Testis-specific Linker Histone H1t Is Multiply Phosphorylated during Spermatogenesis

IDENTIFICATION OF PHOSPHORYLATION SITES*

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During normal spermatogenesis, the testis-specific linker histone H1t appears at pachytene stage becomes phosphorylated in early spermatids and disappears in late spermatids. Using reversed-phase and hydrophilic interaction liquid chromatography, H1t from rat and mouse testes was isolated, subjected to enzymatic digestion, and analyzed by mass spectrometry. We observed different phosphorylated states of H1t (mono-, di-, and triphosphorylated) as well as the unphosphorylated protein. Tandem mass spectrometry and immobilized metal ion affinity chromatography experiments with MS/MS/MS and multistage activation were utilized to identify five phosphorylation sites on H1t from rats. Phosphorylation occurs on both serine and threonine residues, whereas only two of these sites were located on peptides containing the CDK consensus motif (S/T)P.XZ. Rat H1t phosphorylation starts first by phosphorylation of the non-consensus motif SPKS in the COOH-terminal domain, namely at Ser-140 and to a smaller degree at a further nonconsensus motif at Ser-186. This is followed by phosphorylation of Ser-177 and Thr-155, both located in CDK consensus motifs. A single phosphorylation site at Ser-8 in the NH2-terminal tail was also found. Mouse H1t lacks Ser-186 and is phosphorylated at up to four sites. In contrast to somatic linker histones, no strict order of increasing phosphorylation could be detected in H1t. Thus, it appears that not the order of up-phosphorylation but the number of the phosphate groups is necessary for regulated chromatin decondensation, thus facilitating the substitution of H1t by transition proteins and protamines.

Mammalian spermatogenesis is a complex, strictly organized process, including proliferation and differentiation. After several mitotic divisions of spermatogonia, germ cells differentiate into spermatocytes and pass through two consecutive meiotic divisions, resulting in haploid round cells termed spermatids. These spermatids then evolve into highly condensed and transcriptionally inert sperm through a process known as spermiogenesis (for a review, see Ref. 1). In rats, 19 developmental steps have been defined by morphological criteria (2, 3). During the early stages, comprising steps 1–8, round spermatids are transcriptionally active and contain nucleosomal chromatin. The later stages can be roughly divided into three phases. In the elongating phase (step 9 to early step 12) the transcriptional activity of the genome decreases to undetectable levels. The second phase, from late step 12 to step 15, includes the substitution of histones by transition proteins TP1, TP2, and TP4. Steps 16–19 compose the third phase when transition proteins are replaced by protamines Prm1 and Prm2 (4).

The underlying mechanisms that lead to this apparently dramatic reorganization and packaging of the nucleosomal chromatin to a highly condensed chromatin during spermiogenesis are not well understood up to now. The changing histone gene expression pattern seems to play a considerable role (5). In spermatogonia and preleptotene spermatocytes, the linker histones H1a and H1b are expressed (6). When cells enter the meiotic prophase, somatic histones are partially replaced by testis-specific core histone variants, including TH2B, TH2A, and TH3 (6–13) as well as by linker histone variants H1t (14), H1t2 (15), and HILS1 (16).

H1t expression starts in pachytene spermatocytes, constituting as much as 55% of the linker histone associated with chromatin in these cell types and persists until the stage of elongating spermatids. Although H1t has the usual tripartite structure typical of metazoan linker histones, its amino acid sequence is highly divergent from that of other mammalian H1s. De Lucia et al. (17) reported that H1t exerts the lowest condensing effect on rat testis oligonucleosomes of all somatic H1 variants. The high levels of H1t during the pachytene phase could suggest a role in keeping chromatin in a relatively decondensed state, enabling nuclear events during this spermatogenic stage. It persists until it is almost completely replaced by transition proteins in elongated spermatids. Very little is known of the mechanisms controlling the process of histone replacement. Govin et al. (18) proposed a new testis-specific “histone code,” since it appears that before histone replacement a massive chromatin alteration occurs due to extensive incorporation of histone variants as well as to globally specific histone modifications.

Posttranslational histone modifications during spermatogenesis encompass acetylation, methylation, ubiquitination, and phosphorylation (for a review, see Refs. 19 and 20). Histone H4 was found to be hyperacetylated at Lys-5, Lys-8, and Lys-12 during replacement of H1t (21, 22). Monoubiquitinylation at Lys-119 of H2A and hyperacetylation of histone H4 occur a short time before histones disappear in Drosophila (23). His-
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tone ubiquitylation and methylation are involved in meiotic DNA recombination and middle gene expression in yeast (24–26). Temporarily mono-, di-, and trimethylated histone H3 at Lys-4 was identified (27) in elongating spermatids of mice and marmoset. Mono-, di-, and trimethylated histone H3 was detected from leptotene to diplonete spermatocytes in mice (28).

