypsin (1 mg/ml, Sigma) at 24 °C with agitation. Aliquots (1-2 ml) were removed at time intervals and treated with sulfuric acid to a final concentration of 0.2 M to stop the reaction and to extract the acid-soluble proteins as described above.

Some January nuclei preparations were treated with trypsin after digestion with micrococcal nuclease. These trypsin digestions were performed in micrococal nuclease digestion buffer after stopping the nuclease reaction by the addition of EDTA to 20 mM. The same digestion conditions were used as described above, but the reaction was stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor (Sigma) and PMSF to 0.5 mM. Samples prepared in this way were analyzed on 10-30% sucrose gradients as described below.

Gel Electrophoresis—Proteins were analyzed on acetic acid-urea 15% polyacrylamide slab gels (15) containing 6.25% urea prepared and run as previously described (10). Prior to electrophoresis, samples were fully reduced as described by Knefel (16) unless already carboxymethylated. Gels were stained for 2 h in a mixture of 0.1% Coomassie blue and 0.07% Amido black in 7.5% acetic acid and 15% ethanol and destained in the same solvent. DNA was analyzed on 1.5% agarose gels (0.3 X 13 X 16 cm) (pH 6.3). Analyses of micrococcal nuclease limit digests were done on 10% polyacrylamide gels. After electrophoresis, the gels were stained with ethidium bromide (2 μg/ml) in water for 1-2 h and photographed under UV light.

Nucleosome Repeat Length—Agarose gels were calibrated using DNA fragments from HincII and HpaII digests of plasmid pBR322. Negatives of the ethidium bromide-stained gels were then scanned either with a Joyce-Loebl densitometer or a Beckman LS spectro-photometer to obtain the midpoints of the DNA bands in each micrococcal nuclease digest. The repeat length was determined from the slope of the line obtained from linear regression analysis of a plot of DNA length versus band number (17).

Sucrose Density Gradients—Micrococcal nuclease digestes were analyzed on linear 10–30% sucrose gradients (11) or 34 ml containing 10 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 1.0 mM cacodylic acid, and 0.5 mM PMSF, freshly added. Centrifugation was for 20 h at 27,000 rpm in an SW 41 rotor. Gradients were harvested from the bottom and their absorbance profiles at 254 nm were recorded. Fractions containing the mononucleosome peak were pooled, as were those fractions containing higher oligomers. Acid-soluble proteins were extracted from each sample and from the pellet as described above.

Amino Acid Analysis—Amino acid analyses were done as described previously (10). No corrections were made for losses during hydrolysis.

RESULTS

Four testis samples, representing different stages of spermatogenesis, were used throughout this study. These were from early October, at which time some nuclei do not contain any HM, BNP's, late October, at which time the nuclei contain the highly phosphorylated form of these proteins; late November, at which time the nuclei contain HM, BNP's that are largely dephosphorylated; and January, at which time the nuclei (spermat) contain unphosphorylated HM, BNP's (11).

Rate of Micrococcal Nuclease Digestion—In Fig. 1, the course of micrococcal nuclease digestion has been determined for each of the testis nuclei by measuring the release of acid-soluble material. Both early and late October nuclei are digested readily and at similar rates, even though the latter nuclei contain highly phosphorylated HM, BNP's, which are not present in the former. In late November nuclei, the rate of digestion is noticeably slower and is further decreased in the January sperm nuclei.

Average Nucleosome Repeat Length—The DNA from micrococcal nuclease-digested testis nuclei was analyzed on 1.5% agarose gels (Fig. 2) to determine if there was any change in the nucleosomal DNA repeat length during testis development. In these gels, there is an obvious phase difference

FIG. 1. Rate of micrococcal nuclease digestion of testis nuclei at different stages of development. The degree of digestion is represented by the per cent of DNA that is acid-soluble in cold 1 M HClO₄ and 1 mM NaCl. The digestion was performed as described under “Experimental Procedures” at 24 °C with 5 units of enzyme/2A₀₀₅ unit. The testis samples used were from early October (C), late October (A), late November (V), and late January (J).
between the DNA patterns from early October and January. A densitometer tracing of the negative from a similar gel (Fig. 3) shows that the hexamer in January is approximately the same length as the heptamer in October. The gels were calibrated with restriction fragments of known length. The procedure used for the determination of the average nucleo-

![Densitometry tracing](image)

**Fig. 3.** Densitometry tracing of DNA from micrococcal nuclease-digested testis nuclei. DNA was prepared from early October and late January testes nuclei that had been digested with micrococcal nuclease at 24 °C for 5 and 10 min, respectively. The percent acid solubilities were 5% for the October sample and 3% for the January sample. The DNA was electrophoresed as described in the legend to Fig. 2. Densitometry was performed on the negative of the ethidium bromide-stained gel using a Joyce-Loebel densitometer. The bottom tracing represents the HindIII fragments of plasmid pBR322 as described in the legend to Fig. 2, and O represents the origin of the gel.

