SPERMATOGENESIS IN THE MOUSE

I. Autoradiographic Studies of Nuclear Incorporation and Loss of ³H-Amino Acids

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ABSTRACT

Autoradiographic and electron microscope methods were used to correlate changes in nucleoproteins with nuclear fine structure during spermatogenesis in the mouse. Testes were fixed at daily intervals after intratesticular injection with labeled amino acid. [³H]Arginine, lysine, valine, and proline were rapidly incorporated into primary spermatocyte nuclei, retained through subsequent spermatocyte divisions and through spermatid differentiation to step 12 of spermiogenesis, but were lost with spermatid differentiation beyond step 12. Arginine and lysine (not valine or proline) also were rapidly incorporated into certain elongated spermatid nuclei but differed strikingly in their distribution and fate. Nuclei of late step-12 through step-15 spermatids were initially labeled with arginine. This label was retained through subsequent spermatid differentiation and sperm maturation in the epididymis. By contrast, lysine was initially incorporated only into late step-12 and step-13 spermatid nuclei, and was retained only to early step 14 of spermiogenesis. Spermatid incorporation of lysine coincided with the initiation of chromatin condensation in late step-12 nuclei, and loss of lysine coincided with the completion of condensation in step-14 nuclei.

KEY WORDS spermatogenesis · nuclei · nucleoprotein · autoradiography · ultrastructure

In eutherian mammals, the somatic histones present in nuclei of cells in early stages of spermatogenesis are ultimately replaced by protamines, highly basic proteins rich in arginine and cysteine (1, 2, 14, 23). Replacement of the histones involves a series of nuclear protein changes rather than a direct replacement of one protein by another. For example, recent biochemical studies of isolated testicular cells of the rat have demonstrated that at least three histonelike proteins first appear in primary spermatocyte nuclei (4, 9, 13, 17, 27) but are not retained in late spermatid nuclei (10). These results are consistent with previous autoradiographic studies which have demonstrated the uptake of ³H-amino acids into pachytene spermatocyte nuclei of mouse (12, 22), rat (28), and ram (19). Biochemical (3, 8-11, 14, 15, 17, 18, 20, 26, 27) and autoradiographic (12, 19, 22) studies also have indicated that a number of new basic nucleoproteins are synthesized in mammalian spermatids. In the rat, only one of these spermatid proteins, protamine, is retained through spermiogenesis and sperm maturation in the epididymis (10, 14). As yet, however, the exact stages of spermatogenesis in which basic protein replacements occur and in which transient proteins are lost have not been determined. Ad-
ditionally, the relationship of basic protein changes to the morphology of the nuclei in which they occur is not known.

MATERIALS AND METHODS

Tissue Preparation

Sexually mature Swiss albino mice (25-30 g) were injected intratesticularly (0.02 ml) with 20-μCi [3H]arginine (sp act, 23 Ci/mM), [3H]lysine (sp act, 60 Ci/mM), [3H]proline (sp act, 60 Ci/mM), or [3H]valine (sp act, 11.5 Ci/mM) (New England Nuclear, Boston, Mass.). In some experiments, the initial injection with labeled amino acid was followed by an additional injection (chase) containing 1,000-fold higher concentration of unlabeled amino acid. The results were identical with or without a chase, and the data shown in this paper were obtained from experiments without a chase. At various intervals (2 h-23 d) after injection, testes and epididymides were fixed for 1.5 h at 4 °C or 23°C with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The resulting samples were then washed several times with cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol, and embedded in Epon-Araldite.

Autoradiography

Serial sections (0.5, 1.0, and 1.5 μm) were cut with an ultramicrotome (Sorvall MT1 DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), mounted on subbed slides, and dipped in Kodak NTB2 emulsion. After exposure for 1, 2, 3, 4, or 8 wk at 4°C, the slides were developed in Kodak D-19 (4 rain at 19°C) and fixed in Kodak Ektalfo (7 min). After they were washed and dried, the sections were either stained with 1% toluidine blue in 1% borax or viewed unstained under a phase-contrast microscope. Specific stages of spermatogenesis were determined by cell association criteria established for the mouse by Oakberg (24).

To determine which amino acids were incorporated into protein, [3H]arginine, [3H]lysine, or [3H]proline was injected into control (untreated) testes or testes which had received prior (0.5 h earlier) treatment with cycloheximide (Boehringer Mannheim Biochemicals, Indianapolis, Ind.: 100 μg in 0.02 ml). 2 h after injection with a given amino acid, testes were homogenized in 0.15 M Tris buffer (pH 8.0) containing 1.1 M NaCl, 0.1 M 2-mercaptoethanol, 6 M urea, and 1% Triton X-100 (21). The samples were then centrifuged at 20,000 g for 20 min to pellet DNA and insoluble debris. The resulting supernatants were treated (0°C, 20 min) with an equal volume of 20% TCA to precipitate proteins. Incorporation of label into TCA-soluble and TCA-insoluble fractions of testis homogenates was then measured in the control and cycloheximide-treated testes.