Phosphorylated H2B and H3 at Ser-10 correlate with meiotic chromosome condensation in yeast, whereas histone H4 is phosphorylated later during sporulation (29). Phosphorylation of histone H3 starts in rodents at the end of the prophase at the centromeric heterochromatin in the diplotene stage. As experiments with kinase inhibitors demonstrate, this event can be uncoupled from meiotic chromosome condensation (30). Moreover, several specific phosphorylation sites of TP1 and TP2 (31, 32) and human protamines 1 and 2 (33) have been identified.

Although bulk phosphorylation of various testis-specific proteins has been well documented and is known to be important during spermatogenesis, the specific sites of phosphorylation of histone H1t have not been characterized in detail or reported. Therefore, the prime objective of this investigation was to use HILIC2 in combination with MS/MS and MS3 proteomic techniques to identify H1t phosphorylation sites. The present paper demonstrates that phosphorylation occurs mainly on serine residues. Moreover, a single modified threonine was detected. We found five phosphorylation sites in rat H1t, four located in the COOH-terminal tail and one in the NH2-terminal tail. Despite the fact that only two of them are located in the consensus sequences (S/T)P/ACE 2100, an untreated capillary (fused silica, 57 cm total length × 75 μm inner diameter) was used, protein samples were injected by pressure, and detection was performed by measuring UV absorption at 200 nm. Runs were carried out in 0.1 M sodium phosphate buffer (pH 2.0) containing 0.02% hydroxypropylmethylcellulose at a constant voltage (12 kV) and at a capillary temperature of 25 °C (40–43).

Enzymatic Cleavage—Histone H1t fractions (−100 μg) obtained by HILIC fractionation were digested either with α-chymotrypsin (EC 3.4.21.1) (Sigma type I-S; 1:150, w/w) in 100 μl of 100 mM sodium acetate buffer (pH 5.0) for 30 min at room temperature or with endoproteinase Arg-C (EC 3.4.21.1) (sequencing grade, 1:20, w/w; Roche Applied Science) in 100 μl of 0.01M Tris buffer (pH 8.0) and 0.001M phenylmethylsulfonyl fluoride and mixed with 280 μg of alkaline phosphatase (60 units/mg; Sigma). The concentration of solvent B was increased from 0 to 60% B for 5 min, from 60 to 100% for 35 min, and then maintained at 100% for 30 min.

Capillary Electrophoresis—High performance capillary electrophoresis (HPCE) was performed on a Beckman system P/ACE 2100. An untreated capillary (fused silica, 57 cm total length × 75 μm inner diameter) was used, protein samples were injected by pressure, and detection was performed by measuring UV absorption at 200 nm. Runs were carried out in 0.1 M sodium phosphate buffer (pH 2.0) containing 0.02% hydroxypropylmethylcellulose at a constant voltage (12 kV) and at a capillary temperature of 25 °C (40–43).

Incubation of H1 Histones with Alkaline Phosphatase—About 100 μg of whole linker histones from rat testis were dissolved in 47 μl of 0.01 M Tris buffer (pH 8.0) and 0.001 M phenylmethylsulfonyl fluoride and mixed with 280 μg of alkaline phosphatase (60 units/mg; Sigma).

Mass Spectrometric Analysis—Arg-C digests of the fractions H1tp0-p3 were analyzed using nano-HPLC consisting of an UltiMate 3000 system ( Dionex Corp.) connected online to a linear ion trap mass spectrometer (ThermoElectron Finnigan LTQ) equipped with a nanospray ionization source. The nanospray voltage was set at 1.6 kV, and the heated capillary was held at 200 °C. Data-dependent neutral loss MS3 and multistage activation were used for precise localization of phosphorylation. MS/MS and MS3 spectra were searched against a histone database.

