Lastly, the S-30 extracts employed in these studies do contain low levels of nuclease which degrade the input intact STNV [^{125}I]RNA during long-term incubations. 5′-Terminal cap structures will protect mRNAs from certain nuclease degradations (Furuichi et al., 1977). The observed higher extent of 15 U-^{14}C-labeled amino acid incorporation (i.e., long-term translation) by limiting quantities of capped STNV RNA (Figure 3) and the slightly greater pm^{7}G dependent inhibition of long-term translation of capped STNV RNA (Figure 3) probably reflect these endogenous nuclease problems and cap dependent long-term stabilization of STNV RNA.

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H1 Histone Subfractions of Mammalian Testes. 1. Organ Specificity in the Rat†

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ABSTRACT: When H1 histones are extracted from chromatin by 5% (w/v) aqueous trichloroacetic acid, the population obtained from rat testis differs from that found in somatic organs in several ways. A readily observable difference is the presence in testicular extracts of a very prominent component that migrates more slowly than other H1 subfractions during electrophoresis in acetic acid/urea–polyacrylamide gels. We present several lines of evidence that indicate that this H1 species is not a unique product of germinal tissue. Rather, it corresponds to a minor H1 component of many other organs, namely, H1a, the first subfraction to elute during chromatography on Bio-Rex 70. Thus, H1a fractions isolated from rat testis and thymus cannot be distinguished by Bio-Rex 70 chromatography or by electrophoresis under two different conditions in which H1a is resolved from all other subfractions: (i) in the presence of acetic acid/urea; and (ii) in the presence of sodium dodecyl sulfate. While H1a evidently plays a special role during male gamete development, and constitutes about 36% of the total H1 histone present in the adult rat testis, it is present in many other organs and represents about 12% of the H1 population in rat thymus. A further distinctive feature of the rat testis H1 population is the lack of readily detectable H1b, the second subfraction to elute from Bio-Rex 70. The functional requirement(s) underlying these changes in the H1 population is not understood.

H1 histones are known to display microheterogeneity due to differences in amino acid sequence (Kinkade & Cole, 1966; Bustin & Cole, 1968; Rall & Cole, 1971), and at least six subfractions have been identified in various rat organs (Kinkade, 1969; Payim & Chalkley, 1969a,b). The distribution of these subfractions among various organs is not uniform, and this fact suggests that one or more of them may have distinctive functional roles.

For some time it has been thought that one such role might relate to meiosis. Sheridan & Stern (1967) reported over a decade ago that the process of meiosis in the lilly and the tulip is associated with a special H1-like histone that is absent or present in but trace amounts in somatic tissues. This observation has been confirmed (Strokov et al., 1973), and these authors suggested that the meiotic histone might be a com-

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ponent of the synaptonemal complex. An unusual complement of H1 histones has also been found in the sexually mature rat testis. Specifically, this organ contains a prominent H1 component that is distinguished from other rat H1 subfractions by distinctly slower electrophoretic mobility on acetic acid/urea–polyacrylamide gels (Branson et al., 1973; Kistler & Geroch, 1975; Branson et al., 1975; Shires et al., 1975; Platz et al., 1975; Mills et al., 1977). For reasons developed later in this paper, we will refer to this H1 subfraction as H1a.

There is general agreement that the presence of a high level of H1a in rat testis is associated with active spermatogenesis. Thus, it is severely reduced in amount in the very immature (Kistler & Geroch, 1975; Grimes et al., 1975; Shires et al., 1975) or cryptorchid testis (Kistler & Geroch, 1975) or in the testis of a hypophysectomized (Grimes et al., 1975) or germ cell deficient rat (Mills et al., 1977). In addition, fractionation of rat testis cell suspensions by sedimentation at unit gravity has indicated that large amounts of this subfraction are associated with cells undergoing meiosis (spermatocytes) as well as with young haploid cells (round spermatids) (Platz et al., 1975).