**Table I**

Length of DNA in mono- and oligonucleosomes

The size of each DNA fragment was determined from photographs of gels such as the one in Fig. 2. Densitometry was performed on the negatives using a Beckman U5 spectrophotometer programmed for molecular weight determinations. The values shown below are from the gel in which molecular weights could be determined for the largest number of oligomers. The average nucleosome repeat length was obtained from the slope of the line, band size versus band number, by linear regression analysis. The error shown is the standard error of the slope.

<table>
<thead>
<tr>
<th>Band</th>
<th>Flounder testis</th>
<th>Calf thymus</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Early October</td>
<td>January</td>
</tr>
<tr>
<td>2</td>
<td>389</td>
<td>444</td>
</tr>
<tr>
<td>3</td>
<td>565</td>
<td>655</td>
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<tr>
<td>4</td>
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<tr>
<td>7</td>
<td>1349</td>
<td>1359</td>
</tr>
<tr>
<td>8</td>
<td>1547</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>195 ± 2</td>
<td>222 ± 3</td>
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</table>

**Fig. 4.** Polyacrylamide gel electrophoresis of DNA fragments from limit micrococcal nuclease digests of testis and calf thymus nuclei. Lane 1, *HindIII* digest of plasmid pBR322 (38 g); lanes 2, 3, 4, and 5, DNA (6 μg) from early October, late October, late November, and January testes, respectively; lane 6, calf thymus DNA (6 μg). Electrophoresis was at 22 °C and pH 8.3 under non-denaturing conditions on a 10% polyacrylamide gel.

some repeat length is described in the legend to Table I. This method ensures that the lengths determined are not affected by differences that might occur from one sample to another in the extent of digestion at the ends of the fragments (17). The average nucleosome repeat length (Table I) was 195 ± 2 bp in early October chromatin, 222 ± 3 bp in sperm chromatin, and 195 ± 2 bp in calf thymus chromatin. This 14% increase in the average nucleosome repeat length occurs prior to late November (Fig. 2). When testes that were slightly more mature than the late October samples used in Fig. 2 were analyzed in this manner, the ladder pattern was consistently blurred such that a definite nucleosomal repeat length could not be determined (not shown).

**Analysis of Core Particle DNA**—It is believed that subnucleosomal DNA fragments generated by micrococcal nuclease digestion result from specific cleavage of the core particle (18). Therefore, a comparison was made of the subnucleosomal DNA fragments from October, November, and January testis nuclei to see if a reorganization of the nucleosome core was contributing to the increased nucleosomal DNA repeat length. As shown in Fig. 4, all samples have similar sized submonomer DNA fragments and show a marked absence of staining in the 76-bp region.

**Histone H1**—It has been suggested that histone H1 might control the length of the linker DNA between nucleosomes (17, 19). When the amino acid compositions of testis H1 before and after the change in nucleosomal repeat length were de-
termed, no significant difference in the two compositions was observed (not shown). This agrees with the observation that the mobility of H1 on acid-urea gels does not change throughout spermatogenesis (10).

**Digestion of Sperm Nuclei with Trypsin**—Sperm nuclei were digested with trypsin for various periods of time, after which their acid-soluble nuclear proteins were analyzed on acid-urea gels as shown in Fig. 5. The HM, BNP's and histone H1 appear to be the most susceptible to trypsin digestion. At this trypsin concentration, nearly all the HM, BNP's have been cleaved within 1 h, whereas comparable digestion of H1 is attained after 3 h. The core histones are less susceptible to digestion by trypsin, although H3 and H4 are cleaved more readily than H2A and H2B. In chick erythrocyte nuclei, the same order of digestion of the histones by trypsin has been reported (30).