**EXPERIMENTAL PROCEDURES**

*Materials*—Acetonitrile, sodium perchlorate (NaClO4), trifluoroacetic acid, and triethylamine (TEA) were purchased from Sigma. Acetonitrile, sodium perchlorate (NaClO4), trifluoroacetic acid, and triethylamine (TEA) were purchased from Aldrich; and hydroxypropylmethylcellulose (4000 centipoises) was obtained from Fluka (Buchs, Switzerland); EGME was obtained from Aldrich; fluoroacetic acid, and triethylamine (TEA) were purchased from Aldrich. The abbreviations used are: HILIC, hydrophilic interaction liquid chromatography; EGME, ethylene glycol methyl ether; ESI, electrospray ionization; MS, mass spectrometry; HPCE, high performance capillary electrophoresis; RPC, reversed-phase high performance liquid chromatography; TEA, triethylamine; RP-HPLC, reverse phase high performance liquid chromatography; IMAC, immobilized metal ion affinity chromatography.

2 The abbreviations used are: HILIC, hydrophilic interaction liquid chromatography; EGME, ethylene glycol methyl ether; ESI, electrospray ionization; MS, mass spectrometry; HPCE, high performance capillary electrophoresis; RPC, reversed-phase high performance liquid chromatography; TEA, triethylamine; RP-HPLC, reverse phase high performance liquid chromatography; IMAC, immobilized metal ion affinity chromatography.
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data base using SEQUEST (LCQ BioWorks; ThermoFinnigan) and validated manually. The identified peptides were further evaluated using charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides were Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charge ions, and Xcorr > 2.5 for triply charge ions. Only best matches were considered. MS/MS tolerances of +1 Da were allowed. IMAC was performed using the SigmaPrep Spin column kit and the PHOS-Select iron affinity gel from Sigma.

Determination of the chymotryptic peptide masses of the histone H1t subfractions obtained by HILIC was carried out by RP-HPLC coupled online to electrospray ionization mass spectrometry (ESI-MS). Samples (5–10 μg) were analyzed on a Nucleosil 300-5 C18 column (150 × 2 mm inner diameter; 5 μm particle pore size; end-capped; Macherey-Nagel) using solvent A (water containing 0.1% formic acid) and solvent B (85% acetonitrile and 0.1% formic acid). Chromatography was performed within 55 min at a constant flow of 0.1 ml/min. The concentration of solvent B was increased linearly from 5 to 25% during 20 min and from 25 to 80% during 30 min.

RESULTS

The mammalian testis consists of numerous cell types, but previous work has shown that the testis-specific histone H1t protein occurs in midpachytene primary spermatocytes and in early haploid spermatids (steps 1–8), where it is the most enriched H1 variant in these cell types. Fig. 1 reveals the HPCE analysis of linker histones from whole testis of rats aged 10, 30, and 60 days. The H1 pattern obtained reflects the H1 changes from sexually immature (Fig. 1A; 10 days) to pubertal (Fig. 1B; 30 days) and mature (Fig. 1C; 60 days) male rats. Assignment of the peaks in the electropherogram is according to Lindner et al. (43). In 10-day-old rats, the testis consists principally of somatic cells, and the H1 pattern strongly resembles that seen in several somatic tissues (data not shown). It is evident from Fig. 1A that at this age, not even traces of H1t can be detected. The increasing amount of H1t in Fig. 1, B and C, is consistent with the progressive differentiation of germ cells during spermatogenesis, whereas the developmental stage of the spermatids evolves from steps 1 and 2 (10 days) to 6 and 7 (30 days) and 8 (60 days). H1t is found to already be mono- and diphosphorylated in 30-day-old rats. Fig. 1 also shows an increase in the histone variant H1a, which is known to be enriched in testis (44), and in H1.0, an H1-like protein found mainly in mammalian tissues with little or no cellular proliferation. The two H1.0 peaks represent the NH2-terminally unacetylated (ac0) and acetylated form (ac1) (35).

To identify the phosphorylation sites of H1t, whole linker histones of testis from rats aged 60 days were fractionated using RP-HPLC (Fig. 2A). The H1t fraction eluting at about 40 min was collected and further analyzed by HPCE (Fig. 2B). Under these conditions, H1t was further separated into its un-, mono-, and diphosphorylated forms, and moreover, even a triphosphorylated peak was obtained due to the purity of the H1t fraction and the rather large amount of sample applied onto the capillary. To prove the existence of phosphorylated H1t forms, whole linker histones were incubated with alkaline phosphatase. After treatment with the enzyme, the sample was chro-

matographed according to Fig. 2A (data not shown), and the H1t subfraction was subjected to HPCE (Fig. 3). In contrast to Fig. 2B, only a single peak was detectable with a migration time corresponding to the unphosphorylated H1t protein.