In the process of attempting to delineate the roles of specific H1 subfractions in events that are specific to gametogenesis, such as meiosis, or specific to spermatogenesis, such as the replacement of somatic histones by sperm or spermatid-specific chromosomal proteins, it is important to determine whether testicular H1 components are unique or are molecules that find roles in nongerminal tissues as well. In the present report we present evidence that the presence of readily detected H1a in the rat testis represents a quantitative rather than qualitative difference between testis and other organs. In a companion study (Seyedin & Kistler, 1979) we have compared testicular and somatic H1 populations in mice and rabbits.

Materials and Methods

Sources of Animal Tissue. Mature Sprague-Dawley rats were obtained from Sprague-Dawley, Madison, WI. Animals were killed by cervical dislocation, and organs were processed immediately or were frozen in liquid nitrogen and stored at −70 °C.

Preparation of H1 Histone Extracts. The procedure of De Nooij & Westenbrink (1962) was used with all steps performed at 0–4 °C. Our homogenization and washing medium contained 0.12 M NaCl, 0.02 M NaOH, and sufficient acetic acid to bring the pH to 4.0. In some experiments this medium was modified to contain protease inhibitors, in which case the final concentrations of individual components were 0.12 M NaCl, 0.02 M sodium metabisulfite, and 0.3 mM phenylmethanesulfonyl fluoride (added immediately before use from a stock solution in isopropyl alcohol). The pH was adjusted to 4.0 by addition of NaOH as necessary. Normally, crude chromatin was prepared by collecting material that sedimented after centrifugation at 1000*g* for 10 min and then washing this pellet twice by suspension in the homogenization medium followed by centrifugation. The recovery of DNA by this procedure was routinely found to be about 85%. The washed pellet was suspended in ice-cold distilled water and adjusted to a concentration of 5% (w/v) Cl_3CCOOH by adding a suitable volume of a 100% stock solution. Precipitated material was removed by centrifugation, and soluble proteins were freed of Cl_3CCOOH by extensive dialysis against distilled water or by gel filtration through a standardized column of Sephadex G-25 equilibrated and eluted with 1% (v/v) acetic acid. In either case H1 histones were then collected by lyophilization. In some cases H1 histones were extracted directly from a portion of the initial, uncentrifuged homogenates. Cl_3CCOOH was immediately added to the homogenate to give a concentration of 5%, and those proteins that were not precipitated were collected as described above.

Ion-Exchange Chromatography. Bulk H1 histones prepared as described above (20–25 mg) were fractionated on a column (2.5 × 30 cm) of Bio-Rex 70, 100–200 mesh (Bio-Rad, Richmond, CA) at room temperature using a linear gradient (1300 mL) of 7–14% (w/v) guanidine hydrochloride in 0.1 M sodium phosphate at pH 6.5. The guanidine hydrochloride solutions from which the gradient was formed contained the indicated weight of guanidine hydrochloride and a 0.1 volume of a sodium phosphate stock solution (0.5 M NaH_2PO_4, 0.5 M Na_2HPO_4). The final mixture was then adjusted to pH 6.5 by addition of NaOH. The refractive index of the starting solution for the gradient was 1.3475 and that of the final solution, 1.3597. A flow rate of 9 mL/h was established with a peristaltic pump, and fractions of 3 mL were collected. In some cases a smaller column (1 × 25 cm) was used, in which case 5–6 mg of bulk H1 histones was applied and fractionated using a gradient formed from 160 mL of each of the above solutions. Flow was maintained at 2 mL/h.

Polyacrylamide Gel Electrophoresis. Acetic acid/urea gels and those at pH 4.5 were prepared as slabs (0.2 cm thick × 10 cm long), run, and stained with Coomassie brilliant blue R1 as described previously (Kistler & Geroch, 1975). $NaDodSO_4$[1] (British Drug House) containing gels were run as slabs of the above dimensions containing 15% acrylamide (Eastman) following exactly the protocol of Laemmli (1970).