**Release of Nucleosomes from Testis Nuclei**—When nuclei from early October testis were digested to 10% aci solubility with micrococcal nuclease, approximately 70% of the chromatin had been released into the combined supernatants (10,000 × g) by the end of the EDTA wash described under

![Fig. 6](https://www.jbc.org/)

**Fig. 6.** Proteins recovered in the fractionation of early October and January testis nuclei digested to 10% acid solubility with micrococcal nuclease. Lanes 1-5, acid-soluble proteins from nuclease-digested January testis nuclei. Lane 1, proteins recovered from the initial supernatant (10,000 × g) of the nuclease-treated nuclei plus the subsequent 1×10^6 M EDTA wash (10 μg). Lane 2, proteins in the 0.4 M NaCl extract of nuclease-treated January testis nuclei (20 μg). Lane 3, proteins in the pellet from the sucrose gradient in Fig. 9B in which the H1-depleted chromatin was not treated with trypsin (30 μg). Lane 4, proteins in the pellet from the sucrose gradient in Fig. 9B in which the H1-depleted chromatin was treated with trypsin (30 μg). Lane 5, standard proteins from January testis (30 μg). Lanes 6 and 7, acid-soluble proteins (30 μg) recovered in the fractionation of nuclease-digested early October nuclei into supernant and pellet, respectively. Lane 8, standard acid-soluble nuclear proteins from early October testis (30 μg). HMG-T, high mobility group T; HMW BNP, high molecular weight basic nuclear protein.

“Experimental Procedures.” This figure was not influenced by the presence of PMSF. The proteins in the supernatant (Fig. 6, lane 6) are representative of those present in whole chromatin of October testis (lane 8) and in the pellet (lane 7), although somewhat enriched in the high mobility group proteins T and H6. When nuclei from sperm were treated in the same fashion, little A260-absorbing material was released in the presence of PMSF, and the only protein detected in the supernatant fraction was H1 (Fig. 6, lane 1). This observation was extended when the two chromatin digests were layered onto 10–30% sucrose gradients and their sedimentation profiles were obtained (Fig. 7). Early October nuclei readily released mono- and oligonucleosomes into the gradient (Fig. 7A). No such products were solubilized from sperm nuclei (Fig. 7B, 0'). Both of the digested nuclei samples released small fragments of DNA, which are seen at the top of the gradients (Figs. 7, A and B). However, as the micrococcal nuclease-digested sperm nuclei were treated with trypsin (1 μg/ml) for increasing amounts of time, mono- and oligonucleosomes were gradually released (Fig. 7B, 5', 10', and 15').

The proteins in the monomer, oligomer, and pellet fractions were recovered from one set of sucrose gradients that were identical with the 10' set in Fig. 7B and were analyzed on an acid-urea gel (Fig. 8). No intact HM, BNP's appear in the monomer, oligomer, or pellet fractions (Fig. 8) after trypsin
Chromatin Reorganization during Spermatogenesis

Fig. 7. Sucrose gradient profiles of early October and January nuclei digested to 10% acid solubility with micrococcal nuclease. A, early October testis nuclei layered directly onto a 10-30% linear sucrose gradient (11 ml) after micrococcal nuclease digestion. B, January testis nuclei layered directly onto similar gradients after micrococcal nuclease digestion (0') or after 5 min (5'), 10 min (10'), and 15 min (15') of treatment with trypsin as described under "Experimental Procedures." 5', for example, 5 min.

Fig. 8. The protein content of gradient fractions derived from micrococcal nuclease- and trypsin-treated sperm nuclei. January testis nuclei were treated first with micrococcal nuclease and then with trypsin for 10 min and were fractionated on sucrose gradients as in Fig. 7. Acid-soluble proteins were recovered from the mononucleosome (lane 2), oligonucleosome (lane 3), and pellet (lane 4) fractions of the gradient and analyzed on an acetic acid-urea gel. Electrophoresis was for 30 h at 200 V. The standard (lane 1) contained acid-soluble protein from undigested January testis nuclei (60 μg). Other samples contained approximately 40 μg of protein.

Fig. 9. The effect of histone H1 depletion on the release of nucleosomes from sperm nuclei. Micrococcal nuclease-treated nuclei from calf thymus (A) and January flounder testis (B) were fractionated on 10-30% linear sucrose gradients (11 ml) as described under "Experimental Procedures." In B, January testis nuclei were first treated with micrococcal nuclease to 10% acid solubility, depleted of H1 by extraction with 0.4 M NaCl, and then either loaded directly onto the gradient (lower tracing) or first digested for 10 min with trypsin (upper tracing). The positions of RNA markers after sedimentation in identical gradients are shown in A.

digestion, and the pellet contains only a trace of H1. In all three fractions, there are a series of faint bands that migrate midway between the usual migration positions of the HM, BNP's and H1. These bands might be digestion products originating from the HM, BNP's or, possibly, less basic portions of histones, which migrate more slowly in this gel system.