Grain counts were performed on autoradiographs exposed for 1-2 wk. At least 50 randomly selected nuclei were counted for each cell type. Grains were counted over spermatocyte or round-spermatid nuclei showing maximal cross sections, and over elongated and mature spermatids showing full sagittal sections.

RESULTS

Incorporation into Protein

With each amino acid tested, >97% of the total radioactivity of the testis was found in TCA-insoluble fractions of homogenized testes (Table I). These results indicated that labeled amino acids were incorporated into protein. Additionally, when testes were treated with cycloheximide be-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]Arginine</th>
<th>[3H]Lysine</th>
<th>[3H]Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCA soluble</td>
<td>913 ± 225</td>
<td>7,798 ± 2,909</td>
<td>2,061 ± 362</td>
</tr>
<tr>
<td>TCA insoluble</td>
<td>142,491 ± 54,268</td>
<td>232,272 ± 82,042</td>
<td>112,508 ± 45,081</td>
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<tr>
<td>Cycloheximide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCA soluble</td>
<td>2,572 ± 593</td>
<td>13,048 ± 1,341</td>
<td>8,264 ± 1,176</td>
</tr>
<tr>
<td>TCA insoluble</td>
<td>8,547 ± 4,397</td>
<td>16,275 ± 1,452</td>
<td>39,872 ± 8,008</td>
</tr>
</tbody>
</table>

* 2 h after injection with a given 3H-amino acid (20 μCi in 0.02 ml), testes were homogenized on 0.15 M Tris buffer (pH 8.0) containing 1.1 M NaCl, 0.1 M 2-mercaptoethanol, 6 M urea, and 1% Triton X-100. The samples were then centrifuged at 20,000 g for 20 min to pellet DNA and insoluble debris. The resulting supernatants were treated (0°C, 20 min) with an equal volume of 20% TCA to precipitate proteins. TCA-soluble and -insoluble fractions were assayed by scintillation counting.

† All values represent an average of 3-5 determinations ± SEM.

§ Cycloheximide (100 μg in 0.02 ml) was injected 0.5 h before 3H-amino acid injection.
fore injection of label, the level of radioactivity in TCA-insoluble testis fractions was reduced by 75–99% (Table I). This further suggested that the label was incorporated into protein, although the possibility that the cycloheximide treatment might inhibit cellular amino acid uptake was not ruled out.

**Incorporation into Spermatocytes**

The initial incorporation of [3H]arginine, [3H]lysine, [3H]proline, and [3H]valine into spermatocyte nuclei, and the subsequent appearance of these amino acids in nuclei of successive stages of spermatogenesis, were identical. In testes fixed 2 h after a single injection of any one of the four amino acids, primary spermatocyte nuclei were labeled, with pachytene spermatocytes labeled most heavily (Figs. 1A–D and 2a). Nuclei of secondary spermatocytes were only sparsely labeled (Figs. 1A–D and 2b) as were nuclei of early spermatids (steps 1–11; Figs. 1A–D and 2a).

In testes fixed at daily intervals after injection, labeled amino acids appeared in nuclei of successive stages of spermatogenesis. The daily shifts seen in the populations of cells with labeled nuclei coincided exactly with the rate at which spermatocytes would be expected to complete spermatogenesis (5, 25), indicating that the nucleoprotein initially labeled in spermatocyte nuclei was retained as the spermatocytes completed meiosis and entered spermiogenesis (Fig. 3A–D). Label first appeared in nuclei of secondary spermatocytes 1 d after injection (Fig. 1A). 2 d after injection, nuclear uptake of [3H]lysine into late step-12 and step-13 spermatids, but not into step-14 and step-15 nuclei, was observed (Fig. 1B). When the initially incorporated lysine was followed, label appeared in early step-14 spermatid nuclei 1 d after injection (Fig. 3A) but was not seen in spermatids beyond early step 14 on this or subsequent days (Fig. 3B and C). Thus, in striking contrast to [3H]arginine, epididymal spermatid nuclei showed little or no [3H]lysine nuclear labeling, even 13 d after injection (Fig. 3E).

In contrast to both arginine and lysine, little or no [3H]proline or [3H]valine was incorporated into spermatid nuclei (Fig. 1c and d). Additionally, these amino acids were not observed in epididymal spermatozoa even 9 d after label was introduced. This is consistent with the fact that the protamine of mouse spermatozoa does not contain valine or proline residues and that protamine is the major basic nuclear protein of mouse spermatozoa (1, 2).

**Incorporation into Spermatid Nuclei**

When testes were injected with [3H]arginine or [3H]lysine and fixed 2 h later, certain elongated spermatid nuclei, in addition to primary spermatocyte nuclei, appeared labeled. 2 h after injecting testes with [3H]arginine, the nuclei of spermatids in steps 1 through early 12 (Fig. 1A) showed little label. By contrast, the nuclei of spermatids in late step 12 through step 15 (Fig. 1A) showed dense grain accumulations. The nuclei of the last step of spermatid development, step 16, and of epididymal spermatozoa (Fig. 1A) showed only background levels of label. The arginine initially incorporated into nuclei of late step-12 through step-15 spermatids was followed as these spermatids continued to differentiate and subsequently were released into the epididymis. Label first appeared in early step-16 nuclei 1 d after injection (Fig. 3F).

