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NUCLEAR TRANSITION PROTEIN 2 (TP2) OF MAMMALIAN SPERMATIDS HAS A VERY BASIC CARBOXYL TERMINAL DOMAIN

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Summary. Nuclear transition protein 2 (TP2) along with TP1 are major basic chromosomal proteins of rat spermatids during the period of transition from histone-associated to protamine-associated DNA. TP2 isolated by reversed phase high pressure liquid chromatography was cleaved with S. aureus V8 protease to yield two fragments. The complete amino acid sequence of the 27 residue peptide assigned to the carboxyl terminus was established. It contains most of the basic residues of the protein and is likely to be a major site of DNA binding. Thus, TP2 is differentiated from core histones in having its basic domain at the carboxyl rather than amino terminal end.

During spermatogenesis, spermatogonia proliferate mitotically to form spermatocytes, which undergo meiosis to yield haploid spermatids. About midway through spermatid development the round nucleus elongates and then gradually condenses into the very compact state characteristic of spermatozoa. This process is accompanied at the molecular level by a transition in the major basic chromosomal proteins from histones to the so-called spermatid transition proteins. The transition proteins are themselves subsequently replaced by the protamines, which are retained as the principal basic proteins of the sperm nucleus. The transition proteins vary in their degree of characterization, and may involve as many as four proteins in rats (1), mice (2), and rams (3). Shortly after the first transition proteins appear, the spermatid nucleus becomes resistant to breakage by sonication, and this forms the basis for a convenient method to isolate these proteins (4). Among the proteins that can be extracted from sonication resistant nuclei by dilute mineral acid, both TP1 and TP2 are soluble in 5% TCA (4,5). The former is a highly basic small protein of 54 amino acids, which generally (though not invariably) lacks

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Abbreviations: HPLC, high pressure liquid chromatography; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid.

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cysteine and is identifiable in many species (6-8). TP2, by contrast, migrates electrophoretically as though it is of the size range of the core histones, and contains cysteine (4,5). As yet, no information is available about the actual structure of TP2. In this communication we report that rat TP2 can be cleaved by S. aureus V8 protease to yield a large amino terminal domain that contains all the cysteine of the protein and a smaller, highly basic carboxyl fragment, which we have sequenced.

EXPERIMENTAL PROCEDURES

Sprague/Dawley rats were killed by quick cervical dislocation. Tissues were frozen in liquid nitrogen and stored at -70° C. Crude chromatin was prepared in the presence of bisulfite and phenylmethylsulfonyl fluoride as described (9). Sonication resistant spermatid nuclei were prepared as described by Meistrich (10). To extract acid-soluble proteins, chromatin or nuclei were homogenized in cold 0.25 M HCl. After centrifugation (10,000 x g for 15'), the supernatant was adjusted to 3% (w/v) TCA, and precipitated material was discarded following centrifugation. The supernatant fraction was then adjusted to 20% TCA, and precipitated proteins were collected by centrifugation, washed several times with ethanol:ether (1:1), and allowed to dry.

Polyacrylamide gel electrophoresis conditions have been described (9). Conditions for separation of proteins and peptides by high pressure liquid chromatography, amino acid analysis, and amino acid sequence determination by automated Edman degradation are described elsewhere (11). TP2 was modified with (2-bromoethyl)trimethylammonium bromide (Aldrich Chemical Co., Milwaukee, Wis.) in 6 M guanidine HCl, 0.2 M Tris·HCl, pH 8.5 following reduction with dithiothreitol as described by Itano and Robinson (12).

RESULTS AND DISCUSSION

A very convenient way to examine the proteins of late spermatid nuclei is to destroy all other testicular nuclei by sonic irradiation and then collect the resistant nuclei by differential centrifugation (10). When rat testis sonication resistant nuclei were extracted with 0.25 M HCl and the soluble proteins fractionated to recover those soluble in 3% TCA, electrophoretic analysis in either acid/urea gels or SDS gels showed that two major protein bands were recovered (Fig. 1A, lane c; 1B, lane f), in agreement with earlier results of others (4,5). In either gel system the most rapidly migrating band is TP1. (Protamines are not present in these extracts due to insolubility in 3% TCA.) The most prominent upper band is identified as TP2 based on its solubility in 3% TCA and its electrophoretic mobility (4,5). TP2, like TP1, could also be identified in extracts prepared from crude salt washed testicular chromatin. These later extracts proved a convenient starting point for isolation of TP2 though they also