Miscellaneous Procedures. Protein was estimated by the procedure of Lowry et al. (1951) using crystalline bovine serum albumin as standard. DNA was estimated by the diphenylamine procedure described by Burton (1956). Measurements of pH employed an Orion combination glass electrode and were made at room temperature. Unless otherwise stated, where solutions were formed from multiple components, pH measurements refer to completed mixtures. Amino acid analysis was performed on a Durrum analyzer as described previously (Kistler et al., 1975).

H1 Histone Nomenclature. We have followed the tradition of naming individual H1 subfractions in order of their elution from Bio-Rex 70. As the thymus seems to contain all of the clearly identified H1 subfractions of the rat (Kinkade, 1969), we have used thymus as a reference organ in naming H1 subfractions isolated from other organs. As it seems somewhat more euphonious to follow a numeral with a letter rather than with another numeral, we have denoted individual subfractions by lower case letters. Unfortunately, a minor H1 species identified by Panyim & Chalkley (1969a,b) and generally referred to as H1°, has occasionally been termed H1a (Varricchio, 1977). H1° migrates more rapidly on acetic acid/urea gels than the other recognized H1 subfractions (Panyim & Chalkley, 1969a,b). However, it elutes from chromatography on Bio-Rex 70 considerably later than the other H1 subfractions (Panyim & Chalkley, 1969a) and is not seen in the portion of the elution profile shown in our figures.

Results

Verification of the Presence of H1a in Organs Other Than Testis. We have employed extraction of H1 histones by aqueous 5% Cl_3CCOOH (De Nooij & Westenbrink, 1962) as we find that this procedure does not liberate noticeable amounts of the acid-soluble nonhistone proteins HMG1 and HMG2, whose electrophoretic properties in some systems are

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

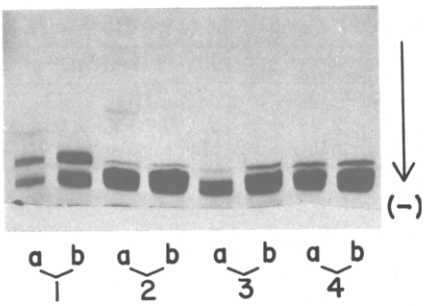

FIGURE 1: Electrophoretic analysis of H1 histones of various rat organs on acetic acid/urea slab gels. For each organ side by side comparison is made between proteins extracted from the initial homogenate (denoted "a") and those extracted from washed chromatin prepared from a separate portion of the same homogenate (denoted "b"). Except where noted, each sample contained 40 μg of protein. Samples are: 1-a (20 μg), 1-b, testis; 2-a, 2-b, lung; 3-a (20 μg), 3-b, small intestinal mucosa; 4-a, 4-b, thymus.

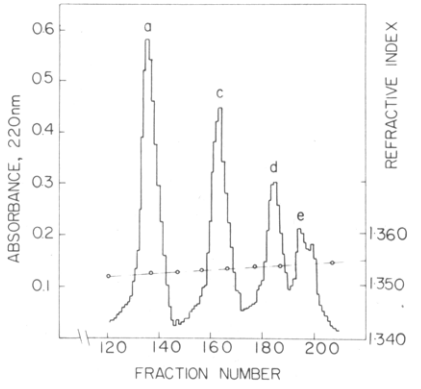

FIGURE 2: Chromatographic separation of rat testis H1 histones from washed chromatin on Bio-Rex 70 (2.5 × 25 cm). Peaks are identified by comparison with those of rat thymus (see Figure 3).

very similar to those of H1 histones. As a check against possible loss or degradation of the H1 species of interest during preparation of chromatin, we compared H1 populations extracted directly from whole homogenates as well as from salt-washed chromatin prepared from the same homogenates. Comparison of the extracts on acetic acid/urea–polyacrylamide gels (Figure 1) showed that lung, small intestinal mucosa, thymus, and kidney (not shown) all contained small amounts of the slowly migrating H1a histone component so prominent in testicular extracts. No quantitative difference is evident between the amounts of H1a present in initial homogenates and that recovered in the washed chromatin. Examination of these same extracts on pH 4.5 gels again showed no differences to result from the washing procedures (not shown). Furthermore, as noted by others (Kinkade & Cole, 1966), there is actually little gain in purity of the H1 preparations that results from the extraction of washed chromatin as compared with the extraction of unfractionated tissue.

