H1 Histone Subfractions of Mammalian Testes. 2. Organ Specificity in Mice and Rabbits†

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ABSTRACT: H1 histones were examined in testes and somatic organs from mice and rabbits. They were extracted from washed chromatin by aqueous 5% (w/v) trichloroacetic acid and analyzed by several electrophoretic systems as well as column chromatography employing Bio-Rex 70. In each species the testicular H1 population contains at least two components that are scarce or absent in the somatic organs examined. The unusual testicular species do not appear to be phosphorylated derivatives. The studies in this and the accompanying report [Seyedin, S. M., & Kistler, W. S. (1979) *Biochemistry 18* (preceding paper in this issue)] confirm that marked changes from the somatic type H1 population are associated with spermatogenesis in mice, rabbits, and rats. However, in terms of electrophoretic and chromatographic behavior, the pattern of change is species specific.

The accompanying paper (Seyedin & Kistler, 1979) indicates that, when H1 histones are extracted from chromatin by aqueous trichloroacetic acid (5% w/v), the population of subfractions from rat testis is distinguished from those of rat thymus chiefly by (i) a markedly greater level of H1a, the first subfraction to elute from Bio-Rex 70 chromatography, and (ii) a greatly diminished level of H1b, the second subfraction to elute. In this report we have asked whether a comparable picture is found when H1 histones of mouse and rabbit testes are compared with somatic organs from each animal.

Shires et al. (1975), examining total chromatin acid soluble proteins on acetic acid/urea–polyacrylamide gels, detected a slowly migrating species of approximately the mobility of rat H1a in testis extracts from the rabbit, mouse, and monkey. Unusual H1-like histones have also been associated with male gamete development in such diverse organisms as lillies (Sheridan & Stern, 1967; Strokov et al., 1973) and roosters (Mezquita & Teng, 1977).

Although a reorganization of H1 components may accompany spermatogenesis in some organisms, there does not appear to be a general biological requirement for such a process. A careful analysis of the histones present at different stages of spermatogenesis in the cricket failed to reveal any H1 species of unusual electrophoretic mobility during either meiotic or postmeiotic stages (Kaye & McMaster-Kaye, 1974). Similarly, histone changes accompanying spermatogenesis in trout have been dissected in great detail by Dixon and his associates (Louie et al. 1973); yet this group has not reported an unusual H1 species accompanying meiotic or other stages of spermatogenesis. Indeed, judging by the behavior of trout testis H1 during Bio-Rex 70 chromatography (Marushige & Dixon, 1971) and the recent report of the complete amino acid sequence of this molecule (MacLeod et al., 1977), trout testis contains only a single H1 species.

In view of the foregoing examples, in which spermatogenesis apparently progresses satisfactorily without the appearance of unusual patterns of H1 species, it is of interest to determine whether the type of H1 changes found in laboratory rodents is characteristic of other mammals as well. In the studies to be described, we have found that rabbit testis H1 histones are indeed differentiated from the population in rabbit thymus, but that the pattern of change is quite different from that found in the rat. We have confirmed the above-mentioned observations of Shires et al. (1975) with respect to mouse testis, but have noted the occurrence of yet an additional apparent H1 species not seen in several other mouse organs.

Materials and Methods

Extraction procedures for H1 histones and chromatographic and electrophoretic techniques were as described in the accompanying paper (Seyedin & Kistler, 1979). Mature CD-1 outbred albino mice were obtained from Charles River, Wilmington, MA. Frozen rabbit thymus and testicles (mature, type 2) were obtained from Pel Freeze Biologicals, Rogers, AR, and stored at −70 °C. In some instances live, breeding-age, male, New Zealand White rabbits were obtained locally and killed by injection of sodium pentabarbital (Nembutal) prior to dissection of various organs. The pattern of H1 histones obtained from fresh and frozen rabbit testes was identical.