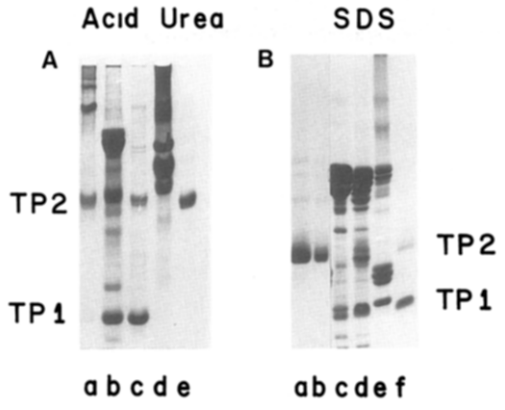

Fig. 1. Polyacrylamide gel electrophoretic analysis of protein samples. (A) Acid acid urea (2.5 M) gel: lane a, TP2 isolated by HPLC and stored for several weeks without sulfhydryl modification; lane b, 3% TCA soluble proteins from testis chromatin; lane c, 3% TCA soluble proteins from sonication resistant nuclei; lane d, calf thymus histones; lane e, TP2 isolated by HPLC and treated with (2-bromoethyl)trimethylammonium bromide. (B) SDS gel: lane a, TP2 isolated by HPLC and treated with (2-bromoethyl)-trimethylammonium bromide; lane b, same as lane a but half the sample volume; lane c, 3% TCA soluble proteins from liver; lane d, 3% TCA soluble proteins from testis; lane e, calf thymus histones; lane f, 3% TCA soluble proteins from sonication resistant nuclei. Both gels contained 15% acrylamide and were stained with Coomassie brilliant blue R.

contain H1 histones and high mobility group (HMG) proteins (Fig. 1A, lane b; 1B, lane d). When an identical extraction was made of liver chromatin (Fig. 1B, lane c) or of testis chromatin from immature (21 day old) rats (not shown), no band with the mobility of TP2 in SDS gels was identified.

The TP2 band present in a TCA fractionated HCl extract of testis chromatin could be isolated by preparative high pressure liquid chromatography on a C-18 reversed phase column (Fig. 2A). When this material was analyzed on either acetic acid urea or SDS gels it migrated as a band that was free of significant contamination. If the purified protein was stored for several weeks, even as a lyophillized powder, it often appeared as a "ladder" of bands when analyzed on acid urea gels (Fig 1A, lane a). Upon reduction with thiols, the same material ran as a single band on either acid urea or SDS gels (not shown). Since TP2 is known to contain cysteine (4,5) it appeared that these bands of slower electrophoretic mobility were due to formation of intermolecular disulfide bonds. In order to prevent formation of crosslinked products, some preparations of the protein were treated with (2-bromoethyl)trimethylammonium bromide to convert cysteine residues to its thialaminine derivative (12). The modified protein

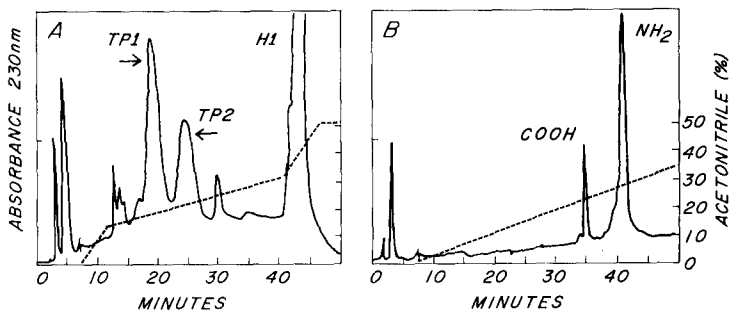

Fig. 2. Isolation of TP2 and its S. aureus peptides by reversed phase high pressure liquid chromatography. (A) 3% TCA soluble proteins from testis. The profile is for a typical preparative run in which a 0.1 ml sample of proteins (corresponding to 10 g of testis) dissolved in 0.1% trifluroacetic acid was applied. The material under the peak labeled "TP2" was collected just downstream of the flow cell, concentrated under a stream of nitrogen in a 40° water bath in a fume hood, and lyophillized (11). About 0.14 mg of TP2 was recovered from such a run (estimated from amino acid analysis), and the products of several runs were pooled (B) A sample of TP2 with modified cysteine residues was treated with 1% its weight of S. aureus V8 protease at pH 4.0 in 75 mM ammonium acetate, 2 mM EDTA at 25° for 8 hr. Samples were applied directly to the HPLC system. For both (A) and (B), flow rate was 1 ml/min. The gradient was formed as described previously (11). Peptides assigned to the amino and carboxyl termini are indicated. If present, the intact protein would elute slightly ahead of the amino terminal peptide.

migrated as a single band on acid urea (Fig. 1A, lane e) or SDS gels (Fig. 1B, lanes a,b).

