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Meiotic synthesis of testis histones in the rat

(spermatogenesis/meiosis/hydroxyurea)

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ABSTRACT **The distribution and synthesis of the testis-specific variants of histones H1 and H2B, TH1 and TH2B, respectively, and of the somatic histones were studied in rat testis cells. Rat testis cells were labeled *in vivo* with intratesticular injections of [^{3}H]lysine. The cells and nuclei were then separated into different developmental classes by velocity sedimentation and the histones were analyzed. TH1 and TH2B, as well as the somatic histones, were present in spermatocytes and round spermatids, but none of them were detectable in elongated spermatids. The synthesis and nuclear accumulation of TH1 and TH2B took place throughout pachytene, as well as in earlier stages, but not in the round spermatids. In addition, there was synthesis during pachytene of a histone that migrates electrophoretically with H2A. However, somatic histone synthesis, with the possible exception of H2A and H2B, was not detectable at the pachytene stage. *In vivo* treatment of rats with hydroxyurea reduced DNA synthesis in the testis to 1% of control values and signficantly reduced the synthesis of H3, H2B, H2A, and H4, with the greatest effect being on H3 and H4. However, the hydroxyurea treatment did not significantly decrease the synthesis of TH1, H1, or TH2B. These results prove that the synthesis of several histones during the meiotic prophase is not dependent upon concurrent S-phase DNA synthesis.**

During spermatogenesis, the germ cell chromatin undergoes an impressive sequence of structural and functional changes. Structural alterations include chromosomal pairing (synapsis) and the formation of the synaptonemal complex during zygotene (1), genetic recombination during pachytene, chiasmata formation during diplotene, and loss of the "beaded" chromatin structure (2) during spermiogenesis, followed by formation of the highly compact sperm nucleus.

Transitions in chromosomal proteins occur concurrently with changes in testis cell morphology and gene expression; such transitions must play some role in regulating chromatin structure and function. In this paper we describe the appearance, synthesis, and turnover of two testis-specific histones, testis H1(TH1) and testis H2B (TH2B); these histones are closely related, by molecular weight and amino acid composition, to the somatic histones H1 and H2B, respectively (3, 4).

MATERIALS AND METHODS

Preparation of Cell Suspensions. The method of preparing a single-cell suspension from the testes of adult Sprague–Dawley rats by treatment with EDTA and trypsin has been described (5, 6).

Cell Separation. Cells were separated by centrifugal elutriation (7) according to a published protocol of buffer flow rates and rotor speeds (6). The buffer used for elutriation was phosphate-buffered saline containing 0.5% bovine serum albumin and 5 mM 2-napthol-6,8-disulfonic acid. We used 2.5×10^9 cells in a 100-ml volume for each elutriator run. After elutriation, each fraction was centrifuged at $500 \times g$ for 15 min, washed with phosphate-buffered saline, and recentrifuged. Air-dried smears were made of each fraction for cytological analysis (8).

Hydroxyurea Treatment of Rats. Either 20-day-old or adult rats were injected intraperitoneally with hydroxyurea (300 mg/kg) 1 hr before an intratesticular injection of [^{3}H]lysine and [^{14}C]thymidine. The animals were given a second hydroxyurea injection at the time of isotope administration and a third injection 1 hr later. Multiple injections of hydroxyurea were necessary because of the fast metabolic clearance of the drug. After the 1.5-hr labeling period, the testes were removed and the nuclei were prepared. The basic proteins were extracted and fractionated into lysine-rich and arginine-rich histones and analyzed as above. The DNA pellet was used to determine ^{14}C specific activity (cpm/mg of DNA).

Preparation and Purification of Nuclei. Nuclei were prepared from whole rat testis according to the method of Platz *et al.* (9). To prepare nuclei from cells after elutriation, the cell pellet, after washing in phosphate-buffered saline, was resuspended in lysing solution [5 mM sodium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$, 0.25% Triton X-100, 0.025% soybean trypsin inhibitor (Worthington Biochemicals), and 5 mM 2-naphthol-6,8-disulfonic acid] at a concentration of 2×10^7 cells per ml or less. This suspension was forced through a 25-gauge needle twice and then diluted with 5 mM sodium phosphate/5 mM $MgCl_2$, pH 6.5, to a concentration not exceeding 5×10^6 nuclei per ml. This preparation was subjected to velocity sedimentation on a Staput apparatus (10) according to a recent protocol (6). For differential counting, nuclei were fixed by resuspension in 35% (vol/vol) acetic acid, recentrifuged, and resuspended in 35% acetic acid containing 0.5% orcein. More than 400 nuclei were counted from each fraction.