Our preliminary study (Kistler & Geroch, 1975) indicated that the slowly migrating H1 species on acetic acid/urea gels is the first H1 component to elute from chromatography on Bio-Rex 70 by the procedure of Kinkade & Cole (1966). As this chromatographic protocol remains the most effective method for resolving the multiple H1 components of several species, we felt it was important to establish that H1a from testis and thymus would, in fact, coelute during such chromatography. Accordingly rat testis H1a was prepared by chromatography of total testis H1 histones (Figure 2). Peak fractions of testis H1a were pooled, separated from buffer components by gel filteration, and lyophilized. Bulk thymus

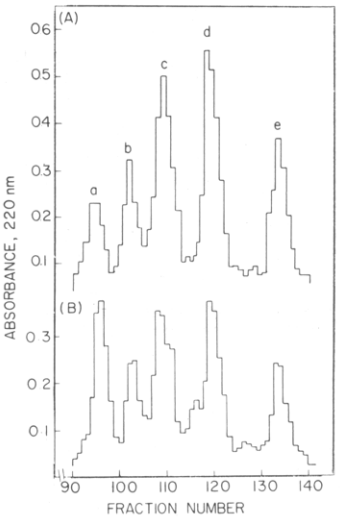

FIGURE 3: (A) Chromatographic separation of rat thymus H1 histones from washed chromatin on Bio-Rex 70 (1.0 × 25 cm). (B) Co-chromatography of 0.5 mg of purified fraction H1a from rat testis with bulk H1 histones of rat thymus.

Table I: Quantitative Distribution of H1 Subfractions in Rat Thymus and Testis[a]

	H1a	H1b	H1c	H1d	H1e
thymus	12%	13%	26%	30%	19%
testis	36%	ND[b]	30%	19%	15%

[a] Individual subfractions are estimated as a proportion of the total using the absorbance values from the chromatographic separation of Figures 2 (testis) and 3A (thymus). [b] Not detected.

H1 histones were then separated on an identical column in the absence (Figure 3A) and presence (Figure 3B) of about 5 mg of added testis H1a. From the increase in component H1a seen in Figure 3B, it is clear that testis and thymus H1a do coelute from this sensitive chromatographic procedure.

An estimation of the quantitative distribution of individual subfractions of both testis and thymus is given in Table I. It is notable that H1a is the principal component of testis H1 species but the least prevalent component of the thymus H1 family. Equally interesting is the lack of readily detectable amounts of H1b in testis preparations.

Analysis of H1 Subfractions by NaDodSO$_4$ Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis has not been reported for individual rat H1 components isolated from Bio-Rex 70 chromatography. In view of the common use of this technique in numerous studies of chromosomal proteins, we analyzed the behavior of bulk H1 histones and certain isolated H1 components on such gels (Figure 4). Component H1c of rat thymus migrates most rapidly and separates from all other components. Component H1a migrates just behind H1c and is also clearly resolved from all other H1 species. The remaining H1 components (H1b, H1d, H1e) migrate essentially indistinguishably under the conditions employed and more slowly than either of the other two H1 components. Separation of unfractionated H1 histones from several organs on NaDodSO$_4$ gels (Figure 4) yields patterns that are clearly interpretable in terms of mixtures of the subfractions from thymus. Testis is distinguished from other organs in that the band of intermediate mobility is very prominent. From comparison with bulk thymus H1 histones and with isolated testis H1a, it is evident that this intermediate band does, in fact, reflect the contribution of H1a to the H1 population. In other experiments (not shown) we documented that each remaining testis H1 component isolated from

