ELECTROPHORETIC ANALYSIS OF LIVER AND TESTIS HISTONES OF THE FROG *RANA PIPIENS*

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ABSTRACT

Histones were extracted from frog livers and testes and analyzed by electrophoresis on long polyacrylamide gels and on sodium dodecyl sulfate (SDS)-containing polyacrylamide gels. Frog histones were found to be similar to those of calf thymus except that frog histone fraction F2A2 showed a marked dependence on the temperature at which the long gels were run, and frog histone fraction F3 could be separated from frog F2B on SDS-containing gels.

Comparisons between frog liver and frog testis histones indicated that the testis contains as its major F1 component a fast migrating species not found in liver. Testis histones also showed less microheterogeneity of fractions F3 and F2A1 than liver histones. These were the only differences observed between liver and testis histones, even when testis histones were prepared from sperm suspensions that were rich in cells in the late stages of spermiogenesis. Thus it seems that, in *Rana*, the electrophoretic properties of the basic proteins of sperm differ from those of somatic cells only in the nature of histone F1 and in the degree of microheterogeneity of fractions F2A1 and F3.

The sperm cells of most organisms contain highly condensed chromatin and are genetically inactive. In many species, sperm also contain basic proteins which differ from the histones associated with the chromatin of somatic cells (3, 9, 10, 18, 29). It is tempting to suggest that at least some of the differences between the basic proteins of sperm and somatic cells are somehow correlated with either the genetic inactivity or with the presence of highly condensed chromatin in sperm. However, there are organisms in which spermiogenesis is reported not to involve detectable changes in histones. For example, in the frog *Rana pipiens*, the sperm have been shown to contain somatic type histones both cytochemically (33) and biochemically (2, 30, 31). However, recent advances in methods for isolating and characterizing histones (22, 23) have led us to reexamine the histones of *R. pipiens* spermiogenic cells to determine if, in fact, there are no differences between sperm and somatic histones.

MATERIALS AND METHODS

Preparation of Tissues

Sexually mature male *R. pipiens* were obtained from Vermont Frog Farms (Albury, Vt.) and were either used as shipped or treated with tetracycline-HCl (5 mg/0.2 ml of water twice per week) following the method of Gibbs et al. (11).

For use, frogs were struck on the head, pithed, and then dissected on ice. Livers were removed and cold 0.9% NaCl-0.01 M Na-oxalate was forced through the tissue by injection with a 22-gauge needle to remove residual blood cells. Livers were used immediately for nucleus isolations or were quick-frozen on dry ice and stored at −20°C. This process usually took 4–5 min.

Testes were dissected and were either quick-frozen immediately or macerated into 10% Holtfreter's solution

and allowed to sit for 5–10 min to release sperm. The testicular material which remained at the bottom after decanting the sperm suspension was quick-frozen for future use and is referred to as testis residue. The sperm suspension was centrifuged at low speed and then frozen on powdered dry ice.

Isolation of Chromatin

Chromatin was isolated as described by Panyim et al. (22) or by a modification of their method: instead of the 2.5-h centrifugation through heavy sucrose, nuclei were suspended in 0.5 M sucrose, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 8.0), and 0.05 M $NaHSO_3$, followed by centrifugation at 8,000 rpm for 10 min.

Isolation of Histones

Histones were extracted from purified chromatin with 0.4 N H_2SO_4 (22). Histones were fractionated as described previously (17, 21).

Polyacrylamide Gel Electrophoresis

High resolution electrophoresis was performed in 2.5 M urea-containing polyacrylamide gels (23). Gels were stained with fast green (12). Sodium dodecyl sulfate (SDS)-containing polyacrylamide gels, pH 7.6 (25) were stained as described previously (13). Gels were scanned using a Gilford model 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Whole calf thymus histones and purified calf thymus F2A1 were used as standards.

