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ISOLATION OF HISTONE TH1-xB FROM RAT TESTIS

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SUMMARY: The lysine-rich histones TH1-xA and TH1-xB, previously detected in rat testis nuclei by two-dimensional gel electrophoresis (Levinger et al. (9)), have been isolated and partially characterized. Histone TH1-xB has an unusually high arginine content for a H1 histone. Although TH1-xA is present in small amounts in testes of rats at 5 d after birth, TH1-xB is not detectable at 5 d or 14 d, but is present at 18 d. Both TH1-xA and TH1-xB are present in nuclei of pachytene primary spermatocytes and early spermatids.

INTRODUCTION: This and other laboratories have reported that the testicular SEC[1] of rats and other species of animals contain several histone subfractions which are absent from somatic cells or are present only in low concentrations (1-7). Among these unusual histone subfractions is a lysine-rich histone of the H1 type [initially (1,2) designated by Branson et al. (12) as X_1, and subsequently (8) as TH1-x]. By application of two-dimensional gel electrophoresis Levinger et al. (9) demonstrated that TH1-x actually consists of two subfractions, designated as TH1-xA and TH1-xB, which move as a single band in acid/urea/polyacrylamide gels but separate in sodium dodecyl sulfate (SDS)/polyacrylamide gels. Inasmuch as the amount of total TH1-x relative to other histone fractions changes dramatically during spermatogenesis (8,10), it is reasonable to conclude that one or both of these histones may play an important role in this process. We report here the isolation and partial characterization of TH1-xA and TH1-xB.

METHODS

Male Sprague-Dawley rats (200-250 g) were the source of testicular SEC for isolation of TH1-xA and TH1-xB, and young rats of this strain were used at 5, 14, and 18 days after birth to determine the approximate time of appearance of TH1-xB.

Isolation of TH1-xA and TH1-xB. Nuclei of testicular SEC were isolated from the testes of 25 rats as described previously (2), with the modification of adding 1

[1] The following abbreviations are used: SEC, seminiferous epithelial cells; TCA, trichloroacetic acid; SDS, sodium dodecylsulfate; PMSF, phenylmethylsulfonyl fluoride.

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mM PMSF to the homogenization medium to inhibit proteolysis. The dried nuclei were homogenized in 100 ml. of 0.05 M sodium citrate, pH 5.8, and centrifuged at 2000 xg for 30 min. The nuclear pellet was extracted two additional times with citrate, and the extracts were discarded. The nuclear pellet then was homogenized in 50 ml of cold 5% TCA and stirred for 30 min at 4° before centrifugation at 2000 xg for 30 min. The pellet was extracted twice more with 25 ml portions of 5% TCA. The pooled TCA extracts were filtered through a 0.45 μm Millipore filter, and the filtrate was treated with TCA to a final concentration of 20% to precipitate H1 type histones (except TH1-xB which was not extracted by 5% TCA). The precipitated H1 histones were collected by centrifugation and then subjected to column chromatography on carboxymethyl cellulose as described previously (2) to isolate TH1-xA which then was purified by a second cycle of chromatography on CM cellulose.

The nuclear residue remaining after the extraction with 5% TCA was stirred with 30 ml of cold 2.5% TCA for 30 min at 4° and then was centrifuged at 2000 xg for 30 min. The residue was extracted twice with 25 ml of 2.5% TCA, and after centrifugation the three TCA extracts were combined and treated with additional TCA to a final concentration of 6%. The precipitate, which contained TH1-xB and small amounts of other proteins, was collected by centrifugation at 2000 xg, and the pellet was washed twice with cold 6% TCA and twice with acetone. The crude TH1-xB then was dissolved in 2.6 M formic acid/70% ethanol and applied to a 0.9 x 55 cm column of carboxymethyl cellulose (Whatman CM52), and eluted with a gradient of 70-30% ethanol in a buffer of 2.6 M formic acid/0.1 M sodium formate. The eluant was monitored turbidimetrically by addition of TCA to the elution fractions to a final TCA concentration of 20% and measurement of absorbance at 400 nm in a Beckman DB spectrophotometer. Fractions from the elution peak containing TH1-xB were pooled, dialyzed against water at 4°, and lyophilized. The partially purified TH1-xB then was chromatographed a second time on carboxymethyl cellulose by an identical procedure to obtain the final sample.

Amino acid analyses were performed on the purified samples of TH1-xA and TH1-xB by the method of Spackman et al. (11) as used previously (2).