For determination of phosphorylation sites, tandem mass spectrometry has become a powerful tool. However, proteolytic digests examined by MS are often likely to fail to detect phosphopeptides, because ionization of phosphorylated peptides in positive ion mode is generally less efficient than ionization of their nonphosphorylated counterparts, resulting in ion suppression effects. As can be seen from Fig. 2B, the main fraction of H1t is unphosphorylated (50.8% of whole H1t), followed by p1 (32.0%), p2 (12.2%), and very little p3 (5.0%). To avoid these unwanted suppression effects, the distinctly phosphorylated forms of H1t were separated from each other and from the unphosphorylated form by means of HILIC. This technique is particularly suitable for separating posttranslationally modified proteins (e.g. acetylated core (45) and H1 histones (46), methylated and deamidated histones (35, 47–49), and phosphorylated H1 proteins (34, 50)). As shown in Fig. 4A, three major and
some minor peaks of H1t were found by using a PolyCAT A column with a triethylammonium phosphate buffer system (pH 3.0) in the presence of 70% acetonitrile. To characterize the HILIC fractions in terms of their phosphorylation state, each peak was digested with chymotrypsin and analyzed by LC-ESI-MS. Determination of the chymotryptic peptide masses of the histone H1t subfractions obtained by HILIC was carried out by RP-HPLC coupled online to ESI-MS. The mass for the unphosphorylated COOH-terminal peptide 107–207 of rat H1t was calculated to be 10,606.5 Da. Mass differences found correlate with the molecular mass of additional phosphate groups (+0, +1, +2, and +3).

HILIC fractions in terms of their phosphorylation state, each peak was digested with chymotrypsin and analyzed by LC-ESI-MS. Under the conditions used, two main peptide fragments were obtained (data not shown), an NH2-terminal fragment (amino acids 1–106) with a calculated mass of 11,005.9 Da and a COOH-terminal fragment (amino acids 107–207) with a calculated mass of 10606.5 Da. In each case, an NH2-terminal peptide mass of 11,048 Da was detected by LC-ESI-MS for fractions p0–p3, indicating acetylation of the NH2 terminus (+42 Da) but no substantial phosphorylation. The masses of the COOH-terminal peptide fragments amino acids 107–207 of p0, p1, p2, and p3 differed by 80 Da each and correspond to the presence of one (p1), two (p2), and three (p3) phosphate groups (depicted in Fig. 4B). The minor peaks eluting between the labeled peaks in Fig. 4A were identified as oxidized forms of H1t. The NH2-terminal fragments of these peaks were found to contain one oxidized methionine (16 Da), and the COOH-terminal fragment was found to contain up to two oxidized methionines. The finding that phosphorylation of H1t takes place in the COOH-terminal part of the protein was not unexpected, since this fragment contains two typical phosphorylation motifs, namely T155PTK and S177PAK (Table 1).
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TABLE 1
Sequence alignment of rat and mouse histone variant H1t

| P06349|H1T_RAT | SetafAlSTLVAPVEKPPATKKGKFGMATKPRGFGSVEKLIPEALSMQBRAGMSL | 60 |
| Q07133|H1T_MOUSE | SetafAlSTLVAPVEKPPATKKGKFGMATKPRGFGSVEKLIPEALSMQBRAGMSL | 60 |
|-------------------------------|-------------------|-------|--------|
| P06349|H1T_RAT | AALKKALAAAGYDEKSRKRLAKRLFVQTVGKGAESKFLSKEAKASNGKK | 120 |
| Q07133|H1T_MOUSE | AALKKALAAAGYDEKSRKRLAKRLFVQTVGKGAESKFLSKEAKASNGKK | 120 |
|-------------------------------|-------------------|-------|--------|
| P06349|H1T_RAT | GKSSTAKKLMGPARKSBKPKSSTKAVKKPARKGSGRKGRTKGAKGQVR | 180 |
| Q07133|H1T_MOUSE | GKSSTAKKLMGPARKSBKPKSSTKAVKKPARKGSGRKGRTKGAKGQVR | 180 |
|-------------------------------|-------------------|-------|--------|
| P06349|H1T_RAT | ARATNSSGKMKMKDRLAAAGRGK | 207 |
| Q07133|H1T_MOUSE | ARATNSSGKMKMKDRLAAAGRGK | 207 |
|-------------------------------|-------------------|-------|--------|

The choice of a suitable enzyme is a prerequisite for identification of phosphorylation sites by tandem MS. Due to the high lysine content of H1t, enzymes like trypsin and Lys-C were found to be suitable only to a limited extent, because the resulting small, hydrophilic phosphopeptides from H1t were either not at all retained or were hardly retained in RP chromatography (data not shown). Therefore, endoproteinase Arg-C was used to digest H1t HILIC fractions p1, p2, and p3. It cleaves predominantly COOH-terminally of arginine, yielding fewer small peptide fragments. In this case, sequence coverage of 88.41% for p0, 84.06% for p1, and 93.94% for p2 was found, comprising 94.90, 89.7, and 97.4% of total Ser, Thr, and Tyr residues and including all potential SP and TP phosphorylation motifs. Combining data from both, Arg-C and Lys-C digest experiments, did not significantly improve sequence coverage.