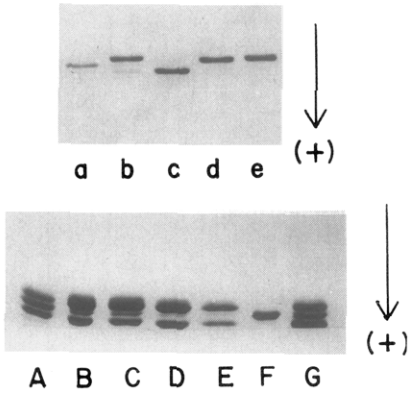

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of H1 histone fractions from rat organs. (Top) Chromatographically resolved H1 components from rat thymus. Lettering corresponds to the peaks identified in Figure 3A. (Bottom) Comparison of bulk H1 histones from several organs with isolated fraction H1a from rat testis. Bulk H1 samples were from testis (A), thymus (B), small intestine mucosa (C), lung (D), kidney (E), and again testis (G). Isolated testis H1a is channel F. Bulk H1 samples were extracted from washed chromatin prepared in the presence of protease inhibitors.

Bio-Rex 70 chromatography displayed the same behavior on $NaDodSO_4$–polyacrylamide gels as did the comparable chromatographic fraction from rat thymus.

Compositional Analysis of H1a. Amino acid analysis of rat testis H1a (Table II) confirms that it is a typical H1 histone. Our analysis is in essential agreement with that of Branson et al. (1975) for the component they denote X_1.

Discussion

In this study we set out to determine whether the unusually prominent H1 subfraction of rat testis, H1a, could be differentiated from the comparable subfraction of other organs by chromatographic or electrophoretic means. All other organs examined have contained detectable amounts of H1a as determined by electrophoresis on acetic acid/urea gels. Furthermore, testis subfraction H1a cannot be diffentiated from H1a of rat thymus by electrophoresis under nondenaturing conditions at pH 4.5 (Kistler & Geroch, 1975), by electrophoresis in the presence of $NaDodSO_4$, or by chromatography on Bio-Rex 70. The simplest interpretation of these results is that the H1a subfractions from various organs are identical. Proof of this hypothesis will of course have to await appropriate chemical studies on H1a from testis and other organs.

Certain nonhistone proteins such as the HMG family (Goodwin et al., 1973) will coextract with H1 histones from chromatin by procedures employing perchloric acid (Johns, 1964; Smith & Stocken, 1973; Sanders & Johns, 1974) and sulfuric acid (Seyedin & Kistler, 1978). In addition, some members of this group, particularly HMG1 and HMG2, have electrophoretic mobilities somewhat similar to those of H1 subfractions. The procedures we have used for extraction of H1 histones do not result in the extraction of either HMG1 or HMG2 in noticeable quantities. Were these proteins to be present, they would migrate distinctly more slowly than H1a on acetic acid/urea gels and more rapidly than any H1 component on $NaDodSO_4$ gels, and they would elute during Bio-Rex chromatography well in advance of the various H1 subfractions (Seyedin & Kistler, 1978, and unpublished). Levinger et al. (1978) have recently detected a new protein in rat testis chromatin which they have denoted TH1-XB. It has electrophoretic mobility identical with that of H1a on acetic acid/urea gels but migrates more rapidly than any standard H1 subfraction in the presence of $NaDodSO_4$. We have independently observed what appears to be the same

Table II: Amino Acid Composition of Rat H1 Histone Fractions[a]

amino acid	mol/100 mol recovered[b] *testis H1a* 24-h hydrolysis	*testis H1a* 48-h hydrolysis	thymus H1c[c]
Asp	3.1	3.5	2.2
Thr	6.4	6.3	5.3
Ser	7.7	6.5	7.9
Glu	5.0	5.6	4.3
Pro	9.6	9.6	9.3
Gly	5.7	6.5	7.9
Ala	19.2	18.9	23.1
Val	9.9	9.9	4.8
Cys	ND[d]	ND	ND
Met	0.0	0.0	0.0
Ile	0.90	1.0	2.2
Leu	4.4	4.5	5.1
Tyr	0.30	0.36	0.4
Phe	0.54	0.64	0.5
His	trace	trace	0.0
Lys	24.4	23.5	23.4
Arg	2.9	2.9	3.2