Extraction and Fractionation of Histones. Basic nuclear proteins were extracted from purified nuclei according to the method described by Platz *et al.* (9).

Testis cell histones were fractionated into lysine-rich and arginine-rich fractions by differential extraction of nuclei according to the methods of Johns (11) and Oliver *et al.* (12), which we modified to accommodate the small amounts of chromatin used in our experiments. An outline of the fractionation is shown in Fig. 1. All procedures were performed at about 0°C, except the H_2SO_4/acetone precipitation, which was performed at −20°C. The centrifugation steps were $12,000 \times g$ for 15 min. The ethanol/HCl contained 10 ml of concentrated HCl, 90 ml of distilled water, and 400 ml of absolute ethanol. For each extraction, 5 ml or more of ethanol/HCl was used for every 15 mg of DNA in the pellet. For the H_2SO_4/acetone precipitation, 3 M H_2SO_4 was added to the supernatant to a final concentration of 0.2 M H_2SO_4, then 4 vol of ice-cold acetone was added and the suspension was mixed.

Electrophoresis. Histones were separated electrophoretically by loading equal amounts of protein on 15% polyacrylamide

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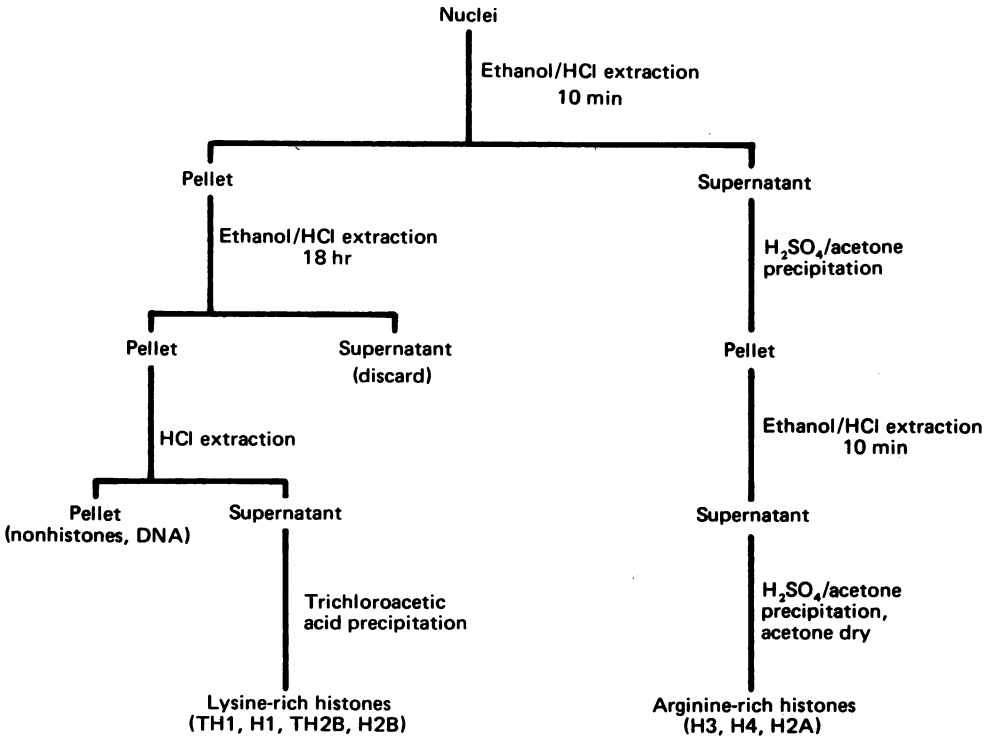

FIG. 1. Procedure for separating lysine-rich and arginine-rich histones.