Phosphatase Treatment

Histone fraction F1 isolated from sperm suspension was treated with *Escherichia coli* alkaline phosphatase as described by Sherod et al. (28).

RESULTS

Electrophoretic Analysis of Frog Liver Histones

Fig. 1 shows desitometer tracings of gels containing histones isolated from frog liver. The various histone fractions were identified by the criteria described by Panyim et al. (22). Confirmation of these identifications was obtained by fractionating liver histones (17, 21). Although completely pure histone fractions were not obtained, the enrichment of particular fractions was sufficient to allow identification.

Separation between frog histone fractions F2B and F2A2 was achieved by analyzing liver histones on long gels run in the cold (Fig. 2) instead of at room temperature. Resolution of these two fractions on gels run in the cold appears to be due to a slight reduction in the relative electrophoretic mobility of F2A2 (Table I). In the cold, the relative mobility of fraction F1 is also increased so that the fastest migrating subfraction of F1 now overlaps with the slowest migrating subspecies of F3 (Fig. 2).

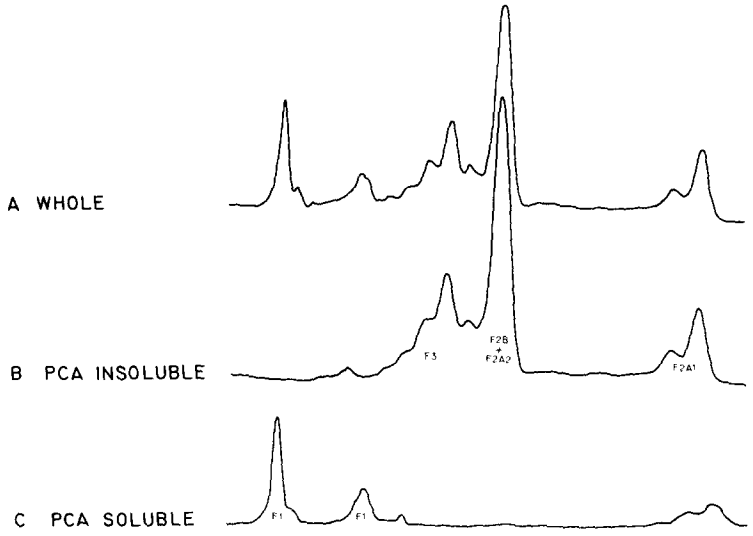

FIGURE 1 Densitometer tracings of long polyacrylamide gels containing histones extracted from frog liver. (A) whole histones; (B) 5% perchloric acid-insoluble histones; (C) 5% perchloric acid-soluble histones plus purified calf thymus F2A1 added as a mobility marker. Electrophoresis at room temperature, 250 V for 26 h.

