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Immunological Evidence for a P2 Protamine Precursor in Mature Rat Sperm

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ABSTRACT High molecular weight proteins in *Rattus norvegicus* that are immunoreactive with an anti-protamine 2 specific antibody but not with an anti-protamine 1 specific antibody are described. These proteins were detected by coupling high-performance liquid chromatography (HPLC) with an enzyme-linked immunosorbent assay (ELISA). Briefly, following HPLC separation of rat sperm nuclear proteins, the HPLC fractions were probed with the antibodies. We estimate that the antibody probes are 100–1000 times more sensitive than UV absorbance measurements. Immunoblot analysis following acid-urea electrophoretic separation of rat sperm nuclear proteins, and of the HPLC fractions, also detected putative protamine 2 precursor proteins. The proteins reactive with the anti-protamine 2 antibody are most likely not mature protamine 2, since they were detected in a region of the chromatogram where we would not expect protamine 2 to migrate based on the chromatographic locations of human and mouse protamine 2. Likewise, the immunoblotting experiments demonstrated that the anti-protamine 2 antibody recognized proteins with slower electrophoretic mobilities than would be expected for a mature protamine 2. An anti-protamine 1 monoclonal antibody, Hup1N, that binds rat protamine 1 is also described. Hup1N allowed for identification of the HPLC fractions that contained rat protamine 1. Finally, we demonstrated that Hup1N binds protamine 1 from a large number of species, suggesting a conserved epitope for Hup1N.

Key Words: Anti-protamine antibody, Immunogram, Protamine immunoblots

INTRODUCTION

Protamines are small, arginine-rich, DNA-binding proteins that replace histones and transition proteins during spermiogenesis and are found in the sperm of vertebrates. Numerous reviews have appeared recently describing protamines (Balhorn, 1989; Risley, 1989), the genes coding for different protamines (Hecht, 1989b), and the structure and expression of mammalian protamines (Hecht, 1989a).

Sperm of many species (e.g., bull, boar, and ram) contain only a single type of protamine, referred to as protamine 1 (P1) (Balhorn, 1989). Two different protamines, P1 and P2, have been detected in only a limited number of species, including mouse (Bellve et al., 1988), hamster (Corzett et al., 1987), and stallion (Pirhonen et al., 1989), and 3 protamine species have been found to be present in the sperm of humans and rhesus monkey (McKay et al., 1986; Balhorn, 1989) (referred to as P1, P2a, and P2b protamine). The complete amino acid sequence of P1 is known from bull (Coelingh et al., 1972; Mazrimas et al., 1988), boar (Tobita et al., 1983), ram (Sautiere et al., 1984), stallion (Belaiche et al., 1987; Ammer and Henschen, 1987), mouse (from cDNA sequence) (Kleene et al., 1985), rat (Ammer and Henschen, 1988), goat (Ammer and Henschen, 1988), rabbit (Ammer and Henschen, 1988), and human (McKay et al., 1985, 1986; Ammer et al., 1986; Gusse et al., 1986). A partial amino acid sequence for Rhesus monkey P1 also is known (Balhorn, 1989). Likewise, the amino acid sequence of P2 is known for mouse (Bellve et al., 1988), for human P2a and P2b (McKay et al., 1985, 1986; Ammer et al., 1986; Gusse et al., 1986; Balhorn et al., 1987), as well as a partial sequence for hamster P2 (Corzett et al., 1987) and stallion P2 (Pirhonen et al., 1990).

Mouse P2 is synthesized as a precursor protein that is processed in late-step spermatids from 106 amino acids to the final 63 amino-acid sequence found in mature sperm (Balhorn, 1989; Yelick et al., 1987). A similar process is thought to occur with human P2 (Balhorn, 1989). P2 has not been reported to occur in sperm from all members of the Rodentia. Electrophoretic analyses indicate that mature P2 is present in sperm of cotton rat, dwarf and Syrian hamster, deer mouse, *Perognathus*, vole, mouse, chinchilla, and nutria but is not detected in Chinese and Armenian hamster, the laboratory rat *Rattus norvegicus*, guinea pig, beaver, and porcupine (Balhorn, 1989). Recent studies by Tanhauser and Heart (1989) and Bunick et al. (1990) have demonstrated that a P2 gene is present in rat and that mRNA coding for a P2 molecule is synthesized, but at reduced levels compared to mouse.

