PARTIAL CHARACTERIZATION OF A NEW BASIC NUCLEAR PROTEIN FROM RAT TESTIS ELONGATED SPERMATIDS

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SUMMARY A basic protein designated TP2 has been isolated from rat testis elongated spermatids. This new protein contains basic and acidic amino acids in relative amounts similar to those in histone F2al but is unusually rich in serine and proline. Techniques which were developed for preparing relatively homogeneous populations of spermatid nuclei were used to demonstrate that TP2 is most abundant and most actively synthesized in spermatids representing steps 12 through 15 of spermiogenesis.

Studies on basic nuclear proteins of rat testis have revealed that three major basic nuclear proteins are synthesized in elongated spermatids (1). One of these, a low molecular weight, very arginine-rich protein designated S1, is present in epididymal sperm as well (2,3). This protein is relatively rich in cysteine, and in mature epididymal sperm essentially all of the sulfhydryl groups are oxidized to disulfides (4,5), making this protein no longer soluble in dilute acid without prior reduction of the disulfide bonds (2,6). A second low molecular weight protein, designated TP, is testis specific (2) and relatively rich in both arginine and lysine but devoid of cysteine. We have recently presented evidence that a third electrophoretic band of protein with a mobility similar to that of histone F2al on acid-urea polyacrylamide gels is present in elongated spermatids and appears to be labeled *in vivo* with [^{35}S] cysteine (1).

The results presented here indicate that this protein, designated TP2, is indeed a unique basic protein which is synthesized in elongated spermatids representing steps 12 through 15 of spermiogenesis.

METHODS Male Sprague-Dawley rats weighing 250-300 gm were used in these experiments. Proteins were labeled *in vivo* for 1.5 hours by intratesticular injection

of 100 μCi [^{3}H] L-arginine (NEN) per testis.

Whole testis nuclei were prepared mechanically by first isolating crude nuclei as described before (1) except for the replacement of sodium bisulfite by 100 μM phenylmethylsulfonylfluoride (PMSF) in the homogenization medium to inhibit proteolytic activity (7). The nuclei were pelleted by centrifugation at 700 x g for 20 minutes. The crude nuclei were carried through the syringing step as described before (1), but they were collected by centrifugatior at 1000 x g for 20 minutes through a cushion of 10 ml of 0.31 M sucrose containing 3 mM $MgCl_2$, 10 mM KH_2PO_4, pH 6.0, and 0.05% Triton X-100. Isolation of elongated spermatid nuclei was accomplished by homogenizing the crude nuclear pellet from 3 rats prepared as described above in 15 ml of distilled water and sonicating for 3 minutes with 15 second bursts of ultrasound at 90% of the maximum power output using the large probe with a Bronwill Biosonik BPIII Ultrasonic System Sonifier, thereby disrupting most cellular components except elongated spermatid nuclei. Purification was accomplished by layering the sample over 10 ml of 1.5 M sucrose and centrifugation at 1000 x g for 30 minutes to pellet the sonication resistant nuclei and then by carrying the sample through two more cycles of homogenization in water and centrifugation through 1.5 M sucrose. Testis cell suspensions were prepared as described previously (1). A fraction enriched in round spermatids was collected by centrifugal elutriation (8) and nuclei from these cells were purified by separation for 8 hours using the Staput technique of velocity sedimentation at unit gravity (1,9). Elongated spermatids were isolated from elutriator fractions by the sonication technique as described above. Nuclei from fraction 1, which corresponded to Staput fraction I (1), was composed of spermatids in steps 16 through 19 (43%) and steps 12 through 15 (57%) while fraction 2, which corresponded to Staput fractions F plus G (1), was composed of steps 12 through 15 (95%) and steps 16 through 19 (5%).