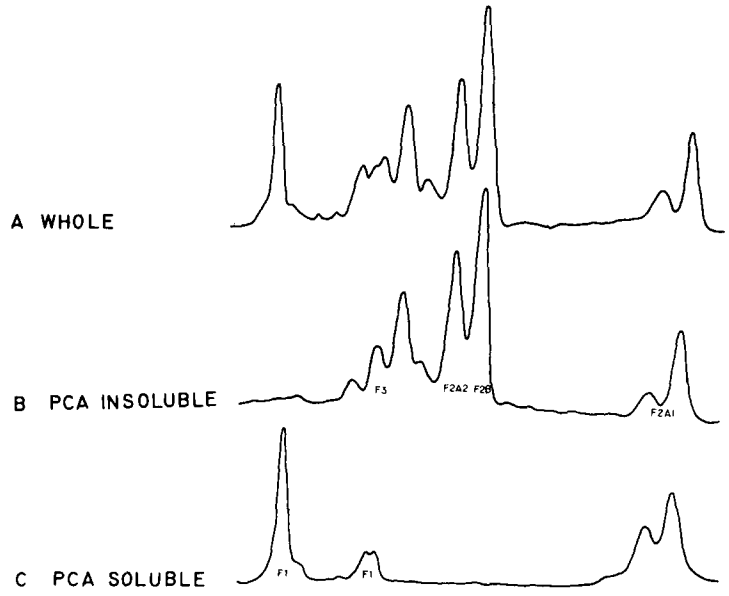

FIGURE 2 Densitometer tracings of long polyacrylamide gels containing histones extracted from frog liver. (A) whole histone; (B) 5% perchloric acid-insoluble histones; (C) 5% perchloric acid-soluble histones plus purified calf thymus F2A1 added as a mobility marker. Electrophoresis at 5°C, 250 V for 28 h.

TABLE I

Relative Electrophoretic Mobilities of Frog Histones in Urea-Acrylamide Gels*

Fraction	Liver gels						Sperm suspension gels					
	Room temp			Cold			Room temp			Cold		
	1A	1B	1C	2A	2B	2C	4A	4B	4C	5A	5B	5C
F2A1												
Faster‡	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Second	0.978	0.980		0.977	0.978		0.978	0.978		0.974	0.977	
F2B	0.847	0.856		0.854	0.856		0.853	0.853		0.854	0.859	
F2A2												
Faster	0.847	0.856		0.836	0.837		0.853	0.853		0.837	0.841	
Second	0.820	0.829		0.811	0.813							
F3												
Fastest	0.806	0.813		0.798	0.800		0.808	0.813		0.799	0.804	
Second	0.790	0.800			0.782							
Third	0.775	0.783		0.766	0.763							
F1												
Fastest									0.805			0.840
Second	0.737		0.748			0.781	0.745		0.745			0.773
Third	0.677		0.687	0.707		0.718	0.683		0.684	0.707		0.711

* Calculated from measurements made on the densitometer tracings.

‡ Mobility of the fastest migrating subspecies of frog F2A1 or of purified calf thymus F2A1 arbitrarily set equal to 1.000.

Fig. 3 A shows a densitometer pattern of frog liver histones run in SDS-polyacrylamide gels. All five frog liver histone fractions are clearly resolved. The only fraction which is heterogeneous on these gels is F1.

Electrophoretic Analysis of Histones of Sperm Suspensions

The procedure used to remove sperm from testes resulted in a heterogeneous mixture of cells including sperm, spermatids, and other cell types. All sperm suspensions were examined microscopically to insure a predominance of later stages. In no case were sperm suspensions used which contained less than 50–60% elongated stages (sperm, late spermatids).

Figs. 4 and 5 show densitometer tracings of long gels containing histones prepared from sperm suspension. Except for histone F1, the major histones of sperm suspension have electrophoretic mobilities indistinguishable from those of liver histone fractions (Table I). Liver F1 contains two distinct subfractions (Figs. 1 C and 2 C). F1 of sperm suspensions contains small amounts of the two F1 subfractions of liver, but the major F1 subspecies of sperm suspensions is absent in liver. This F1 fraction of sperm suspension is heterogeneous and has an electrophoretic mobility overlapping that of histone F3 in gels run at room temperature (Fig. 4). On gels run in the cold (Fig. 5), the major F1 of sperm suspension overlaps fraction F2A2.