[a] H1a from rat testis was obtained as described in Results but was subjected to a second cycle of chromatography on Bio-Rex 70 under identical conditions. The final preparation was free of contamination as judged by electrophoresis on acetic acid/urea gels and pH 4.5 gels. [b] No corrections have been made for hydrolytic losses. [c] Values from Kinkade (1969) for rat thymus component 4 of his terminology. [d] Not determined.

protein. It is further differentiated from H1a by a slower mobility during electrophoresis at pH 4.5 and is not extracted from chromatin in more than trace amounts by the procedure of De Nooij & Westenbrink (1962; see Figure 3 of Kistler & Geroch (1975)). Accordingly it does not behave like a classical H1 histone, and it is not present in the preparations we have studied. In extracts in which this protein is present, it elutes from Bio-Rex chromatography approximately coincident with subfraction H1d (Seyedin & Kistler, unpublished).

H1a is a typical H1 histone both with respect to its extraction from chromatin and with respect to its amino acid composition. Compositional analyses are available for rat subfractions H1c, H1d (components 3 and 4, Kinkade, 1969; components I and II, Sluyser & Hermes, 1973), and H1° (Panyim & Chalkley, 1969a). Comparison with the composition of rat thymus H1d (Table I, data from Kinkade, 1969) indicates that the most marked differences between the two rat H1 components lie in a reduction of alanine and an increase in the valine content of testis H1A as compared with thymus H1d. Earlier studies of H1 subfractions from several species have noted that there is often a correlation between increased valine content and early elution from Bio-Rex 70 (or Amberlite IRC-50; Bustin & Cole, 1968; Kinkade, 1969). Recently Smerdon & Isenberg (1976) have documented the presence of a minor component of calf thymus H1 histones that both migrates slowly on acetic acid/urea gels and elutes early from Bio-Rex 70. This component (subfraction 1a of their terminology) was not isolated in the pure state, but from the composition of the mixtures of their subfractions 1a and 1b it is clear that 1a must have both reduced alanine and increased valine compared with other calf thymus H1 components. Calf thymus subfraction 1a is, however, clearly not identical with rat testis H1a. Like other calf thymus H1 subfractions, it contains conspicuously more lysine and less arginine than do rat H1 components. It would be interesting to know if component 1a of the calf is more prominent in the mature bull testis.

Our studies indicate that, with the possible exception of H1b, rat testis contains the same set of H1 subfractions found in

the thymus and those other organs that have been examined carefully by chromatographic procedures: liver, spleen, and kidney (Kinkade, 1969). The apparent absence of H1b must indicate its scarcity in germinal cells, which comprise the greater amount of the testis (Roosen-Runge, 1956). Whether it is present or absent in particular subpopulations of testicular cells will be difficult to determine as this rat H1 subfraction is the only one that cannot yet be identified unambiguously by one or another electrophoretic system.

Previous studies have established that in the rat (Marushige & Marushige, 1975) as in salmonid fish (Marushige & Dixon, 1969, 1971) H1 histones seem to be the last somatic type histones to be replaced by spermatid or sperm-specific basic nuclear proteins. Separations of various nuclear (Kumaroo et al., 1975) or intact cell populations (Platz et al., 1975) have further indicated that it is chiefly fraction H1a that is retained in late spermatocytes and early spermatids. Whether this change in the H1 population relates chiefly to nuclear events during meiosis or to preparation for the chromosomal protein transitions that occur in spermatids or, perhaps, to the cessation of RNA synthesis that occurs during spermatid development (Monesi, 1971) remains to be established.

Added in Proof

We have recently purified the protein identified as TH1-XB by Levinger et al. (1978). Its amino acid composition indicates that it may be a member of the H1 family despite its failure to be extracted from chromatin by aqueous 5% Cl_3CCOOH. Work is in progress to explore more fully its tissue distribution and its possible H1 nature.

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