As a result, individual phosphorylation sites of the H1t fraction (p1–p3) were identified using nanoflow liquid chromatography coupled to an LTQ mass spectrometer by applying data-dependent MS3 for peptides showing a neutral loss of 98 Da (single charged), 49 Da (double charged), or 33 Da (triple charged) due to the loss of phosphoric acid. Exact identification of the specific phosphorylation sites was performed with multitarget activation, a combination of MS/MS and MS3 fragmentation that retains the informative fragments from the precursor ion. Table 2 lists the phosphopeptides identified by tandem MS of Arg-C digests of HILIC fractions p1–p3 of rat H1t without IMAC enrichment (Table 2, top) and with IMAC enrichment for rat (Table 2, middle), and mouse H1t (Table 2, bottom). Without using IMAC, the monophosphorylated HILIC fraction p1 from rat was found to be primarily phosphorylated at Ser-140 and to a minor extent at Ser-186. Interestingly, neither phosphorylation site is located within a CDK consensus motif. HILIC fraction p2 showed the same phosphopeptides as p1. In addition to these, two more sites, namely Ser-177 and Thr-155, were found, both located in CDK consensus motifs. Furthermore, to a very small extent, an NH2-terminal peptide 1–23 was detected with a phosphorylated serine at position 8. HILIC fraction p3, which was obtained in very small amounts, was analyzed after IMAC enrichment only. Application of IMAC to fractions p1–p3 revealed an increased number of phosphorylated peptides; additional new modification sites, however, were not observed. Because of the ability of IMAC to enrich low abundance phosphopeptides, all four modification sites were detected in fraction p1. Fractions p2 and p3 did not differ from each other but exhibit a Ser-8 phosphorylation not detectable in p1.

In contrast to somatic H1 histones, which were found to be nonrandomly phosphorylated during the cell cycle (34), no such strict order of increasing up-phosphorylation could be detected in H1t. To confirm this result, we also investigated mouse H1t, the analogous testis-specific histone protein to rat H1t. Both proteins exhibit a very similar amino acid sequence with 93.2% homology (Table 1). Distinctly phosphorylated H1t fractions from mice were isolated in the same way as described for rats (i.e. RPC followed by HILIC separation) (data not shown). The separation pattern was very similar to rat H1t, showing an unphosphorylated main fraction (46.2% of whole H1t), followed by p1 (31.2%), p2 (17.2%), and p3 (5.4%). The HILIC fractions were digested with Arg-C, and resulting phosphopeptides were enriched with IMAC and analyzed with the same MS method applied for rat H1t. The results are summarized in Table 2 (bottom). Compared with rat H1t, identical sites were found to be phosphorylated, with one exception; Ser186 is replaced by proline, and, therefore, phosphorylation is not possible at this position. Despite the lack of Ser186, no other, additional phosphorylation site could be detected.

DISCUSSION

Little is known about the relevance of phosphorylation of testis-specific proteins during spermatogenesis. In rat TP2, the major phosphorylation sites are Ser-109 and Thr-101, which greatly reduce its DNA condensation property (51). TP1 from
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TABLE 2
Post-translational modifications identified by nanospray-MS/MS from rat and mouse H1t

Shown are the identified phosphorylation sites obtained without IMAC enrichment for rat H1t (top), by using IMAC enrichment for rat H1t (middle), and by using IMAC enrichment for mouse H1t (bottom). Observed amino acid sequences, the ion species (m/z), the charge state, and the cross-correlation score (Xcorr) are shown. S*, phosphoserine; T*, phosphothreonine; S#, acetylserine.