^{125}I Labeling of H1 Histones. Lactoperoxidase catalyzed iodination was performed according to the protocol of Marchalonis (1969). Trial studies indicated that incorporation of label into H1 histones was considerably enhanced under acid conditions, and the following procedure was adopted in which the final pH of the reaction mixture was 3.9. H1 histones (250 μg) were lyophilized from acetic acid in a small polypropylene tube, and the following were added in the order given: 10 μL of 1 M acetic acid, 9 μL of 0.1 N NaOH, 10 μL of 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.2, 1.5 μL of $Na^{125}I$ (100 μCi, 1–2 × 10^3 Ci/mmol, New England Nuclear, Boston, MA), 10 μL of lactoperoxidase (1.5 mg/mL, Sigma, St. Louis, MO), and 1.5 μL of 8.8 mM H_2O_2. The mixture was kept at room temperature for 60 min with occasional stirring, and the reaction was terminated by addition of 50 μL of 50 mM 2-mercaptoethanol. Unlabeled KI (100 μL of 1 mg/mL) was added, and labeled protein was separated from unbound iodine by gel filtration through a column (1 × 20 cm) of Sephadex G-25 equilibrated and eluted with 1% acetic acid. In a typical application of this procedure, the specific activity of the iodinated H1 histone fraction from rabbit testis was 2 × 10^4 cpm/μg.

Results

Bulk H1 histones were prepared from both mouse and rabbit organs in the presence of protease inhibitors by the procedure

† From the Department of Chemistry and School of Medicine, University of South Carolina, Columbia, South Carolina 29208. *Received October 2, 1978.* This work was supported by National Institutes of Health Biomedical Research Support Grant 1 S07 RR07160 and by Grant HD 10793 from the U.S. Public Health Service.

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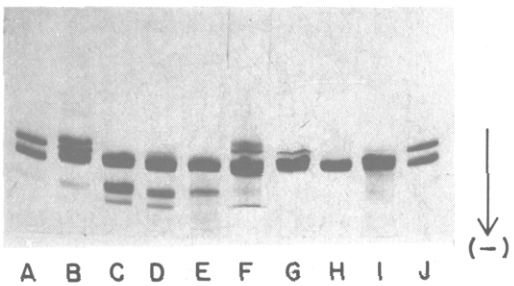

FIGURE 1: Electrophoretic analysis on acetic acid/urea slab gels of bulk H1 histones extracted from washed chromatin prepared in the presence of protease inhibitors. Individual samples represented from 15–25 μg of protein. (A) Rat testis, (B) mouse testis, (C) mouse kidney, (D) mouse liver, (E) mouse small intestine, (F) rabbit testis, (G) rabbit thymus, (H) rabbit liver, (I) calf thymus, (J) rat testis. In no case were more slowly migrating components seen. The more rapidly migrating band present in all rat and mouse samples has been identified as H1° of Panyim & Chalkley (1969).

of De Nooij & Westenbrink (1962) as described in the accompanying paper (Seyedin & Kistler, 1979). Preparations were then examined electrophoretically on acetic acid/urea slab gels (Figure 1). In this system rat testis H1 species migrate to give a doublet pattern (A and J, Figure 1) in which the faster of the two stained bands contains a mixture of up to four subfractions (principally H1c, H1d, and H1e in rat testis). The slower component of the doublet contains only subfraction H1a when bulk H1 histones are extracted by the De Nooij & Westenbrink procedure. It is this intense band of H1a that distinguishes samples of rat testis H1 histones from those of other organs. Preparations from four mouse organs were compared. Despite the fact that, for example, mouse mammary tissue is known to contain five chromatographically resolvable H1 components (Hohman & Cole, 1971), H1 histones from mouse kidney, liver, and small intestine (C, D, E, Figure 1) migrated as a single band [with the exception of H1° (Panyim & Chalkley, 1969), which is responsible for the rapidly migrating band seen in both mouse and rat tissues (Seyedin, unpublished)]. Mouse testis H1 histones (B, Figure 1), in contrast, gave rise to *two* intensely staining, slowly migrating bands that are not evident in other mouse extracts. Close inspection of the original gels indicated that other organs do contain traces of material that may correspond to the more rapid of these two bands, but that they appear devoid of the slower component.

Rabbit H1 histones, although also known to consist of at least five chromatographically separable components (Bustin & Cole, 1968), again ran largely as an unresolved mixture on acetic acid/urea gels (Figure 1). Testis (F, Figure 1) differs from the two other organs examined (thymus and liver; G, H, Figure 1) by having two minor, slowly migrating components. The faster of these two minor species is readily detectable in the thymus, while the slower is not seen in either thymus or liver.

The results of this electrophoretic examination thus indicate that the population of mouse testis H1 histones is markedly different from that of other organs, while rabbit testis H1 histones are distinguished from, for example, thymus H1 species only by the presence of a very minor, slowly migrating component.