We have previously developed a set of monoclonal antibodies (mAbs), some that specifically bind human

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P1, some that recognize P2, and others that cross-react with P1 and P2 (Stanker et al., 1987). Hup2b, a P2 specific antibody, has been shown to bind proteins present in rat sperm (Bunick et al., 1990). In this communication, putative P2 precursor proteins are identified by (i) analyzing rat nuclear proteins separated by HPLC for immunoreactivity with Hup2b and (ii) by analyzing immmunoblots following acid-urea gel electrophoresis of whole-rat protamine and of HPLC fractions. A new monoclonal antibody (Hup1N) that binds rat P1 has been isolated. This antibody readily detects rat P1 in the HPLC fractions and on immunoblots following electrophoretic separation.

MATERIALS AND METHODS

Sperm Sources

Human serum was collected as part of a local program involving anonymous, healthy donors, 25–45 years of age. Bull, ram, and boar semen were purchased from breeding services (bull, American Breeders Service, DeForest, WI; boar, Swine Genetics International, Cambridge, IA; and ram, Dwane Garner, Dept. Animal Science, University of Nevada-Reno). The cauda epididymidis of adult Rhesus monkeys (California Regional Primate Center, University of California, Davis, CA) and male rats (retired *Rattus norvegicus* breeders purchased from Simonsen Labs, Gilroy, CA) were teased in 0.01M Tris-HCl buffer pH 8.0, 0.15 M sodium chloride (Tris-HCl-saline), and the released sperm were filtered through nylon mesh.

Protamine Isolation and Fractionation by HPLC

Whole protamine (i.e., the mixture of protamines 1 and 2) was isolated from sperm nuclei using a modification of the procedure for the mouse (Balhorn et al., 1977) as described by Mazrimal et al. (1986). All of the protamines used in this study (listed in Table I) were isolated by the above HPLC method. The purified protamine from each species was lyophilized, weighed, and adjusted to a stock concentration of 1 mg/ml. The isolated protamines were evaluated for purity by acid-urea gel electrophoresis (Panyim et al., 1971). The rat protamines were reduced at 21°C with dithiothreitol (10 mg/mg protamine) in 6 M guanidine hydrochloride, 2 mM ethylenediaminetetraacetic acid, and 0.01 M Tris-HCl buffer pH 8.0 for 1.5 hr, and subsequently dialyzed against 2 l of 10 mM hydrochloric acid 1 hr prior to chromatography. The proteins were separated by HPLC on a silica-based, 5-μm Nucleosil (Macherey-Nagel, Duren, Germany) RP-C18 column (7.5 mm × 200 mm) using a multistep acetonitrile gradient (run at a rate of 2 ml/min for a total of 90 min) and the ion-pairing agent trifluoroacetic acid. The aqueous buffer was water containing 0.1% TFA (buffer A) and the organic solvent was aqueous 30% acetonitrile, 0.1% TFA (buffer B). The proteins were injected in 2.0 ml of buffer A and the gradient was started at 40% buffer B, 60% buffer A. The proteins were eluted using a linear gradient run from 40–67% buffer B (10 min), a 20-min isocratic step at 67% buffer B, a second gradient from 67–77% buffer B (10 min), a 15-min isocratic step at 77% buffer B, and a final gradient from 77–87% buffer B (10 min). The histones were subsequently eluted from the column as a single peak by increasing the buffer B concentration to 100%. Protein elution was monitored at 214 nm. Two-milliliter fractions were collected throughout the fractionation.

TABLE 1. Percent Cross-Reactivity* of Hup1N to Protamines From Different Species

Species	% Cross-Reactivity
Dog (beagle)	105
Boar	82
Bull	75
Chinchilla	139
Cotton rat	79
Cynomologus monkey	70
Syrian hamster	82
Rattus norwegicus	90
Human	73[a]
Mouse	71
Mule deer	73
Ram	77
Rabbit	0[b]
Rhesus monkey	100
Vole	4

*Percent cross-reactivity was calculated by comparing the area under the ELISA titration curves, assigning 100% activity to the reaction with human protamine.
[a]Human is being compared with human protamine from a different antigen preparation.
[b]0% cross-reactivity represents less than 0.5% activity.