gels containing 2.5 M urea (13). The gels were stained in 0.1% amido black and the relative protein contents of the different bands were determined by densitometric scanning at 600 nm on a Gilford spectrophotometer. The radioactivity profile of each gel was determined by slicing the gel into 2-mm sections with a Gilson Aliquogel fractionator, depolymerizing the slices with 0.4 ml of 15% (wt/vol) H_2O_2, and scintillation counting with 10 ml of Aquasol (New England Nuclear). The cpm from the gel slices were positioned accurately on the densitometric scan by using two thin reference lines of India ink that were injected into each gel before scanning and slicing. The ink in the gel caused a sharp spike to appear on the densitometric tracing and, after gel fractionation, the ink particles in the scintillation vial identified the exact fraction number corresponding to the spike. The two ink marks in each gel, therefore, served to index the scan for plotting the rest of the fractions. The specific activities of each histone were determined by measuring the area under each curve.

Radioactive Labeling of Proteins. Each rat testis was labeled by an intratesticular injection of 100 μCi of L-[^{3}H]lysine [specific activity 20–40 Ci/mmol; New England Nuclear (1 Ci = 3.7 × 10^{10} becquerels)] in a volume up to 100 μl. Animals were killed 1.5 hr after injection. In some experiments, [^{14}C]-thymidine (10 μCi; specific activity 40 mCi/mmol; New England Nuclear) was also injected intratesticularly.

Protein and DNA Assays. Protein was measured by the method of Lowry *et al.* (14) and DNA according to the method of Burton (15).

RESULTS

Cell and Nuclear Separation. Fractions of elutriated cells containing up to 82% pachytene spermatocytes were pooled, as were fractions containing over 80% round spermatid cells (steps 1–8). Nuclei of these cells were further purified by the Staput method. The results of differential counts of nuclei are presented in Table 1. Sample I was used for an analysis of round spermatid histones and sample II for pachytene histones. Sample III, which was taken from a fast-sedimenting band in the round spermatid Staput fraction (3.25 mm/hr), contained primarily spermatogonia and early primary spermatocytes, with contamination by round spermatids.

Histone Fractionation. Because it is not possible to affect a clear separation between histones H3 and TH2B by this electrophoresis system, it was necessary to first separate them into the lysine-rich (containing TH2B) and arginine-rich (containing H3) fractions. Liver histones were used first in order to demonstrate that H3 stays in the arginine-rich fraction. Further evidence that negligible TH2B contaminates the arginine-rich fraction is given below.

The various histones found in a mature rat testis are shown in Fig. 2. The histone variants of H1 (TH1) and H2B (TH2B) were found, in addition to all five somatic histones. Gels of testis histones after fractionation into arginine-rich and lysine-rich histone classes demonstrated that TH1 and TH2B are found in the lysine-rich fraction. TH1 and TH2B are the most abundant histones in the testis and make up over 30% of the total histones.

Table 1. Percentage composition of three nuclear fractions after elutriation followed by Staput separation

Cells	Sample I	Sample II	Sample III
Spermatogonia*	3	1	29
Early primary spermatocytes†	1	1	53
Pachytene‡	0	95	0
Round spermatids	96	0	17
Late spermatids	0	0	0
Unidentified	0	3	1

* Includes spermatogonia and preleptotene primary spermatocytes.
† Includes leptotene, zygotene, and early pachytene spermatocytes.
‡ Includes mid- to late-pachytene and diplotene spermatocytes.