Examination of H1 species by Bio-Rex 70 chromatography can reveal marked differences in subfraction populations that are not seen on acetic acid/urea gels. For example, such chromatography reveals that rat testis is extremely poor in component H1b, an observation that is not possible in any of several electrophoretic protocols (Seyedin & Kistler, 1979). Because of the ready availability of large amounts of tissue

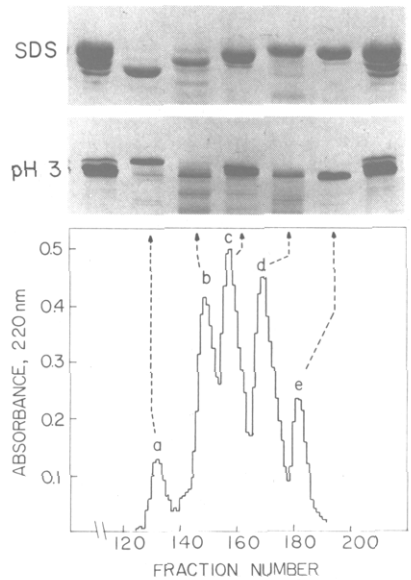

FIGURE 2: Chromatographic separation of rabbit thymus H1 histones on Bio-Rex 70 (2.5 × 25 cm) and electrophoretic analysis of chromatographically resolved fractions. In this case resolved components were subject to a long period of dialysis to eliminate salts, and it appears that several suffered some degree of proteolytic degradation as evidenced by the faster migrating components on the electrophoretic gels. It seems likely, however, that the most slowly migrating components are the undegraded molecules. Acetic acid/urea gels are denoted by the label "pH 3". The electrophoretic pattern to the extreme left and right of each gel slab represents a sample of the unfractionated H1 histones applied to the Bio-Rex 70 column.

from rabbits, we chose to examine the separation of rabbit testis and thymus H1 histones by Bio-Rex 70 chromatography. Resolved fractions were isolated and analyzed electrophoretically to permit correlation of the separation patterns obtained by the two techniques. Chromatography of rabbit thymus H1 histones (Figure 2) yielded five well-separated peaks with the quantitative distribution expected for this organ (Bustin & Cole, 1968, 1969). Examination of individual fractions by electrophoresis revealed that component H1a migrates alone as a slow band on acetic acid/urea gels, while all remaining components migrate as a faster, unresolved complex. On gels containing $NaDodSO_4$,[1] components H1a and H1b migrate as resolved entities, but the remaining three components do not separate significantly under the conditions employed.

H1 histones extracted from rabbit testes exhibited a chromatographic profile (Figure 3) that is distinctly different from that of the rabbit thymus histones. In contrast to the pattern observed with rat testis (Seyedin & Kistler, 1979), the first component to elute from Bio-Rex 70 (H1a) was actually decreased in amount compared with thymus extracts. Qualitatively, the pattern is much more complex than that found for thymus. Two additional species emerge in the peak "b" and peak "d" regions, which we have arbitrarily labeled "b_1" and "d_1". Quantitatively, peak "c" is no longer the dominant H1 subfraction. Analysis of isolated chromatographic fractions by electrophoresis indicated (i) that fraction "a", like its counterpart from thymus, runs more slowly on acetic acid/urea gels than other H1 components; (ii) that the "fine structure" revealed by chromatography did not correspond to components of unusual electrophoretic mobility in either $NaDodSO_4$ or acetic acid/urea gels; and (iii) that the

[1] Abbreviation used: $NaDodSO_4$ (SDS used in figures), sodium dodecyl sulfate.

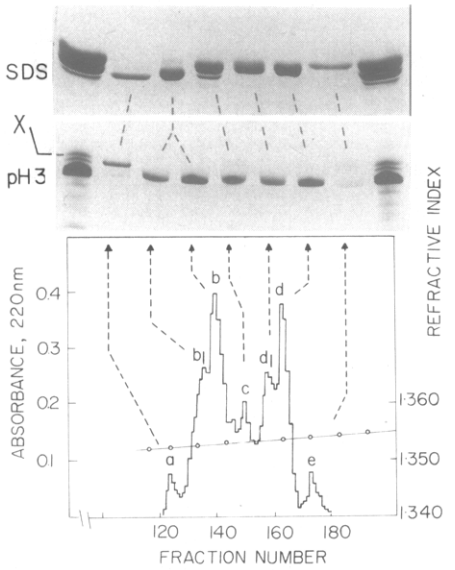

FIGURE 3: Chromatographic separation of rabbit testis H1 histones on Bio-Rex 70 (2.5 × 25 cm) and electrophoretic analysis of chromatographically resolved fractions. Individual components were freed of salts by gel filtration prior to collection by lyophilization. Acetic acid/urea gels are denoted by the label "pH 3". Fractions "b_1" and "b" were run as a mixture on the $NaDodSO_4$ (SDS) gel slab. The electrophoretic pattern to the extreme left and right of each gel slab represents a sample of the unfractionated H1 histones applied to the Bio-Rex 70 column.