| Sample       | Phosphorylation sites | Phosphopeptides                                      | m/z          | Charge state | Xcorr |
|--------------|-----------------------|------------------------------------------------------|--------------|--------------|-------|
| Rat H1t p1   | Ser-140              | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 960.43       | +3           | 4.82  |
|              |                      | S* PKSSKT VVKKPK ATPTKGS GSR                        | 855.71       | +3           | 4.53  |
| Rat H1t p2   | Ser-186              | ATNS’NSGSK VM MQTD L R                               | 726.09       | +3           | 3.44  |
|              |                      | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 960.65       | +3           | 4.54  |
|              |                      | S* PKSSKT VVKKPK ATPTKGS GSR                        | 855.55       | +3           | 3.27  |
|              | Ser-177              | GLQQRK*S PAKAN                                       | 473.88       | +3           | 3.21  |
|              | Ser-186              | ATNS’NSGSK VM MQTD L R                               | 1088.46      | +2           | 4.46  |
|              | Thr-155              | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 960.65       | +3           | 2.32  |
|              | Ser-8                | S* EPAAS* STLV PAV PEK PAT KR                        | 811.30       | +3           | 3.23  |
| Rat H1tp1    | Ser-140              | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 960.60       | +3           | 5.58  |
|              |                      | ASR*S PKSSKT VVK                                        | 528.47       | +3           | 2.53  |
|              | Ser-177              | GGLQQRK*S PAKAN                                       | 474.05       | +3           | 3.38  |
|              |                      | KTKGAK GLQQRK*S PAKAN                                 | 559.42       | +3           | 3.14  |
|              | Ser-186              | ATNS’NSGSK VM MQTD L R                               | 726.56       | +3           | 3.73  |
|              | Thr-155              | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 855.90       | +3           | 5.16  |
|              | Ser-8                | S* EPAAS* STLV PAV PEK PAT KR                        | 863.18       | +3           | 3.15  |
| Rat H1tp2    | Ser-140              | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 960.65       | +3           | 5.09  |
|              |                      | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 855.78       | +3           | 3.67  |
|              | Ser-177              | GLQQRK*S PAKAN                                       | 474.06       | +3           | 2.97  |
|              |                      | GGLQQRK*S PAKAN                                       | 559.47       | +3           | 2.97  |
|              | Ser-186              | ATNS’NSGSK VM MQTD L R                               | 726.50       | +3           | 3.57  |
|              | Thr-155 + Ser-140    | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 987.16       | +3           | 3.39  |
|              | Ser-8                | S* EPAAS* STLV PAV PEK PAT KR                        | 863.18       | +3           | 3.15  |
| Rat H1tp3    | Ser-140              | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 960.64       | +3           | 5.28  |
|              |                      | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 516.80       | +3           | 2.59  |
|              | Ser-177              | GGLQQRK*S PAKAN                                       | 559.84       | +3           | 3.74  |
|              |                      | KTKGAK GLQQRK*S PAKAN                                 | 678.99       | +3           | 3.15  |
|              | Ser-186              | ATNS’NSGSK VM MQTD L R                               | 726.56       | +3           | 3.68  |
|              | Thr-155 + Ser-140    | ASR*S PKSSKT VVKKPK ATPTKGS GSR                     | 987.47       | +3           | 4.79  |
|              | Ser-8                | S* EPAAS* STLV PAV PEK PAT KR                        | 863.43       | +3           | 3.47  |
| Mouse H1tp1  | Ser-140              | ASR*S PKSSKT AVKKPK ATPTKAS GSR                      | 974.86       | +3           | 3.38  |
|              |                      | SPKSKT AVKKPK ATPTKAS GSR                            | 807.06       | +3           | 4.01  |
|              | Ser-177              | KTKGAK GVQRK*S PAKAN                                  | 674.04       | +3           | 2.97  |
| Mouse H1tp2  | Ser-140              | ASR*S PKSSKT AVKKPK ATPTKAS GSR                      | 974.80       | +3           | 5.47  |
|              |                      | ASR*S PKSSKT AVKKPK ATPTKAS GSR                      | 637.20       | +3           | 3.55  |
|              | Ser-177              | KTKGAK GVQRK*S PAKAN                                  | 537.16       | +3           | 3.51  |
|              |                      | S* PKSS KTAVKKPK ATPTKAS GSR                         | 807.09       | +3           | 4.31  |
|              | Thr-155              | S* PKSS KTAVKKPK ATPTKAS GSR                         | 807.21       | +3           | 4.62  |
|              | Ser-8                | S* EPAAS* STLV PAV PEK PK PK ASR                     | 863.80       | +3           | 2.70  |
| Mouse H1tp3  | Ser-8                | S* EPAAS* STLV PAV PEK PK PK ASR                     | 974.95       | +3           | 5.55  |
|              | Ser-140              | ASR*S PKSSKTAVKKPK ATPTKAS GSR                       | 637.22       | +3           | 3.81  |
|              |                      | ASR*S PKSSKTAVKKPK ATPTKAS GSR                       | 870.23       | +3           | 4.28  |
|              | Thr-155              | TKAVKKPK ATPTKAS GSR                                 | 665.59       | +3           | 4.67  |
|              | Ser-177              | GVQRK*S PAKAN                                         | 469.44       | +3           | 3.37  |
|              |                      | GAGKVQRK*S PAKAN                                      | 554.83       | +3           | 3.23  |
|              |                      | GAGKVQRK*S PAKAN                                      | 554.83       | +3           | 3.23  |
|              |                      | GAGKVQRK*S PAKAN                                      | 554.83       | +3           | 3.23  |
|              | Ser-8                | S* EPAAS* STLV PAV PEK PK PK ASR                     | 863.57       | +3           | 3.67  |