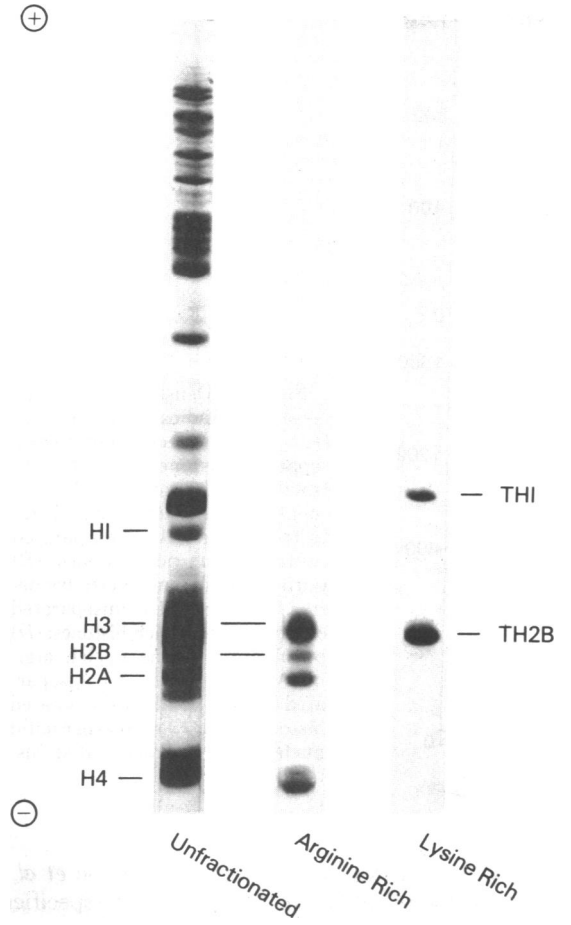

FIG. 2. Photograph of 2.5 M urea/polyacrylamide gels (13) after electrophoresis of fractionated and unfractionated testis histones. The gels were stained in 0.1% amido black. The slight difference in electrophoretic mobility between TH2B and H3 is apparent.

Histone Content and Synthesis in Specific Spermatogenic Cells. The results of histone analysis of purified round spermatid or pachytene spermatocyte nuclei are shown in Fig. 3. It is clear that, in both pachytene spermatocytes and round spermatids, all histone species are present, including the somatic histones and the testis-specific histones, TH1 and TH2B. There is, however, a significantly diminished amount of H1 in pachytene.

The incorporation of [^{3}H]lysine into testis histones is also shown in Fig. 3. The synthesis of the arginine-rich histones (H3, H2A, and H4) was confirmed by using a [^{3}H]arginine label. Synthesis and nuclear accumulation of all histone fractions occurred in the unseparated testis nuclei. But when purified pachytene nuclei were examined (Fig. 3 *B* and *D*), synthesis and nuclear accumulation of somatic histones H1 and H3 was not detectable whereas the rates of synthesis of TH1 and TH2B appeared to be high. The specific radioactivities of TH1 and TH2B were twice as high in the pachytene as in the unfractionated nuclei. Therefore, pachytene spermatocytes are one cell type responsible for the synthesis of TH1 and TH2B. The incorporation of [^{3}H]lysine into protein in the H2A region of the gel was also higher in pachytene than in unseparated testis nuclei. A large peak of radioactivity was associated with TH2B in the lysine-rich fraction (Fig. 3*B*), but there was negligible radioactivity in the TH2B region of the arginine-rich fraction (Fig. 3*D*). This result proves that negligible TH2B contaminates the arginine-rich fraction. Synthesis of a protein migrating with the H2B found in the arginine-rich fraction is also observed in pachytene cells (Fig. 3*D*).

Fig. 3*F* shows that there was essentially no [^{3}H]lysine incorporation into histones in purified round spermatid nuclei. Although round spermatids contain the somatic histones, as well as TH1 and TH2B, there was negligible synthesis of any histone. The small amount of radioactivity under the histone peaks in Fig. 3*F* was only 5% of the specific radioactivity found in pachytene nuclei, and was probably the result of minor contamination by spermatogonial and early primary spermatocyte nuclei.

To rule out the possibility that the reduction in [^{3}H]lysine incorporation into round spermatid histones was due to an increase in the endogenous lysine pools in round spermatids, we also assayed the nonhistone nuclear proteins. The specific activities of [^{3}H]lysine incorporated into the histones and nonhistone nuclear proteins of pachytene spermatocytes and round spermatids were compared (Table 2). Histone specific activity dropped to low levels in round spermatids, but there was only a slight drop in the specific activity of the nonhistones. Thus, the absence of histone synthesis in round spermatids is not the result of a low [^{3}H]lysine specific activity in the lysine pool.