**Chromatin Condensation**

Electron microscope studies demonstrated that the nuclei of spermatids at steps 1 through early 12 (Fig. 4a) contain large amounts of dispersed chromatin. Chromatin condensation is initiated in late step-12 nuclei (Fig. 4b) and continues through step 14 (Fig. 4c). Condensation is essentially completed by late step 14 and undergoes no apparent change through step 16 (Fig. 4d). These observations are consistent with those made previously (6).

**DISCUSSION**

The studies presented here show that primary
FIGURE 2 Autoradiographs showing incorporation of $[^3H]$lysine into spermatocyte nuclei and subsequent loss of label. Testes were fixed at intervals after injection with $[^3H]$lysine. Results were similar with $[^3H]$arginine, $[^3H]$valine, or $[^3H]$proline. Bar, 10 μm. All micrographs × 1200. (a) Testis fixed 2 h after injection. Nuclei of pachytene spermatocytes (arrow) are heavily labeled (Fig. 2a). Note that step-6 spermatid nuclei (arrow head) show little or no label at this time (Fig. 2a). Stained with toluidine blue. 

(b) Testis fixed 2 h after injection. Nuclei of secondary spermatocytes show little or no label. Stained with toluidine blue. (c) Testis fixed 1 d after injection. Nuclei of secondary spermatocytes are now labeled. Stained with toluidine blue. (d) Testis fixed 5 d after injection. Nuclei of spermatids through step 6 (shown) are now labeled. Stained with toluidine blue. (e) Testis fixed 11 d after injection. Nuclei of step-12 spermatids are now labeled. Unstained phase-contrast micrograph; silver grains appear as white dots. (f) Testis fixed 13 d after injection. Nuclei of step-13 spermatids (shown) and later steps of spermatid development show little or no label. Phase-contrast micrograph.

FIGURE 1 Distribution of $[^3H]$-amino acids in spermatocyte (●—●), spermatid (○—○), and caput epididymal sperm (□) nuclei after intratesticular injection with (A) $[^3H]$arginine, (B) $[^3H]$lysine, (C) $[^3H]$proline, or (D) $[^3H]$valine. Fixation 2 h after injection. Spermatogenic cell types (abscissa): leptotene (L), zygotene (Z), pachytene (P), secondary spermatocytes (S), spermatid steps (1–16), and caput epididymal spermatozoa (E). Roman numerals (abscissa) indicate stages of the cycle of the seminiferous epithelium (24). Grain counts were performed on at least 50 randomly selected nuclei for each cell type. Mean numbers of grains are plotted (ordinate). SE (not plotted) were not ≥ 1.8 for any cell type.
spermatocyte nuclei incorporate the intratesticular injected amino acids \(^{[3}H\)arginine, \(^{[3}H\)lysine, \(^{[3}H\)valine, and \(^{[3}H\)proline, thus confirming previous autoradiographic studies of mammalian spermatogenesis (12, 19, 22, 28). Our studies further demonstrate that, at intervals after injection, the initially incorporated labeled amino acids appear in nuclei of successive stages of spermatogenesis.
spermatogenesis. These results indicate for the first time that nucleoprotein initially labeled in primary spermatocyte nuclei is retained through subsequent meiotic stages and through spermatid differentiation to step 12 of spermiogenesis, but is lost with spermatid differentiation beyond step 12. Interestingly, loss of this protein occurred at the time of the initiation of chromatin condensation, in step-12 nuclei.

Intratesticularly injected \[^{3}\text{H}]\text{arginine and }^{3}\text{H}l\text{ysine, but not }^{3}\text{H}v\text{aline or }^{3}\text{H}p\text{roline, also were rapidly incorporated into certain elongated spermatid nuclei, but differed strikingly in their initial distribution and in their fate. }^{3}\text{H}\text{Arginine was initially incorporated into nucleoprotein(s) of late step-12 through step-15 spermatids. As expected from previous studies (2, 7, 16), the labeled arginine appeared in successively more mature spermatid steps at intervals after initial injection but was simultaneously lost from nuclei of earlier steps. Ultimately, only epididymal spermatozoa appeared labeled. Unexpectedly, and in marked contrast to arginine incorporation, }^{3}\text{H}l\text{ysine was initially incorporated into nucleoprotein(s) of late step-12 and step-13 spermatids, retained only through early step 14, and then lost.}
The initial incorporation of lysine into spermatid nucleoprotein coincided with the initiation of chromatin condensation in late step-12 nuclei. Eventual loss of this lysine-labeled protein, in step-14 spermatids, coincided with the completion of chromatin condensation. This close correlation between chromatin condensation and lysine uptake and loss suggested the possibility that the lysine-labeled nucleoprotein(s) may play a role in the process of chromatin condensation.

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J. F. Mayer is a predoctoral candidate, and this investigation constitutes part of his doctoral dissertation.

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