Antibodies

Hup2b, a P2 specific monoclonal antibody (mAb), was previously described (Stanker et al., 1987). Additional hybridomas were produced as described (Stanker et al., 1987). Briefly, BALB/c mice were immunized with a preparation of whole human protamine (i.e., containing human P1, P2a, and P2b) in complete Freund's adjuvant (1:1). Spleen cells from immunized mice were fused with SP2/0 myeloma cells. The resulting hybridomas were screened for anti-protamine activity using an enzyme-linked immunosorbent assay (ELISA) (Stanker et al., 1987). Cells from ELISA-positive wells were expanded and cloned twice by limiting dilution to ensure monoclonal origin. MAbs were produced in ascites tumors and were isotyped using immunoglobulin heavy-chain- and light-chain-specific anti-serum (Southern Biotechnology Assoc., Birmingham, AL). The ELISA used to determine cross-reactivities of Hup1N and Hup2b were performed as described (Stanker et al., 1987). In all of the experiments the 96-well microtiter wells were coated with the same amount of protamine (100 μl of a 1 μg/ml solution of protamine in distilled water). The percent cross-reactivity was calculated by comparing the area under the ELISA titration curves, assigning 100% activity to the

reaction with human protamine. The variation observed between different titrations of human protamine over a 6-month period reflects the variation (approximately ±25%) in this assay.

Immunograms (ELISA of HPLC Fractions)

Immunograms were generated by analyzing HPLC fractions with an ELISA as follows. The HPLC fractions were lyophilized, dissolved in distilled water, and then used as antigen in an ELISA as described above. Briefly, 100 μl of antigen was applied to a 96-well round-bottom microtiter plate (Nunc, HB plates, Nunc Inc. Naperville, IL), diluted 2-fold, and allowed to air dry. The plates were then incubated in 0.01 M phosphate buffer, pH 9.5, containing 3% ovalbumin and 10 μg/ml calf-thymus DNA for 1 hr to block reactive sites on the plastic and to neutralize the charge on the protamine molecules. The antigen-coated and blocked plates were then probed with the anti-protamine Mab by adding 100 μl of mAb (1:500) to each microtiter well, followed by a 1-hr incubation at 37°C. The plates were then washed (0.05% Tween-20/H_2O) and incubated with a peroxidase-conjugated anti-mouse immunoglobulin (a 1:200 dilution, Sigma Chemicals, St Louis, MO) for 1 hr at 37°C. Finally, the plates were washed as above, and the amount of anti-protamine antibody bound to the wells was detected using 2,2 azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) as the substrate.

Gel Electrophoresis and Blotting

The protamines and other sperm basic nuclear proteins were separated by electrophoresis on acid-urea (Panyim et al., 1971) disc (10 cm) and slab gels (10 cm × 10 cm × 1 mm). For the slab gels the TEMED concentration was increased 6-fold to facilitate polymerization. A stacking gel was not necessary. The samples were applied in 0.9 N acetic acid, 20% sucrose, and 0.5 M mercaptoethanol, and run at 180 V for 45 min. Pyronin Y (0.05 M in 15% sucrose, 0.375 M potassium acetate) was used as a tracking dye. Immediately after electrophoresis, the proteins were electroblotted onto Immobilon filters (Millipore, Bedford, MA) in 0.009% acetic acid at 50 V for 15 min. The blots were stained overnight in 0.2% Coomassie Blue (in 10% acetic acid, 45% methanol) and subsequently destained by diffusion, or they were dried and probed with P1- and 2-specific antibodies. The antibody-probed filters were first blocked with 5% bovine serum albumin in Tris-HCl-saline/Tween, containing 20 μg/ml calf DNA. They were then probed with the P1- and P2-specific antibodies, and subsequently were washed and probed with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma, St. Louis, MO), diluted 1 drop/10 ml buffer in 0.01 M Tris-HCl pH 7.4; 0.9% saline, 0.1% Tween-20 (Tris-HCl-saline-Tween). After washing again with Tris-HCl-saline-Tween, the staining was amplified using the ABC reaction (Vector Laboratories, Burlingame, CA) and the protein bands were visualized using 4-chloro-1-napthol.