Histone synthesis in sample III, which contained 17% round spermatids, 29% spermatogonia, and 53% early primary spermatocytes, is illustrated in Fig. 4. As demonstrated above, the round spermatids do not synthesize histones, so all synthesis detected in this fraction must be a result of incorporation of [^{3}H]lysine into histones by spermatogonia, early primary spermatocytes, or both. This result demonstrates that the synthesis of TH1 and probably TH2B (TH2B and H3 were not fractionated in this case) begins before pachytene. The unseparated control for this experiment is shown in Fig. 3*E*.

Histone Synthesis in Hydroxyurea-Treated Rats. To test the effect of blocking DNA replication upon the synthesis of the testis-specific histones, 20-day-old rats were treated with hydroxyurea during labeling with [^{3}H]lysine. Immature rats were chosen for this experiment because their testes contain a greater proportion of spermatogonia than do testes of adults and, therefore, more readily detectable levels of somatic histone synthesis

Testes from 20-day-old rats contained both somatic histones and testis-specific histones, although TH1 and TH2B were relatively less abundant than they are in adults (Fig. 5). When compared to untreated controls, hydroxyurea-treated rats synthesized significantly less H3, H2B, H2A, and H4 (Fig. 5 *B* and *D*; Table 3). These same hydroxyurea-treated animals incorporated [^{14}C]thymidine into DNA at 1% of control values. In contrast, the reduction from controls in synthesis of TH1, H1, and TH2B was minimal and not statistically significant. The results of seven experiments on 20-day-old rats are given in Table 3. Therefore, under the conditions of these experiments, hydroxyurea has a differential effect on the synthesis of the various histones.

Table 2. Specific activity of histones and nonhistone chromosomal proteins in pachytene and round spermatid nuclei

	Specific activity, cpm/μg protein		
Proteins	Unseparated testis nuclei	Pachytene nuclei	Round spermatid nuclei
Histones (acid-soluble)	35	48	4.4
Nonhistones (acid-insoluble)	4.4	6.9	5.0

The "histone fraction" represents the acid-soluble proteins and consists primarily of histones (see Fig. 3*F*).