Basic nuclear proteins were extracted with 0.25 M HCl, precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 25% and washed with acidified acetone (200 ml acetone plus 0.1 ml concentrated HCl) and acetone. In some experiments all protein disulfides were reduced essentially by the method of Coelingh et al. (6) before acid extraction of the nuclei. The new spermatidal basic protein TP2 was partially purified by using a modification of the protein isolation procedure described by Kistler et al. (2). Elongated spermatid nuclei were extracted twice with 0.4 N H_2SO_4, and the extract was mixed with an equal volume of 10% TCA to precipitate S1 and most of the contaminating histones. After centrifugation, the testis specific proteins TP and TP2 were precipitated from the supernatant by adding TCA to a final concentration of 20% and were acetone washed.

Protein samples dissolved in 7 M urea, 0.28 M 2-mercaptoethanol, and 0.9 N acetic acid were electrophoresed on 0.6 x 15 cm gels of 15% polyacrylamide at pH 2.7 (10). After staining and destaining, the band containing the new spermatidal protein was sliced from the gel and hydrolyzed with 6 N HCl (11) and analyzed on a Beckman Model 120 C amino acid analyzer. As a control, stained gel slices containing histone F2a1 were treated in the same manner.

RESULTS AND DISCUSSION Proteins extracted from elongated spermatids (Figure 1) reveal the presence of a new spermatidal basic protein fraction, which we have designated as TP2, with a mobility similar to that of histone F2a1 during electrophoresis at pH 2.7. Although this protein migrates as a doublet, both bands appear to have the same amino acid composition. Experiments are in progress to determine whether the heterogeneity in this protein may be

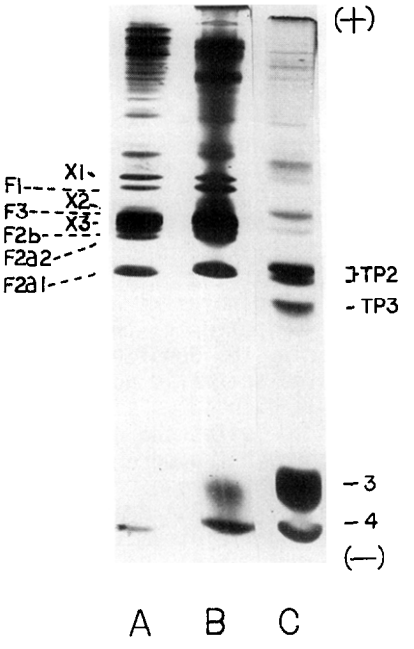

FIGURE 1. Electrophoretic patterns of testis basic nuclear proteins on acid-urea gels (10). (A) The profile of basic proteins from mechanically prepared unfractionated rat testis nuclei (50 µg); (B) Basic proteins from unfractionated testis nuclei in which the proteins were reduced with 2-mercaptoethanol before acid extraction (80 µg); (C) Basic proteins extracted with 0.25 M HCl from elongated spermatid nuclei representing steps 13 through 19, (70 µg). Note that all three basic spermatidal proteins exist in an acid extractable state in sonication resistant nuclei without prior reduction of protein disulfides.

due to side chain modification of the amino acid residues such as phosphorylation or acetylation.

As shown in Table I, the amino acid composition of the new basic protein is different from that of histone F2a1. For example, TP2 contains 22 mole percent serine and 13 mole percent proline while histone F2a1 contains only 3 mole percent and 2 mole percent of these amino acids respectively. Although cysteine was not determined directly, we reported previously that the new protein appears to contain cysteine (1) based on the presence of a peak of [^{35}S] in the region of this electrophoretic band in polyacrylamide gels of late spermatid proteins isolated after intratesticular injections of [^{35}S] cystine.

TABLE I

AMINO ACID COMPOSITION OF BASIC PROTEINS RECOVERED FROM POLYACRYLAMIDE GELS

The testis specific spermatidal protein TP2 was isolated by extraction of purified elongated spermatid nuclei with 0.4 N H_2SO_4. The protein fraction insoluble between the trichloroacetic acid concentrations of 5% and 20% was separated on polyacrylamide gels (10). The stained bands were sliced from the gels, and prepared for amino acid analysis essentially as described by Houston (11).