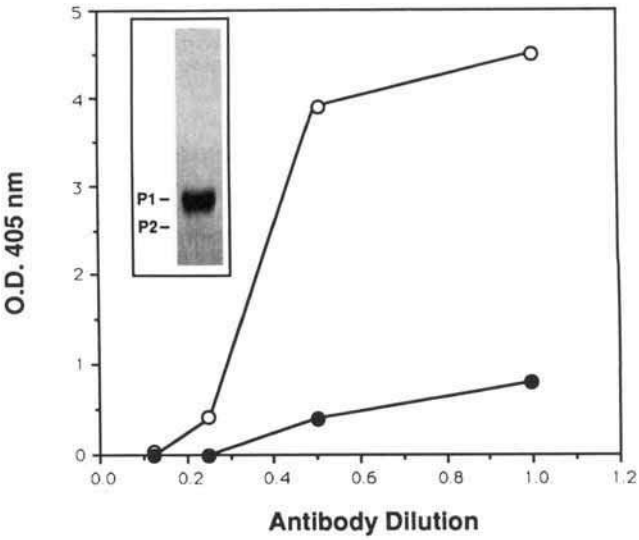

Fig. 1. ELISA analysis of the reactivity of mAb Hup1N to human P1 vs. a mixture of human P2a plus P2b. Empty circles, Hup1N with P1 as antigen; filled circles, Hup1N with a mixture of P2a and P2b as antigen. Tissue culture fluid from the hybridoma was diluted in a 2-fold fashion as indicated in the figure. **Inset:** Immunoblot of whole human protamine separated on an acid-urea gel and probed with Hup1N. The positions of human P1 and P2 are indicated on the left.

RESULTS

Hybridoma Isolation

The anti-P1 mAbs we previously described (Stanker et al., 1987) all recognize human protamine and showed little if any cross-reactivity with protamine from other species. Thus, we initiated the following experiments to generate mAbs that would bind P1 from other species, in particular, mouse and rat P1. Following 2-cell fusion experiments (using human whole protamine as the immunogen), an anti-P1-specific antibody-producing hybridoma (referred to as Hup1N) was isolated. The direct-binding ELISA experiment shown in Figure 1 demonstrates the specificity of Hup1N for human P1. The weak reactivity observed for Hup1N with the P2 antigen (Fig. 1) probably reflects a small amount of P1 contaminating the P2 sample. The P2 preparation used in these ELISAs was evaluated by acid-urea gel electrophoresis followed by staining with amido Schwartz. If P1 was present at less than a few percent of the total protein, it would not be readily detected on these gels. However, the immunoassay has a much greater sensitivity. An ELISA can easily detect a few percent contamination. In other studies of mouse P2 precursor proteins (Balhorn, unpublished observation), proteins not readily visible on the acid-urea gels were easily detected with the antibodies. Immunoblot analyses of human protamines separated by electrophoresis on acid-urea gels probed with Hup1N (Fig. 1, inset) indicated