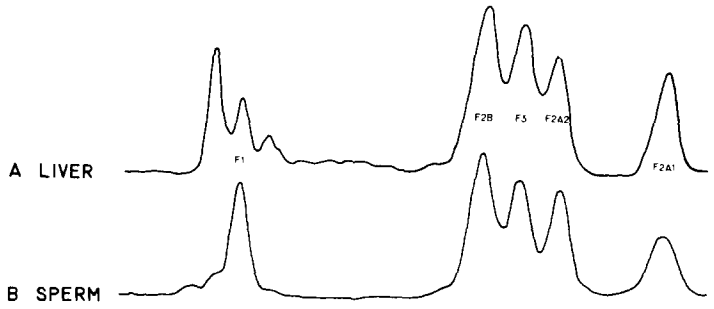

FIGURE 3 Densitometer tracings of SDS-polyacrylamide gels, pH 7.6, containing (A) whole frog liver histones; (B) whole sperm suspension histones. The frog liver histone fractions have been identified by coelectrophoresis of whole frog liver histones with the purified histone fractions. The identity of fraction F3 has been further confirmed by treatment with [*N*-^{14}C]ethylmaleimide (32) and by the fact that it can be oxidized to a slower migrating form upon storage. The amount of the fastest migrating subspecies of liver F1 is somewhat variable. It may be a product of limited proteolytic degradation. Electrophoresis at room temperature, 80 V for 8.5 h.

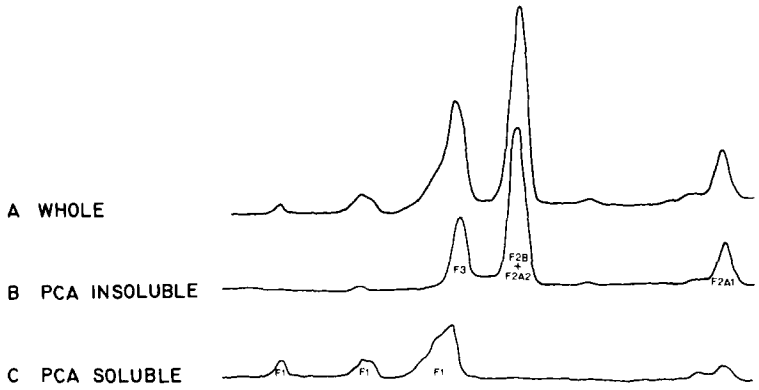

FIGURE 4 Densitometer tracings of long polyacrylamide gels containing histones extracted from frog sperm suspension. (A) whole histones; (B) 5% perhcloric acid-insoluble histones; (C) 5% perchloric acid-soluble histones plus purified calf thymus F2A1 added as a mobility marker. The histone fractions present in each peak were identified either by perchloric acid solubility (F1) or by comparison with liver histones (F2A1, F2A2, F2B, F3). Electrophoresis at room temperature, 250 V for 26 h.

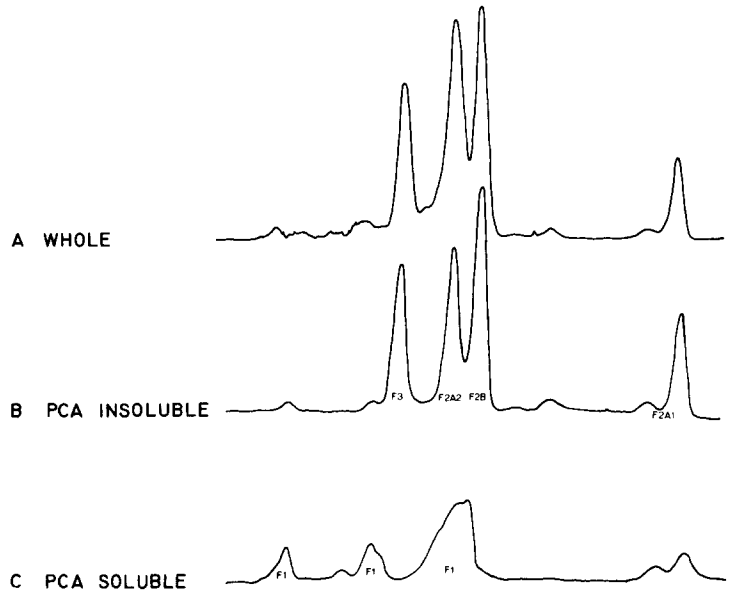

FIGURE 5 Densitometer tracings of long polyacrylamide gels containing histones extracted from frog sperm suspension. (A) whole histones; (B) 5% perchloric acid-insoluble histones; (C) 5% perchloric acid-soluble histones plus purified calf thymus F2A1 added as a mobility marker. The histone fractions present in each peak were identified either by perchloric acid solubility (F1) or by comparison with liver histones (F2A1, F2A2, F2B, F3). Electrophoresis at 5°C, 250 V for 28 h.