The amino acid composition of the purified protein is similar to that previously reported for TP2 (Table I) (5). Two attempts to perform

Table I. Amino Acid Composition of TP2 and its Amino and Carboxyl Terminal Peptides resulting from S. aureus V8 protease digestion

Amino Acid	Intact Protein	NH_2 V8 Peptide	COOH V8 Peptide
	moles/100 moles		
Aspartate	4.0	4.7	0.5
Threonine	6.5	7.4	3.5
Serine	17.7	21.2	11.3
Glutamate	6.0	6.6	0.7
Proline	11.7	16.2	0
Glycine	5.0	4.3	7.9
Alanine	4.2	2.9	7.4
Cysteine	4.2	4.6	0
Valine	1.9	0	5.4
Methionine	2.0	2.9	0
Isoleucine	0	0	0
Leucine	2.3	2.4	0
Tyrosine	1.8	1.3	3.0
Phenylalanine	0	0	0
Histidine	8.4	11	3.5
Lysine	10	6.4	20.6
Arginine	14.4	8.3	36
Tryptophan	0	ND	ND

Values are averages of at least three determinations in each case. Cysteine was determined as the thialamine derivative (12) or as cysteic acid following performic acid oxidation of a sample of unblocked protein (15). Tryptophan was estimated from the UV specturm of the purified protein. No corrections have been made for hydrolytic losses. ND = not determined.

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GLU-LYS-VAL-SER-LYS-ARG-LYS-ALA-VAL-ARG-ARG-ARG-LYS-ARG-THR-HIS-ARG-ALA-

 *  *  *              *  *       *
LYS-ARG-ARG-SER-SER-GLY-ARG-ARG-TYR-LYS
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Fig. 3. Amino acid sequence of the carboxyl terminal peptide derived from TP2 by digestion with S. aureus V8 protease. Basic residues are shown with an asterisk above. The initial glutamic acid residue is not present in the peptide itself and is placed by the specificity of the protease (13). The sequence was determined twice with identical results using about 40 nmol of peptide.

automated Edman degradation on the intact protein, modified or not with the sulfhydryl reagent, yielded no amino acid derivatives, suggesting that the amino terminus is blocked. Accordingly a search was made for proteolytic digestion conditions that would yield a small number of fragments. S. aureus V8 protease used under conditions in which cleavage is specific for the carboxyl side of glutamic acid residues (13) appeared to bisect the protein, yielding two large fragments that could be isolated readily by reversed phase chromatography (Fig. 2B). Following digestion, no traces of the starting material could be observed either by reversed phase chromatography (Fig. 2B), or SDS gel electrophoresis (not shown). Even after prolonged digestion, no additional fragments were produced. Compositional analyses of the two peptides indicated that the first to elute from the C-18 column was extremely rich in basic residues while the second contained all the cysteine of the protein but was relatively depleted of arginine and lysine (Table I). Several attempts to analyze the sequence of the nonbasic peptide failed, and we assume that it represents the amino terminal fragment. Automated Edman degradation of the basic peptide yielded a continuous sequence of 27 amino acids, notable for its highly basic and hydrophillic character (Fig. 3). The assumption that the basic peptide is the carboxyl terminal fragment of the protein was confirmed by treatment of the intact protein with a preparation of Carboxypeptidase B that contained some Carboxypeptidase A type activity. Only arginine, lysine and tyrosine were released, as expected from the sequence of the basic peptide. Barring the possible occurrence of a small internal peptide that has gone unnoticed in our analysis, it appears that TP2 has only one accessible glutamic acid residue, which lies at or near the beginning of the highly basic carboxyl terminal domain.

The TP2 band sometimes appeared as a doublet following electrophoresis in either acid urea or SDS systems, independent of whether the protein had been treated to modify cysteine side chains. Since both components of the band are cleaved by S. aureus V8 protease to give similar or identical products by HPLC or SDS gel analysis, and since analysis of the carboxyl terminal peptide did not give indications of sequence heterogeneity, we suspect there may be some heterogeneity due to sequence variation or to side chain modification(s) in the amino terminal portion of the protein. While the apparent size and amino acid composition of TP2 bear a resemblance to histone H4 (4,5), the structural information presented here indicates that the organization of TP2 is quite different from any of the core histones. Core histones have their major basic, DNA binding domains at the amino terminal rather than carboxyl terminal ends (14). Furthermore, a computer comparison of the carboxyl terminal peptide of TP2 with the core histones failed to detect any striking regions of homology.

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