H1-depleted Sperm Chromatin—Histone H1 can be removed from flounder sperm nuclei by 0.4 M NaCl, whereas the HM, BNP's are only removed by salt concentrations of at least 1 M (11). This property of the basic nuclear proteins made it possible to test the contribution of H1 to the cross-linking of sperm chromatin.

Micrococcal nuclease-treated sperm nuclei were washed with 1 mM EDTA, followed by 0.4 M NaCl. These treatments removed the bulk of H1 and some H2A and H2B (Fig. 6, lanes 1 and 2). This H1-depleted chromatin (Fig. 6, lane 3) was divided into halves. One-half was loaded without further treatment onto a 10-30% linear sucrose gradient, while the other half was first digested with trypsin. Nucleosomes and higher oligomers were released from the trypsin-treated sample but not from the other (Fig. 9). The smaller DNA fragments that appeared at the top of the gradients in Fig. 7 and in the calf thymus standard in Fig. 9 have been mostly removed during the washings of the sperm chromatins with EDTA and 0.4 M NaCl (Fig. 9B). In this way, it was demonstrated that H1 is not required for the cross-linking of nucleosomes in flounder sperm, whereas in nuclease-digested sea urchin sperm, release of nucleosomes is achieved by the removal of H1 (21).

Discussion

The nucleosome is retained as the major structural element of chromatin throughout spermatogenesis in the winter flounder. This was predicted from the observations that somatic-type histones were retained in the sperm in the same proportions as found earlier in spermatogenesis (10) and is confirmed here by micrococcal nuclease digestion. Previously, there was no indication of how the HM, BNP's interacted with the sperm chromatin. They appeared to be DNA-binding proteins from their composition (10) and strength of binding to chromatin...
Two contrasting modes of interaction were considered. In one, the HM, BNPs interacted uniformly with the chromatin and were added on top of the existing nucleosomal structure. In the other, the HM, BNPs replaced a percentage of the nucleosomes in the manner of the nucleohistone to nucleoprotamine transition that occurs in developing trout testes (4). Two observations reported here support a uniform mode of interaction. First, the nucleosomal DNA repeat length is increased throughout most, if not all, of the chromatin. Second, there is no release of nucleosomes from sperm chromatin after micrococcal nuclease has cleaved the DNA linking them together, although release can be subsequently effected by a trypsin treatment that fragments the HM, BNPs.

Since the nucleosomal core structure is apparently unchanged (Fig. 4), the increase in nucleosomal DNA repeat length probably occurs in the linker region, as it does in sea urchin sperm and condensed chicken erythrocyte nuclei. In the latter two systems, the increase in DNA length is associated with additional DNA-binding capacity. In sea urchin sperm, this takes the form of an H1 that is considerably more basic than the somatic H1s and a basic NH2-terminal extension on H2B. The condensed erythrocyte nucleus contains an additional H1-like histone, H5 (22, 23). However, it has not been proven that this histone is responsible for an increase in repeat length (24). In winter flounder sperm, the H1 histones are not significantly different in their amino acid composition from those present in immature testis nuclei prior to the increase in nucleosomal repeat length. However, additional DNA-binding capacity is present in the HM, BNPs.

The average molecular weight of the HM, BNPs is approximately 110,000 (10) and is comparable to the combined molecular weights of the octomer of core histones. However, on the basis of densitometry, the HM, BNPs make up only 20–25% of the acid-soluble proteins in sperm nuclei (10), the remainder being histones. On this basis, there are not enough HM, BNPs to be present in stoichiometric amounts on the nucleosomes, and a uniform interaction with the chromatin could only be achieved if each of these proteins interacted with 3–4 or more nucleosomes. Such an interaction with several nucleosomes would effectively cross-link the chromatin and explain why micrococcal nuclease digestion alone fails to release mono- and oligonucleosomes.

Another consequence of the reorganization of testis chromatin as described here would be a complete change in nucleosome phasing along the DNA. There is increasing evidence that the positioning of nucleosomes along the DNA is not random (25), but may be defined by DNA sequence. Such signals for the positioning of nucleosomes are presumably overridden in the chromatin of terminally differentiated cells and could conceivably help repress the expression of their genes. By the same token, we have noticed that flounder sperm chromatin is digested more slowly by micrococcal nuclease than prespermatid chromatin. This might simply be due to the extreme condensation of the chromatin at this stage and/or the binding of HM, BNPs to linker DNA. However, it could also be due to a redistribution of preferred micrococcal nuclease digestion sites of the kind described by Keene and Elgin (25) between core and linker regions.

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