TABLE II

Relative Amounts of the Major Fractions of Frog Liver and Sperm Suspension*

Fraction	Liver gels‡				Sperm suspension gels‡			
	1	2	3 A	Avg	4	5	3 B	Avg
F2A1	0.147	0.149	0.134	0.143	0.103	0.129	0.137	0.123
F2B		0.217	0.279	0.248		0.260	0.283	0.272
	0.410				0.480			
F2A2		0.207	0.143	0.175		0.221	0.182	0.202
F3	0.231	0.236	0.216	0.227	0.205	0.174	0.202	0.194
F1	0.212	0.191	0.227	0.210	0.213	0.217	0.195	0.208

* The total area under each densitometer tracing was arbitrarily set equal to 1.000 and the relative amount of each histone was determined as a fractional value. From 1 to 7% of the various tracings did not correspond to any particular fraction and are excluded from the analysis.

‡ Numbers refer to figures in the paper which were actually measured.

When the histones of sperm suspension are analyzed on SDS-polyacrylamide gels (Fig. 3 B), four of the five major histone bands are indistinguishable from those of liver (Fig. 3 A). However, the F1s of sperm suspension and liver differ. Liver F1 is usually resolved into two (sometimes three) peaks in SDS gels; F1 from sperm suspension consists largely of a single peak with an electrophoretic mobility similar to that of the middle F1 subfraction of liver (Fig. 3 B).

Quantitative analysis of the amounts of the histone fractions extracted from liver and from sperm suspension indicate that the two tissues contain similar amounts of the five major histone fractions (Table II). However, sperm suspension contains less of the (presumably acetylated) slower migrating subfractions of histones F3 and F2A1 than liver (compare Figs. 2 B and 5 B).

We have also compared histone F1 extracted from liver with that extracted from whole testes,

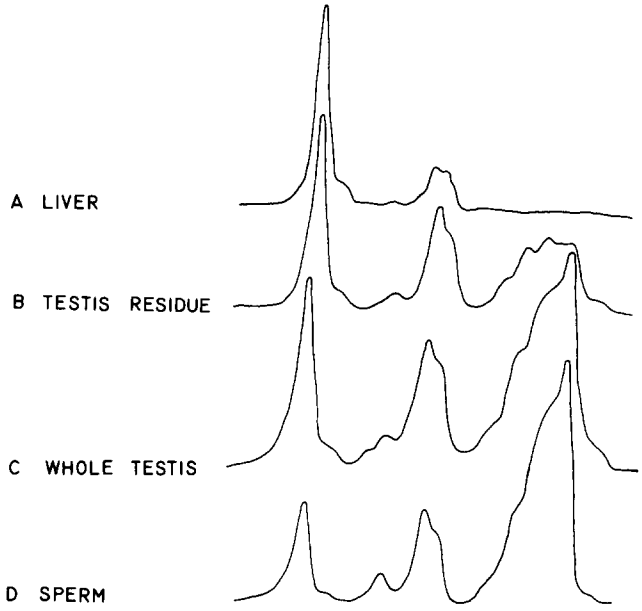

FIGURE 6 Densitometer tracings of the upper portions of long polyacrylamide gels containing 5% perchloric acid-soluble fractions extracted from: (A) liver; (B) testis residue; (C) whole testis; (D) sperm suspension. Electrophoresis at room temperature, 250 V for 25 h.

from sperm suspension, and from the residue that remained after decanting the sperm suspension from the macerated testes. The percentages of cells in late stages of spermiogenesis in these three testicular preparations should vary as follows: sperm suspension > whole testes > testes residue. The relative amount of the major F1 fraction of sperm suspensions varies in a similar manner, while the amount of the fraction which is the major component of liver F1 decreases with increasing numbers of cells in late stages (Fig. 6; Table III). The faster migrating, heterogeneous F1 subfraction(s) of liver seems to make up a similar percentage of the total F1 of liver and of the three testicular preparations.