ram elongating spermatids is known to be mono- and diphosphorylated in the very conserved sequence 29–42 (31). Proteamins are highly phosphorylated as soon as they are synthesized and become dephosphorylated after they are deposited on chromatin. Phosphorylation of basic regions of proteamins in several species has been proposed to regulate interaction between protamine and chromatin during the assembly of nucleoprotein complexes (52).

Lennox and Cohen (5) observed H1t to be phosphorylated in mice already in 1984. Meistrich and co-workers (53) separated cell suspensions from stage-synchronized rat testis by centrifugal elutriation. Using acid-urea-Triton gels, they found unmodified H1t in step 1–3 spermatids, roughly 50% modified H1t in steps 9 and 10, and nearly all of the H1t to be modified in steps 11 and 12.

In order to identify the H1t phosphorylation sites, the histone subtype was extracted from rat and mouse testis, purified via RP-HPLC, and separated into its differently phosphorylated forms by means of HPCE and HILIC. Four fractions were obtained corresponding to the un-, mono-, di-, and triphosphorylated forms of H1t. Each phosphorylated fraction was subjected to enzymatic digestion, phosphopeptide enrichment, and mass spectrometric characterization. This allowed the identification of five phosphorylation sites in rat H1t and four phosphorylation sites in mouse H1t. However, not all of these sites are phosphorylated at the same time, since the maximum
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TABLE 3

CDK consensus motifs of rat somatic H1 histones and in vivo phosphorylation sites found in testis-specific rat and mouse H1t

| H1 subtype | NH2 terminus | COOH terminus | No. of CDK motifs |
|------------|--------------|---------------|------------------|
| Somatic    |              |               |                  |
| rH1.1      | Thr-148      | Thr-165       | Ser-180          | Thr-201 |
| rH1.2      | Thr-145      | Thr-153       | Ser-172          |         |
| rH1.3      | Thr-17       | Thr-146       | Ser-153          | Ser-172 |
| rH1.4      | Thr-17       | Thr-145       | Ser-153          | Ser-171 |
| rH1.5      | Ser-17       | Thr-133       | Ser-150          | Ser-168 |
| Testis-specific |        |               |                  |
| rH1t       | Ser-8        | Ser-140       | Thr-155          | Ser-177 |
| mH1t       | Ser-8        | Ser-140       | Thr-155          | Ser-177 |

phosphorylation level is p3. Two sites only are located within the conserved CDK consensus motifs T155pTK and S177pPAK. In contrast to the two consensus motifs present in H1t, somatic H1 histones from rat display either one (H1.2), four (H1.1), or five (H1.3, H1.4, and H1.5) CDK sites. The positions of these consensus motifs in the NH2- and COOH-terminal tails are shown in Table 3. It is important to note that despite the lack of one or two motifs in certain subtypes (H1.1 and H1.2), localization of the CDK motifs in the NH2- and COOH-terminal domains is nearly identical in all five somatic H1 subtypes (e.g. Ser-180 (H1.1), Ser-172 (H1.2), Ser-172 (H1.3), Ser-171 (H1.4), and Ser-168 (H1.5)). This may be of interest, since Contreras et al. (54) reported that the CDK sites in somatic H1s seem to be important for dynamic nuclear mobility. Moreover, after studying the effects of phosphorylation of the carboxyl-terminal domain of H1 on the secondary structure of DNA-bound histones, Roque et al. (55) suggested that effects of phosphorylation are mediated by specific structural changes and are not simply a consequence of the charge. Among others, the authors investigated the capacity of unphosphorylated and partially and hyperphosphorylated carboxyl-terminal domain to aggregate DNA fragments by band shift gel electrophoresis. As a result, the unphosphorylated and hyperphosphorylated COOH terminus aggregated DNA fragments to a very large extent. However, weakly phosphorylated forms significantly decreased aggregation capacity. IR spectroscopy revealed that, as compared with hyperphosphorylated forms, partial phosphorylation of somatic linker histone carboxyl-terminal domain (mono- and diphosphorylated states) caused a loss of defined structure, which in a similar manner might explain the relaxing effect of phosphorylated H1t on chromatin structure.