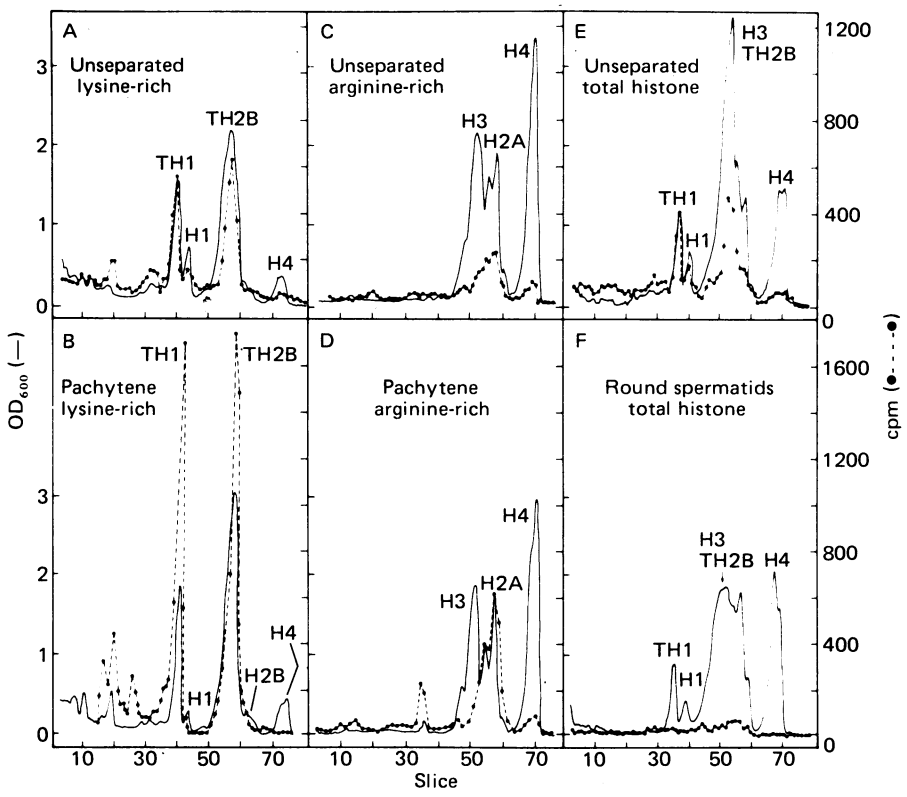

FIG. 3. Densitometric scans (—) and radioactivity profiles (•----•) of electrophoretically separated histones that were extracted and fractionated from separated and unseparated rat testis nuclei. (*A*) Unseparated control, lysine-rich histones; (*B*) purified pachytene nuclei, lysine-rich histones; (*C*) unseparated control, arginine-rich histones; (*D*) purified pachytene nuclei, arginine-rich histones; (*E*) unseparated control, total unfractionated histones; and (*F*) round spermatid nuclei, total unfractionated histones.

DISCUSSION

The testis contains, in addition to the somatic histones, several unique histones and other basic nuclear proteins. Recently, attention has been directed toward the basic protein transitions that occur during nuclear condensation and elongation. In the rat, it has been shown that acid-soluble nuclear proteins are found, as well as synthesized, only in elongated spermatids (9, 16–18). Furthermore, the synthesis and turnover of these proteins is highly stage specific. In addition to the basic nuclear proteins in elongated spermatids, the rat testis contains other unique histones. Two of them, TH1 and TH2B [originally designated X1 and X3, respectively, by Branson *et al.* (3)], appear to be found only in the testis. The tissue specificity of TH1 is not certain, because Kistler and Geroch (16) reported that some other tissues contain small amounts of a protein that migrates similarly to TH1. TH1 is similar in amino acid composition to H1 (3, 19), but the two histones have significant differences in their lysine and valine contents. TH2B is a variant of H2B (4), as demonstrated by amino acid analysis, molecular weight determinations, and chemical fractionation. The main difference between them is that TH2B contains cysteine.

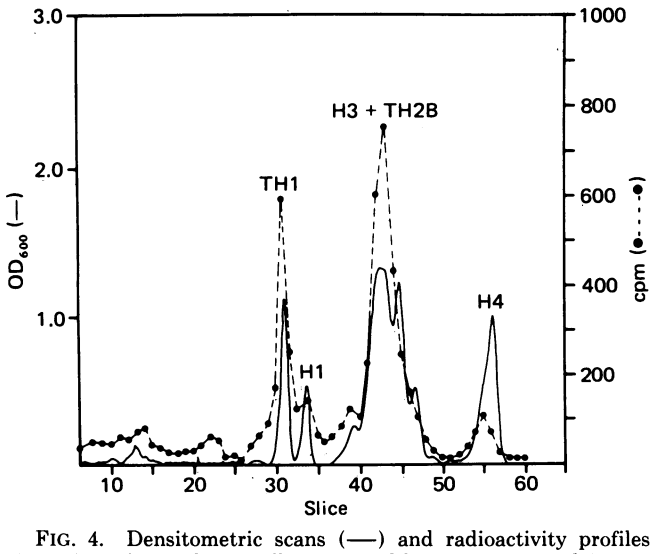

FIG. 4. Densitometric scans (—) and radioactivity profiles (•----•) of electrophoretically separated histones extracted from a fraction containing over 80% early primary spermatocytes and spermatogonia. The histones were *not* separated into arginine-rich and lysine-rich fractions before electrophoresis.