The heterogeneity of the fastest migrating subfraction of testis F1 is due to the presence of covalently bound phosphate groups. Treatment of testis F1 with alkaline phosphatase converts this broad peak to a single, sharp peak having a relative electrophoretic mobility the same as that of the fastest migrating subspecies of the broader peak (Fig. 7).

TABLE III

Relative Amounts of F1 Subfractions in Frog Liver and Testis Histones*

F1 fraction	Liver	Testis residue	Whole testis	Sperm suspension
Fastest		0.418	0.575	0.684
Second	0.268	0.276	0.227	0.187
Third	0.732	0.306	0.198	0.128

* Values obtained from gels in Fig. 6 and expressed as fractional values of total F1.

DISCUSSION

Frog Liver Histones

Not unexpectedly, the histones isolated from frog liver nuclei are similar to those of other vertebrates (22) when analyzed on long polyacrylamide gels at low pH. A difference between frog histones and those of calf thymus was observed concerning the altered mobility of frog F2A2 on long gels run in the cold. The mobility of fraction F2A2 (relative to unacetylated F2A1) changes from approximately 0.838 at room temperature to about 0.852 in the cold. In the cold, frog F2A2 migrates more slowly than frog F2B, while calf thymus F2A2 migrates faster than calf F2B. While the basis for this temperature dependence is not clear, these observations do suggest that varying the temperature at which electrophoresis is performed can provide a means of resolving different histone factions in some organisms.

Frog histones also differ from calf thymus

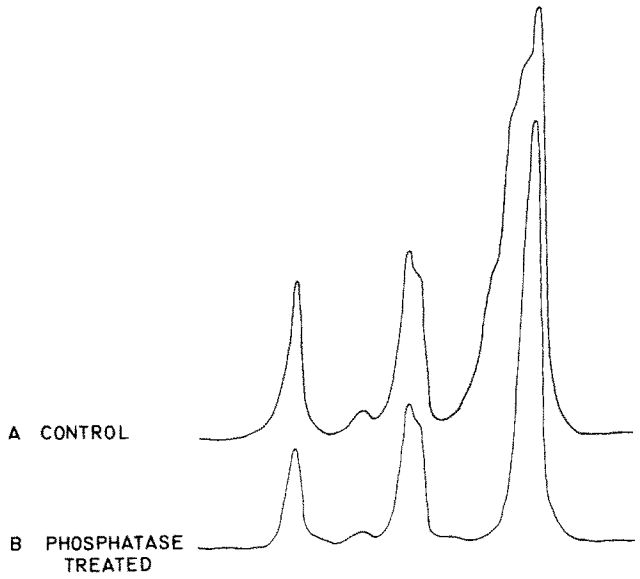

FIGURE 7 Densitometer tracing of long polyacrylamide gels containing F1 extracted from sperm suspension. (A) control: (B) treated with alkaline phosphatase for 8 h. Electrophoresis at 5°C for 4 h at 300 V, then for 20.5 h at 200 V.

histones in their behavior on SDS-acrylamide gels. The relative electrophoretic mobility of frog F2B on these SDS gels was 0.83, the same as that of the unresolved mixture of calf thymus F2B and F3. The relative mobility of frog F3 in these SDS gels is 0.88, thereby indicating a difference in the relative electrophoretic mobilities of calf and frog F3. This is rather surprising since histone F3s of different organisms have the same mobilities on urea-polyacrylamide gels at low pH (26) and the amino acid sequences of F3s from diverse sources are remarkably similar (4, 8, 15). Since histones show anomalous behavior on SDS-containing polyacrylamide gels (25, 14, 16), the molecular basis for this difference between calf and frog F3 is not clear.