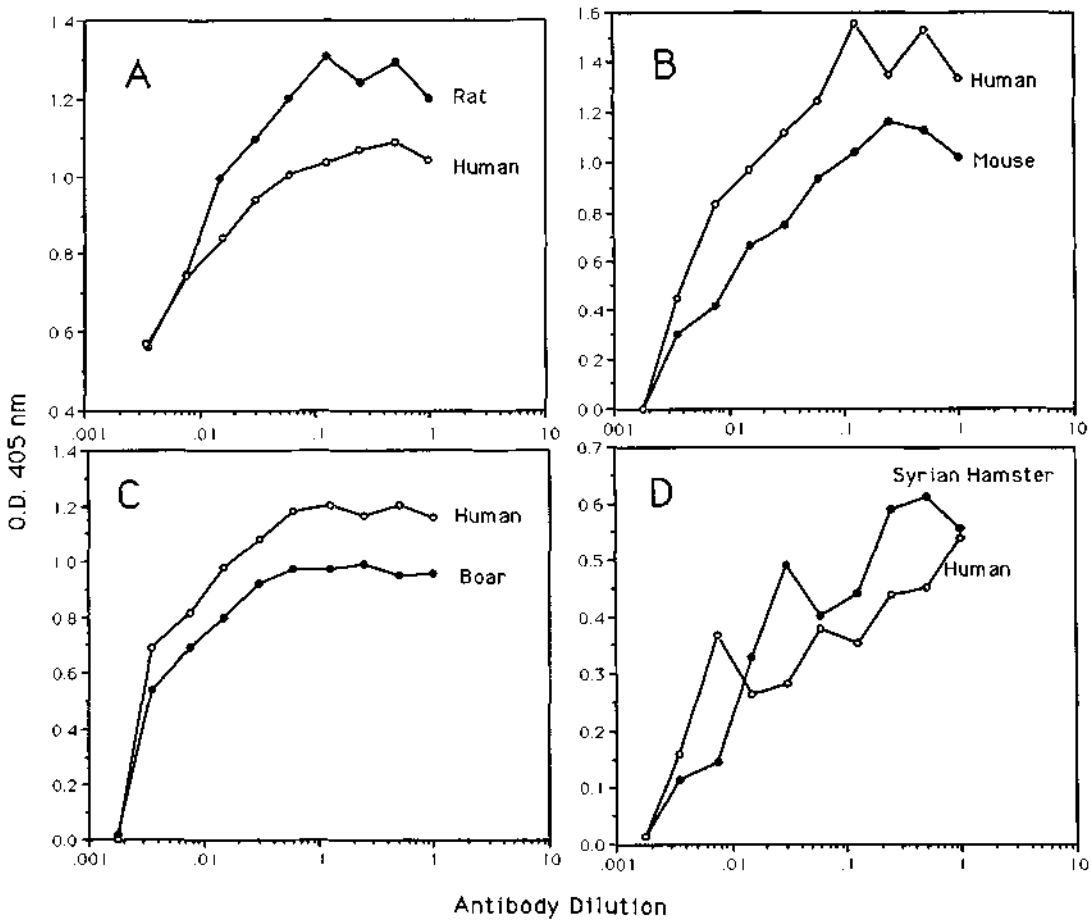

Fig. 2. ELISA analysis of Hup1N on a **(A)** rat protamine, **(B)** mouse protamine, **(C)** boar protamine, and **(D)** Syrian hamster protamine. In each case immunoreactivity to the various protamines was directly compared in companion experiments to reactivity to human protamine (empty circles in each panel). In each experiment tissue culture fluid from the Hup1N hybridoma was used in a 2-fold dilution series, as indicated in the figures.

that Hup1N reacted only with human P1. Immunoblot experiments with mouse protamine (C57B1/6) also suggest that Hup1N was P1 specific (data not shown).

The cross-reactivity of Hup1N with protamine from 15 species is summarized in Table I. In each case the percent cross-reactivity was calculated by comparing the area under the ELISA titration curve observed when unfractionated protamine mixtures from each species were tested vs. the titration curve obtained when human protamine was used as antigen. Representative titration curves of Hup1N on the protamine from 4 species are shown in Figure 2. These data suggest that Hup1N binds to a highly conserved region of the protamine molecule.

Immunoanalysis of Fractionated Rat Protamines

We have previously described Hup2b, an anti-P2 monoclonal antibody (Stanker et al., 1987). The ELISA data, shown in Figure 3, demonstrate that Hup2b binds P2 from mouse as well as it binds human protamine. In contrast, Hup2b displayed only a limited reactivity with the protamine preparation from *R. norvegicus* sperm (Fig. 3). Integration of the areas under these curves suggests that Hup2b has approximately 40% cross-reactivity to *R. norvegicus*. These data are in agreement with earlier studies (Bunick et al., 1990). In an effort to define more clearly the rat protein(s) that mAb Hup2b binds, we extracted the nuclear proteins from rat sperm, separated the proteins by HPLC, and probed each HPLC fraction with both Hup2b and Hup1N.