The rat testis histone F2a1 obtained by electrophoresis of acid soluble protein from whole rat testis nuclei was analyzed in the same way. Values obtained are similar to those in the literature for this histone from other tissues or organisms.

Amino Acid	Moles/100 Moles of amino acids recovered	
	Rat Testis F2a1	TP2[1]
Lysine	8.9	8.7
Histidine[2]	(1.9)	(5.2)
Arginine	13.1	14.2
Aspartic acid	6.2	5.0
Threonine	7.7	7.9
Serine	3.0	22.2
Glutamic acid	7.8	6.6
Proline	1.9	13.4
Glycine	15.9	6.1
Alanine	5.7	4.5
Valine	8.7	2.3
Methionine[3]	(0.3)	(0.9)
Isoleucine	5.9	0.3
Leucine	9.0	2.1
Tyrosine[3]	(0.05)	(Trace)
Phenylalanine	2.2	0.8

[1]The values reported in Table I for the relative amounts of the amino acid residues in TP2 are averages of the relative amounts of the amino acid residues that were determined for each of the bands in the electrophoretic doublet.

[2]The chromatographic peak from the analyzer corresponding to histidine was partially overridden by a large peak corresponding to ammonia.

[3]There was some loss of tyrosine and methionine during acid hydrolysis.

In contrast to these differences there are similarities between TP2 and histone F2a1 with respect to the relative contents of lysine, arginine, aspartic acid, glutamic acid, and threonine and the ratios of basic to acidic amino acids. Filtration on Sephadex G-100 indicated that the molecular weights of TP2 and histone F2a1 are approximately equal. The similarities in molecular weights

TABLE II

RELATIVE AMOUNTS OF THE VARIOUS BASIC NUCLEAR PROTEINS AS A PERCENT OF THE TOTAL BASIC NUCLEAR PROTEINS

The polyacrylamide gels including those shown in Figure 1 were scanned at 550 nm and the relative amounts of the various proteins were estimated from the densitometric tracings. Values shown are percent of the protein with respect to total basic protein (histones plus spermatidal proteins). Basic proteins were extracted from nuclei in the 0.25 M HCl except where noted.

	NUCLEAR TYPE							
PROTEIN TYPE	Whole Testis (Mechanical Preparation) Gel From Figure 1A	Whole Testis Reduced before acid Extraction Gel From Figure 1B	Whole Testis (Control for Round Spermatid Separation) Gel From Figure 2A	Round Spermatids Steps 1-8 Gel From Figure 2B	Elongated Spermatids (Steps 12-19) Gel From Figure 1C	Elongated Spermatids (Steps 12-19) (Reduced before Acid Extraction)	Elongated Spermatids (Elutriator Fraction 1) Gel From Figure 2D	Elongated Spermatids (Elutriator Fraction 2) Gel From Figure 2C
Total Histone[a]	96	85	95	100	12	13	30	60
TP2	--	--	--	--	6	8	12[b]	30
(a)	--	--	--	--	4.3	5.3	--	--
(b)	--	--	--	--	1.7	2.7	--	--
TP3	0	0	0	0	4	5	5	3
Band 3 plus Band 4[c]	4	14	5	0	85	81	65	37

[a]The estimates for total histone include F2a1 and TP2 since they are both present and co-migrate on acid-urea polyacrylamide gels.

[b]This value is an overestimation because the presence of contaminating F2a1 obscures TP2.

[c]Band 3 and Band 4 have been identified as S1 and TP respectively (1).

and charges of these two proteins at pH 2.7 explain their almost identical electrophoretic mobilities on acid-urea gels.