Frog Testis Histones

The electrophoretic mobilities of histones F2B, F3, F2A2, and F2A1 of frog sperm suspension are indistinguishable from those of their counterparts in liver on all of the gel systems examined here. All five of the histone fractions are present in similar amounts in liver and in sperm suspension, although there are less slower migrating subspecies of F2A1 and F3 in sperm suspension than in liver. These subspecies probably represent acetylated forms of the major (parent) species of these molecules (7, 32). Easton and Chalkley (9) have shown that fractions F3 and F2A1 of sea urchin sperm are free of microheterogeneity due to secondary modification by processes such as acetylation or phosphorylation. The small amounts of the (presumably) acetylated subspecies of F3 and F2A1 found in the histones of sperm suspensions studied here could be due to contamination of the sperm suspension with early spermatogenic cells or with somatic cells of the testes. If this is so, then microheterogeneity of these fractions is also reduced or absent in frog sperm. However, it will be necessary to prepare pure preparations of fully mature frog sperm to demonstrate this conclusively.

When examined on low pH urea-acrylamide gels, testis F1 contains a fraction which migrates considerably faster than any F1 fraction found in liver. The differences between testis F1 and liver F1 probably are not due to secondary modification since modifications which cause changes in mobility as great as those observed between liver and testis F1s have not been described. It is also unlikely that the rapidly migrating testis F1 is a product of limited proteolytic degradation of a slower F1 since the mobility of testis F1 on SDS-containing gels is similar to that of liver F1. Also, we have taken considerable care to isolate histones from healthy frogs under conditions which are known to inhibit proteolysis (1), and have never seen any indications that proteolysis was occurring in preparations isolated from testis ma-

terials. The fastest migrating testis F1 probably represents a tissue-specific fraction (see references 5, 6, 19, 24, and 27 for other examples of tissue specificity of F1) which has a higher charge to mass ratio than liver F1. The heterogeneity of the fastest migrating testis F1 is due to phosphorylation which is a common feature of the metabolism of histone F1 and which has been reported to occur in spermatogenic tissues in other organisms (20).

It is of course possible that there are differences between frog and testis histones which are not detected by the electrophoretic methods used here. Nonetheless, this study describes one of the smallest degrees of difference known to occur between the histones of sperm and of somatic cells when examined by this highly sensitive technique.[1] Extracts of testes in which at least 50–60% of the cells represent late stages in spermiogenesis (sperm, late spermatids) contain histones which differ significantly from those of liver only in fraction F1 and in the microheterogeneity of fractions F2A1 and F3 (and possibly F2A2). Zirkin (34) has reported that no structural changes occur in the chromatin (condensation is not considered to be a structural change in the chromatin itself) during sperm formation in *Rana*. It is possible, therefore, that the absence of distinct ultrastructural changes in chromatin during frog spermiogenesis is correlated with the minimal changes in basic proteins which are described here. The more marked changes in basic proteins which occur during spermiogenesis in other organisms may then be related to more extensive structural alterations of chromatin (18).

Finally, our results suggest that if histones play any role either in causing or maintaining the genetic inactivity and condensation of chromatin in *Rana* sperm, that role can only be associated either with histone F1 or with the cessation of processes that secondarily modify histones. It should be noted, however, that the precise roles, *if any*, of different histone fractions or of processes which secondarily modify histones in either chromatin condensation or in the control of genetic activity are still obscure (13, 16).

[1] It should be noted that other studies using less sensitive methods than the high resolution gel techniques used here have also concluded that sperm formation can occur with little or no change in basic proteins (see reference 30, for example). However, until these studies are repeated using more sensitive methods, it is not possible to determine whether these other organisms show differences between sperm and somatic histones which are as small as (or smaller than) those reported here.

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