Isolation of Nuclei of Pachytene Cells and Spermatids: Nuclei of testicular SEC were fractionated by sucrose density gradient centrifugation as described previously (12) to obtain a "bottom" fraction which was enriched in pachytene primary spermatocytes and a "top" fraction which was enriched in early to middle stage spermatids. These fractions were separately recentrifuged through the same sucrose gradient for further purification. The pachytene nuclei contained only 9% contamination by nuclei of other cell types as determined microscopically, and the spermatid fraction had only 5% contamination.

One and Two-dimensional Gel Electrophoresis: One-dimensional disc gel electrophoresis is acid/urea/polyacrylamide gels was by the method of Panyim and Chalkley (13) as described previously (2,8). One-dimensional disc gel electrophoresis in SDS/polyacrylamide with a spacer gel was performed as described previously (9) based upon the method of Laemmli (14) as modified by Thomas and Kornberg (15), but the duration of electrophoresis was twice that used previously (9). Two-dimensional gel electrophoresis was conducted as described previously (9) with the exception that the time of electrophoresis in the acid/urea/polyacrylamide tube-gels (first dimension) and in the SDS/polyacrylamide slab-gels was doubled to permit better separation of the H1 histones.

RESULTS: When histones are extracted from nuclei of testicular SEC of mature rats with 0.2 N H_2SO_4 and then subjected to electrophoresis in SDS/polyacrylamide gels, five H1 species are observed (Fig. 1B). Two-dimensional gel electrophoresis of SEC of mature rats reveals six H1 species (Fig. 2C). Note that the species previously (9) designated as H1A is partially separated into two subfrac-

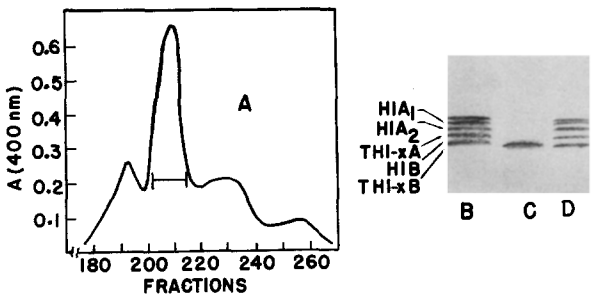

Fig. 1A. Chromatographic purification of TH1-xB on carboxymethyl cellulose (Whatman CM 52, eluted with 70-30% ethanol gradient in 2.6 M formic acid/0.1 M sodium formate). Fractions were 3 ml. The fractions included in the bracket were pooled and rechromatographed on CM cellulose to yield a single peak (not shown here). B-D are electrophoretic patterns of the H1 histones of testicular SEC in SDS polyacrylamide gels. Migration was downward toward the positive electrode. Only the upper sections of the gels containing the H1 histones are shown here. B. H1 histones of testicular SEC of the mature rat. C. Pattern for purified TH1-xB. D. Pattern for H1 histones from nuclei of early to middle spermatids of mature rat.

tions ($H1A_1$ and $H1A_2$) as a result of the doubled time of electrophoresis in the two dimensions. Two-dimensional electrophoresis of histones extracted from testicular SEC of rats five days after birth shows the presence of six H1 species (Fig. 2A), but TH1-xB is absent and another subfraction (H1C) is observed above TH1-xA. At 14 days after birth TH1-xB is not seen in the two-dimensional patterns (not shown here), but TH1-xB is found at 18 days (Fig. 2B). At 18 days H1C is present, but usually in smaller relative amount than at 5 days. At 18 days a total of 7 H1 species can be seen.

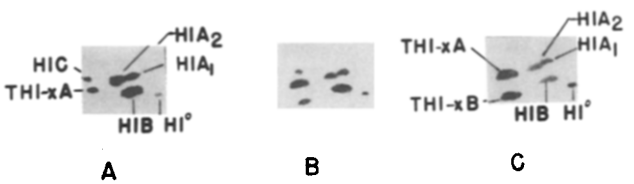

Fig. 2. Two-dimensional polyacrylamide electrophoretic analysis of H1-type histones of seminiferous epithelial cells of testes of rats of different ages. Migration in the first dimension (horizontal) was from left to right in acid/urea/polyacrylamide gels. Migration in the second dimension (vertical) was from top to bottom in SDS/polyacrylamide gels. Only the segments of the gels containing the H1 histones are shown here. A. 5 days after birth. B. 18 days. C. Mature rats.