In order to localize TP2 in rat testis, several purified nuclear fractions were prepared. A fraction composed of 94% round spermatid nuclei, 5% other round nuclei, and 1% elongated spermatid nuclei appeared to contain the somatic and testis-specific histones described previously (12,13) as shown in Table II. On the other hand, nuclei from elongated spermatids (99% representing steps 13 through 19) appeared to contain mainly the spermatidal proteins designated TP2, band 3 and band 4 as shown in Figure 1C and Table II. These data suggest that the replacement of histones by one or more of the spermatidal proteins occurs prior to step 13 of spermiogenesis. The largest relative quantity of TP2 appears in the elongated spermatid nuclei from elutriator

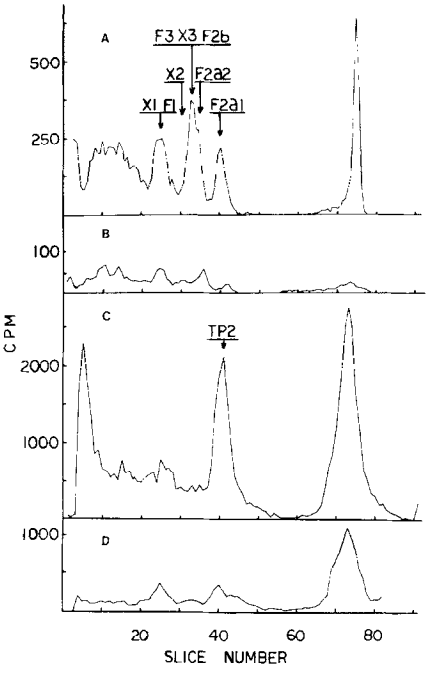

FIGURE 2. Radioactivity profiles of rat testis basic nuclear proteins labeled in vivo with [^{3}H] arginine and separated electrophoretically on acid-urea gels. CPM are normalized to 50 µg total protein per gel. (A) Unfractionated testis nuclei which served as a control for the round spermatid nuclei separation; (B) Round spermatid nuclei representing steps 1 through 8, prepared as described in the Methods; (C) Sonication resistant spermatid nuclei (fraction 2) representing steps 12 through 15; (D) Sonication resistant spermatid nuclei (fraction 1) representing steps 16 through 19.

fraction 2 (Table II), which consists almost exclusively of step 12-15 spermatid nuclei. No trace of TP2 has been found in rat epididymal sperm, even after reduction (14).

It was noted that while the protein migrating as band 3 in Figure 1C is not extracted from mechanically prepared nuclei with HCl (Figure 1A), it is extracted from these nuclei when the proteins are first reduced with 2-mercaptoethanol (Figure 1B). On the other hand all three spermatidal basic proteins including band 3 are at least partially extracted from sonicated spermatid nuclei by HCl without prior reduction (Figure 1C, Table II).

Using velocity sedimentation to separate spermatids in different stages

of development, we have found that the synthesis of TP2 appears to occur predominantly in step 12 to 15 spermatids as indicated by the higher levels radioactivity in TP2 in the sonication resistant nuclei from elutriator fraction 2 after in vivo labeling for 1.5 hours with [^{3}H] arginine (Figure 2C).

Relatively little synthesis of TP2 appears to occur in round spermatids representing earlier steps of spermiogenesis (Figure 2B) or in elongated spermatids more enriched in later stpes of spermiogenesis (Figure 2D). A control sample of unfractionated nuclei is shown in Figure 2A to demonstrate the normal labeling pattern of testis nuclear basic proteins.

Another stained band of acid soluble protein, which migrates slightly ahead of TP2 and is designated TP3 in Figure 1C, appears to be present in relatively higher proportions in elongated spermatids (steps 16 through 19) (Table II), and we reported previously that there was a peak of [^{35}S] in the region of this electrophorectic band after intratesticular injections of [^{35}S] cysteine (1). We have not observed this band in preparations of rat epididymal basic proteins. Further characterization of this protein is in progress.

The apparent replacement of the histones by the spermatidal proteins during spermiogenesis in the rat occurs concomitantly with nuclear elongation and "packaging" of the DNA in the condensed spermatid nucleus (15). This correlation indicates that TP and TP2 could be involved in the early steps of nuclear condensation and genetic inactivation, and it appears that S1, which is rich in cysteine residues, may replace TP and TP2 at a later time and subsequently form a network of cross-linked arginine-rich protein.

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