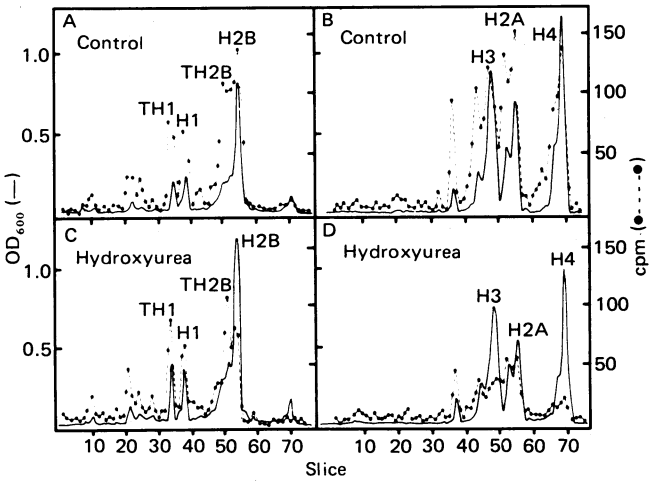

FIG. 5. Densitometric scans (—) and radioactivity profiles (•----•) of electrophoretically separated histones that were extracted and fractionated from control and hydroxyurea-treated nuclei isolated from 20-day-old rat testes. Histones and DNA were labeled by intratesticular injection of [^{3}H]lysine and [^{14}C]thymidine. (*A*) Control, lysine-rich histones; (*B*) hydroxyurea-treated, lysine-rich histones; (*C*) control, arginine-righ histones; and (*D*) hydroxyurea-treated, arginine-rich histones.

Table 3. Percent inhibition of testis histone synthesis in 20-day-old rats by *in vivo* hydroxyurea treatment

Histone	% of control specific activity ± SEM*	P†
TH1	86.1 ± 14.8	NS
H1	81.0 ± 12.2	NS
X2	67.0 ± 6.8	<0.005
H3	51.4 ± 6.7	<0.001
TH2B	93.4 ± 13.7	NS
H2B	64.7 ± 9.7	<0.025
H2A	70.4 ± 11.7	<0.05
H4	22.1 ± 6.4	<0.001
DNA	2.1 ± 0.8	

* Based on the results of seven separate experiments. The specific activity of each histone was determined by calculating the ratio between the areas under the radioactivity and protein curves.

† Probability that the mean is significantly different from 100% as calculated by a two-tailed Student's *t* test. NS, difference from 100% not significant.

In immature animals, TH1 is first detectable in the testis 7 days after birth, while TH2B is not observable until the 11th day (17). This observation, when correlated with studies of testicular histology, suggests that TH1 is first present in spermatogonia and TH2B first appears in primary spermatocytes. But the exact stages of appearance of these proteins cannot be determined by developmental studies because of limitations in the sensitivity of detection methods and because of the heterogeneous cellular composition of the testis. However, we are now able to use velocity sedimentation techniques to prepare fractions that are about 95% pure in nuclei from an individual cell type (6). In this study we were able to routinely prepare up to 10^8 pachytene or round spermatid nuclei from normal adult rats by two successive steps of velocity sedimentation: centrifugal elutriation of cells followed by Staput separation of nuclei. These procedures provided enough material for analysis of basic protein content and synthesis.

The results demonstrate that both pachytene and round spermatid nuclei contain somatic histones, as well as the testis histones TH1 and TH2B. In each of these cell types, the most abundant histones are the testis-specific forms. TH1 and TH2B are synthesized during the pachytene stage, although several somatic histones show negligible synthesis. The principal exception is the apparent synthesis of H2A during pachytene.

Analysis of histones in round spermatids revealed that negligible synthesis occurs during this stage, although all histones, both somatic and testis-specific, are present. This then, is a relatively quiescent stage with regard to basic nuclear protein synthesis even though nonhistone synthesis continues at a similar rate as in pachytene cells. Basic nuclear protein synthesis begins again after nuclear elongation commences (6, 17, 18, 20).

We also have evidence that the synthesis of TH1 and TH2B occurs in early primary spermatocytes and possibly in spermatogonia. Due to the presence of both spermatogonia and early primary spermatocytes in that fraction, it was not possible to determine if just one or both cell types synthesize TH1, TH2B, or both. The somatic histone synthesis detected in this fraction was presumably due to the presence of S-phase cells.

In most somatic cells, histone synthesis is tightly coupled to DNA replication (21, 22), and agents that block DNA replication also block histone synthesis (23, 24). One exception has been the synthesis of histones during early cleavage in the sea urchin (25). We report here that, during the pachytene primary spermatocyte stage of spermatogenesis, histone synthesis is not coupled to DNA replication. The synthesis of TH1, TH2B, and H2A takes place during that stage, while DNA synthesis proceeds at less than 1% of the rate of S-phase synthesis (26, 27). Furthermore, when DNA synthesis is inhibited by hydroxyurea treatment, synthesis of at least four histones continues. This suggests that histone synthesis in spermatocytes is regulated differently from that in some somatic cell systems.

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