When H1 histones are extracted with 5% TCA from testicular SEC, or nuclei derived from SEC, of mature rats, two-dimensional gel electrophoresis of these histones reveals all of the species shown in Fig. 2C with the exception of TH1-xB which is absent or present only in a trace. Consequently, it seemed probable that the TH1-x isolated previously (2) and designated as X_1, was actually TH1-xA. This was verified. When TH1-xA was isolated as described above, this histone subfraction migrated in the one-dimensional and two-dimensional gels in the position of TH1-xA (not shown here), and the amino acid composition (Table 1) agreed with that reported (2) for X_1.

The observation that TH1-xB is not extracted by 5% TCA from SEC or their nuclei led to the development of the procedure described here for the isolation of TH1-xB. Rechromatography on CM cellulose of the pooled fractions of the main peak of Fig. 1A gave a single elution peak (not shown here), and one-dimensional SDS gel electrophoresis of the histone recovered from the eluates corresponding to this peak gave a single band in the position of TH1-xB (Fig. 1C). Homogeneity also was indicated by two-dimensional gel electrophoresis of this sample (not shown here). The amino acid analysis of TH1-xB differs significantly from that of TH1-xA (Table 1). TH1-xB has an unusually high content of arginine for a H1 histone, and the presence of methionine also should be noted.

By both one-dimensional SDS gel electrophoresis and two-dimensional electrophoresis we have found both TH1-xA and TH1-xB in nuclei of pachytene primary spermatocytes (data not shown here) and early to middle stage (round) spermatids (Fig. 1D) isolated by density-gradient centrifugation as described in Methods. By these electrophoretic methods we also have found small amounts (relative to other H1 fractions) of TH1-xA in nuclei of liver, kidney, and small intestine of the rat (not shown here), but TH1-xB was not detected among the H1 histones from these tissues. Histones apparently corresponding to $H1A_1$, $H1A_2$, H1B, and H1C were found in these somatic tissues in various relative proportions.

DISCUSSION: Although histones TH1-xA and TH1-xB have practically identical electrophoretic mobilities in the acid/urea/polyacrylamide gel system, their

Table 1
Amino Acid Composition of Histones TH1-xA and TH1-xB. Values were determined on 24 h hydrolysates and were not corrected for losses.

Amino Acid	Moles per 100 moles of total amino acids	
	TH1-xA	TH1-xB
Lysine	22.2	19.9
Histidine	0	0
Arginine	2.5	6.9
Aspartic Acid	2.3	4.7
Threonine	5.0	5.2
Serine	9.5	10.3
Glutamic Acid	4.6	5.4
Proline	9.5	5.9
Glycine	7.9	9.5
Alanine	21.1	15.9
Cysteine	0	0
Valine	7.3	5.0
Methionine	0	1.3
Isoleucine	1.6	1.3
Leucine	5.4	7.0
Tyrosine	0.5	0.5
Phenylalanine	0.6	1.2

mobilities in the SDS/polyacrylamide gel are sufficiently different to permit separation. It is now apparent that the X_1 species isolated previously (2) is actually TH1-xA. Histone X_1 was shown previously (2) to give a single band in SDS/polyacryamide gels. It is now apparent that TH1-xB was lost in the previous isolation (2) since TH1-xB is not extracted from nuclei or chromatin by 5% TCA.

Histone TH1-xA is present in nuclei of testicular SEC at 5 days after birth when Sertoli cells and spermatogonia are present but before the appearance of primary spermatocytes (based upon the histologic data of Clermont and Perey (16). However, TH1-xA increases considerably in amount relative to some other H1 histones at the time of appearance of primary spermatocytes (14 to 18 days after birth). Thus, this histone subfraction clearly seems to be associated in some manner with the events of spermatogenesis preceding the first meiotic division. However, TH1-xA is certainly not unique to the testis since small amounts of this H1 histone appear to be present in nuclei of some somatic cells of the rat. On the other hand, TH1-xB does appear to be unique to the testicular SEC, and it becomes detectable in immature rat testis between 14 and 18 days when primary spermatocytes are appearing in significant numbers. Both TH1-xA and TH1-xB are

found in nuclei of pachytene primary spermatocytes and early to middle stage spermatids. In contrast, histone H1C is present in nuclei of testicular SEC of young rats (5-18 d), but this histone subfraction is not found except in trace amounts in nuclei of SEC of mature rats.

Histone TH1-xB also is rather unique for a H1 histone in having significant amounts of methionine and arginine. Thus, there might be some reason to question whether it is a H1-type histone. However, the high lysine content and the apparent molecular weight based upon electrophoretic mobility in SDS/polyacrylamide gels are in agreement with the H1 classification. The role of TH1-xA and TH1-xB in spermatogenesis must be clarified in future work.

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