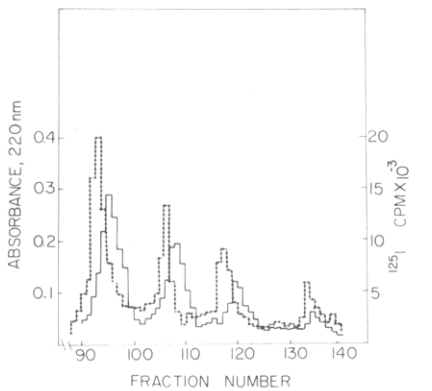

FIGURE 4: Cochromatography of ^{125}I-labeled and noniodinated rat testis H1 histones on Bio-Rex 70 (1 × 25 cm). Absorbance (—); radioactivity (- - -).

most slowly migrating band observable on acetic acid/urea gels (denoted "X" in Figure 3) probably elutes with fraction "e", although insufficient material was recovered to make this identification certain. In other experiments (not shown), we mixed portions of isolated fraction "a" with bulk testis H1 histones and then analyzed the mixture by electrophoresis on acetic acid/urea gels. The band that increased in stained intensity was that which runs between the bulk of unresolved H1 fractions and component "X".

We hoped that further insight into the similarities and differences between thymus and testis H1 histones in the rabbit could be obtained by cochromatography of samples from both organs. Radiolabeling of one set of histones by iodination with ^{125}I would allow simultaneous comparison of a tracer amount of one sample with a carrier portion of the second. Initial experiments carried out with testis H1 histones from the rat demonstrated that all four fractions clearly resolved by Bio-Rex 70 chromatography could be labeled, apparently in proportion to their relative abundance (Figure 4). However, the iodination process resulted in an altered chromatographic behavior such that the labeled components eluted about two fractions

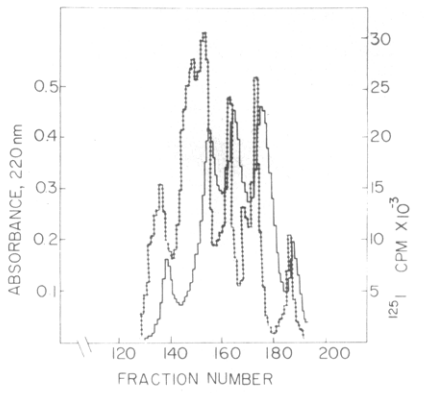

FIGURE 5: Cochromatography of ^{125}I-labeled rabbit testis H1 histones and noniodinated rabbit thymus H1 histones on Bio-Rex 70 (2.5 × 25 cm). Absorbance (—); radioactivity (- - -).

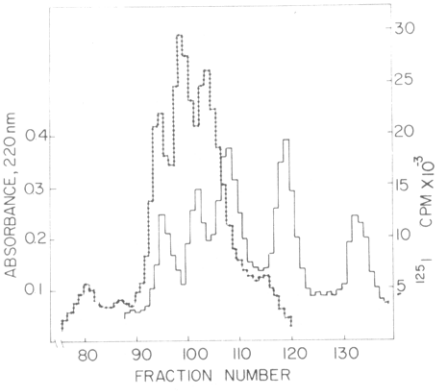

FIGURE 6: Cochromatography of ^{125}I-labeled rabbit thymus H1 histones and noniodinated rat thymus H1 histones on Bio-Rex 70 (1 × 25 cm). Absorbance (—); radioactivity (- - -).

before the unmodified proteins.