Although H1t displays the usual tripartite structure of linker histones, its primary structure is highly divergent as compared with that of the somatic histones H1.1–H1.5. Nevertheless, the distribution of the phosphate groups identified on the H1t molecule strongly resembles that of somatic H1s during mitosis (Table 3). Four of the phosphorylated sites in rat and three in mouse H1t are located in the COOH-terminal part of the protein, which is required for high affinity binding of histone H1 to chromatin. Recently, Hendzel et al. (56) showed that phosphorylation of two consensus motifs, namely Thr-152 and Ser-183, in human H1.1, dramatically destabilizes H1 binding. We detected H1t phosphorylation of Thr-155 and Ser-186, which could affect DNA binding in a similar way as described for H1.1. Interestingly, in contrast to H1.1, where Ser-183 is located within a CDK consensus motif, H1t phosphorylation at Ser-186 in rat testis takes place in the same region but even in a nonmotif sequence. Moreover, we found phosphorylated Ser-140 and Ser-177 in mouse and rat H1t. These modification sites are also equivalent to phosphorylation motifs in various somatic H1 subtypes from different species, like mice and humans.

In contrast to the COOH-terminal domain, the NH2-terminal domain of linker histones is unstructured and does not bind chromatin (57, 58). Nevertheless, phosphorylation in this region takes place on several different H1 isoforms from mouse (59) and humans (34, 59–61). Ser-1, for example, appears to be phosphorylated in mouse and human H1.1, H1.2, and H1.5 and in mouse H1.4. We could not prove this phosphorylation site in either rat or mouse H1t. Instead, a single phosphorylation site at Ser-8 in the NH2-terminal domain was detected in both species. Alignment of various H1 subtypes of mouse showed that only H1t has a serine at position 8, whereas human H1.5 is known to have mitosis-specific phosphorylation at Thr-10 (34). Although at the present time, the NH2-terminal part of linker histones does not appear very important for DNA binding, phosphorylation is a widespread modification also in this region, and its function in chromatin reorganization remains to be further elucidated.

Histone H1t is expressed exclusively in spermatocytes and was considered to have a specific function in the dramatic reorganization of mammalian chromatin during spermatogenesis. The task of its posttranslational modification may be to establish an open chromatin structure for the replacement of histones with transition proteins and protamines. However, the functional significance of H1t has been questioned, because mice lacking histone H1t are fertile and show normal spermatogenesis (62–64). Redundancy of the linker histones and compensation for H1t by other H1s may hide detection of defects caused by the loss of H1t. Knock-out mice reveal unexpected functional redundancy, also of other testis genes (e.g. Tnp2 (65), proacrosin (66), H1.1 (67), and sperm mitochondria-associated cysteine-rich protein Smcp (68)). Triple knock-out lines, Acr/H1t/Smcp and Tnp2/H1t/Smcp, however, exhibited drastic reductions in mice fertility (69). The similar distribution of phosphorylation sites of somatic H1 variants and of H1t, although some of the corresponding CDK motifs are lacking in H1t, might explain why H1t can be replaced with somatic linker histones.

Previous studies reported strong cyclin B-dependent CDC2 kinase activity in pachytene spermatocytes from mouse testis and the ability of this enzyme to phosphorylate histone H1 (70). The level of CDC2-cyclin B complex is rather high in young pachytene spermatocytes and culminates in late spermatocytes. The kinase requires the consensus sequence (S/T)PXZ and could be responsible for the phosphorylation of Thr-155 and Ser-177 in H1t.

Phosphorylation of H1t and hyperacetylation of H4 occur during the same steps of spermiogenesis as chromatin conformational changes and nuclear reshaping (53). It has been postulated that increased acetylation of the core histone tails may be important in these processes, since it destabilizes nucleo-
some and chromatin structure, thereby facilitating recruitment of factors and complexes further involved in histone replacement (18). It clearly appears that before histone replacement, a massive chromatin alteration occurs due to extensive incorporation of histone variants and to globally specific histone modifications. It is very plausible that specific linker histone phosphorylation, as found for H1t, contributes to these extensive chromatin changes during spermiogenesis.

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