While this change in chromatographic behavior means that iodinated components will not serve as exact markers for the location of their unlabeled forms, their displacement is both slight and consistent. Therefore, rabbit testis H1 histones were iodinated, and a trace amount of this preparation was mixed with a sample of unlabeled thymus H1 histones and chromatographed. From the results (Figure 5), it is clear that all testis H1 components that were resolved previously (compare Figure 3) were labeled. Careful comparison of each radioactive and nonradioactive peak from Figure 5 again indicates that the testis components comprising fractions "b" and "d" contain two species, while thymus samples contain but single resolvable components in comparable peaks.

With the availability of iodinated H1 populations, it was possible to compare precisely the elution patterns of rat and rabbit thymus H1 histones from Bio-Rex 70 (Figure 6). While each species contains a clearly resolvable family of five components in its thymus, the two families of H1 histones clearly elute quite differently during the chromatographic separation.

Discussion

Analysis of the testicular H1 histones of mice and rabbits has uncovered a complex situation.

Mouse testes have reproducibly yielded extracts that show two prominent slowly migrating components on acetic acid/urea gels. One of these, perhaps the faster, may correspond to component H1a of rat testis [see the accompanying paper, Seyedin & Kistler (1979)]; however, neither is as prevalent in somatic organs as is rat H1a. A more decisive comparison

between rat and mouse will have to await analysis of mouse testis H1 fractions by Bio-Rex 70 chromatography. It is known from earlier work (Hohmann & Cole, 1971) that mouse tissues, like those of the rat, contain at least five resolvable subfractions.

Rabbit testis presents a markedly different situation. Analyzed by acetic acid/urea gels, rabbit testis H1 histones contain two minor bands of slow mobility, of which the faster clearly corresponds to the established somatic subfraction, H1a. In contrast to the rat (Seyedin & Kistler, 1979) and perhaps the mouse, H1a is not more prominent in the testis than in a somatic organ such as the thymus. The slower of the minor bands ("X" of Figure 3) may correspond to the similar band from mouse testis, but is not nearly so prominent. Rat testis appears to lack this component (Seyedin & Kistler, 1979). Resolution of rabbit H1 histones by Bio-Rex chromatography, however, reveals a dramatic difference between thymus and testis H1 populations, which results from the appearance of two additional testis H1 species eluting in about the positions of rabbit H1b and H1d. Further, subfraction H1c is markedly reduced in amount compared with its level in thymus.

One obvious question to ask of any of the unusual testis H1 components resolved either electrophoretically or chromatographically is whether they result from some type of posttranslational modification of one or more of the other H1 subfractions. Phosphorylation is the best characterized modification of H1 histones that might be likely to result in the generation of species of new electrophoretic or chromatographic behavior. In this respect a few points can be made. The presence of a single phosphate seems not to alter the chromatographic elution pattern of H1 histones on Bio-Rex 70 (Langan et al., 1971), although the presence of multiple phosphates, such as may be required for mitosis, does lead to altered elution patterns (Gurley et al., 1975). However, these hyperphosphorylated species normally occur only during the relatively brief phase of the cell cycle involving actual mitosis. This period is sufficiently short that, in logarithmically growing cell cultures, hyperphosphorylated species cannot be detected (Gurley et al., 1975). On the other hand, Dixon and his colleagues have shown that, during spermatogenesis in trout, multiple phosphorylated forms of H1 are readily detectable by starch gel electrophoresis (Louie & Dixon, 1973). Treatment with bacterial alkaline phosphatase has often proven effective at the removal of phosphates on H1 histones (Sherod et al., 1970; Lake et al., 1972; Louie & Dixon, 1973). We have treated both mouse testis H1 histones and tracer amounts of iodinated rabbit testis H1 histones with bacterial alkaline phosphatase for periods of 24 h under conditions in which dephosphorylation is reported to occur (Sherod et al., 1970). No change was seen in the electrophoretic behavior (mouse) or chromatographic behavior (rabbit) of the H1 extracts (unpublished observations). Earlier studies failed to detect altered electrophoretic mobility of rat testis H1a after alkaline phosphatase treatment (Kistler & Geroch, 1975). Accordingly, we feel that the unusual patterns seen for both mouse and rabbit testis H1 histones are not due to the presence of phosphorylated derivatives.

The present study indicates that an unusual distribution of H1 histones accompanies spermatogenesis in a variety of mammals. However, it appears somewhat too early to make a useful generalization regarding the pattern by which the H1 population of germinal cells will differ from that of somatic tissues. The patterns found for the rat and rabbit bear little resemblance to one another. It remains to be established which, if either, of these two species is the more general model for mammalian spermatogenesis.

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