A representative HPLC chromatogram for whole-rat protamine is shown in Figure 4 (upper panel). Peak 1 can occasionally be resolved into 2 peaks. Thus peak 1 was arbitrarily split into 2 portions at its apex: the leading half (labeled peak 1a) and the trailing portion (labeled peak 1b). An electrophoretic analysis of chromatographic peaks 1a, 1b, and 2 is shown in Figure 5. Only P1 was observed in peak 1a (lane 2), while peak 1b contained P1 in addition to a number of proteins that have a slower mobility in the gel than does P1 (lane 3). Peak 2 contains several proteins, including a trace of

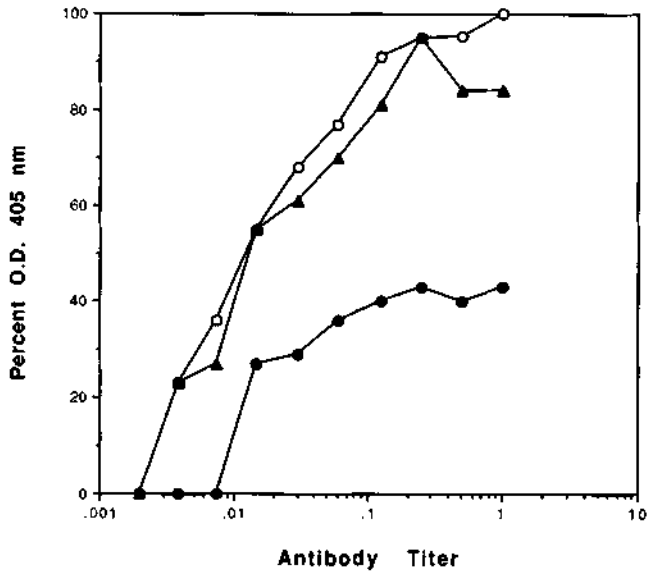

Fig. 3. Representative ELISA data showing the reactivity of Hup2b to nonfractionated extracts of human, mouse, and rat sperm protamine. Empty circles, human protamine preparation; filled triangles, mouse protamine; filled circles, rat protamine.

P1. In addition peak 2 contains at least 2 higher molecular weight proteins (Fig. 5, lane 4) that have an HPLC elution time and electrophoretic mobility similar to the non-disulfide-linked P1 dimer recently isolated from bull sperm (Ashmore et al., 1991). The final peak of the chromatogram represents histone protein (data not shown).

The HPLC fractions were analyzed with Hup1N and Hup2b in separate ELISA experiments (Fig. 4, lower panel). Hup1N detected P1 in peaks 1a and 1b, as well as the putative nonsulfhydral dimer of P1 and monomeric P1 in peak 2. Treatment of rat protamine with hydrochloric acid is known to result in a loss of P1 from peak 2 (Ashmore et al., 1991). In experiments in which the protamine preparation was treated with acid prior to chromatography, this peak was greatly reduced and no immunoreactivity with Hup1N was observed in the region of the chromatogram corresponding to peak 2, confirming the loss of P1 in peak 2. Only the protamine in peak 1 was immunoreactive in these acid treatment experiments (data not shown). More interesting is the observation with Hup2b. Hup2b did not react significantly with proteins comprising HPLC peak 1. However, a weak reactivity with fractions comprising the trailing edge of peak 1 was observed with Hup2b (Fig. 4). In contrast, Hup2b clearly recognized proteins present in peak 2. The identity of the Hup2b reactive proteins was analyzed in immunoblot experiments.

Immunoblots of both whole-rat protamine and pooled HPLC fractions from peaks 1 and 2 were probed with Hup1N and Hup2b (Fig. 6). Hup1N only detected monomeric P1 in peak 1a (lane 2). In peak 1b Hup1N detected P1 as well as 3 proteins with slower migrations than monomeric P1 (lane 3). Hup1N detected the putative P1 dimer and monomeric P1 in peak 2, as well as proteins having a slower migration than the P1 dimer (lane 4). Also shown in Figure 6 are replicate filters probed with Hup2b. Hup2b detected no proteins in peak 1a (lane 5) and weakly detected proteins in peak 1b having intermediate mobilities (lane 6). In peak 2, however, Hup2b clearly detected a series of proteins with slower gel migration rates than monomeric P1 (Fig. 6, lane 7). Some of these same proteins were weakly de-

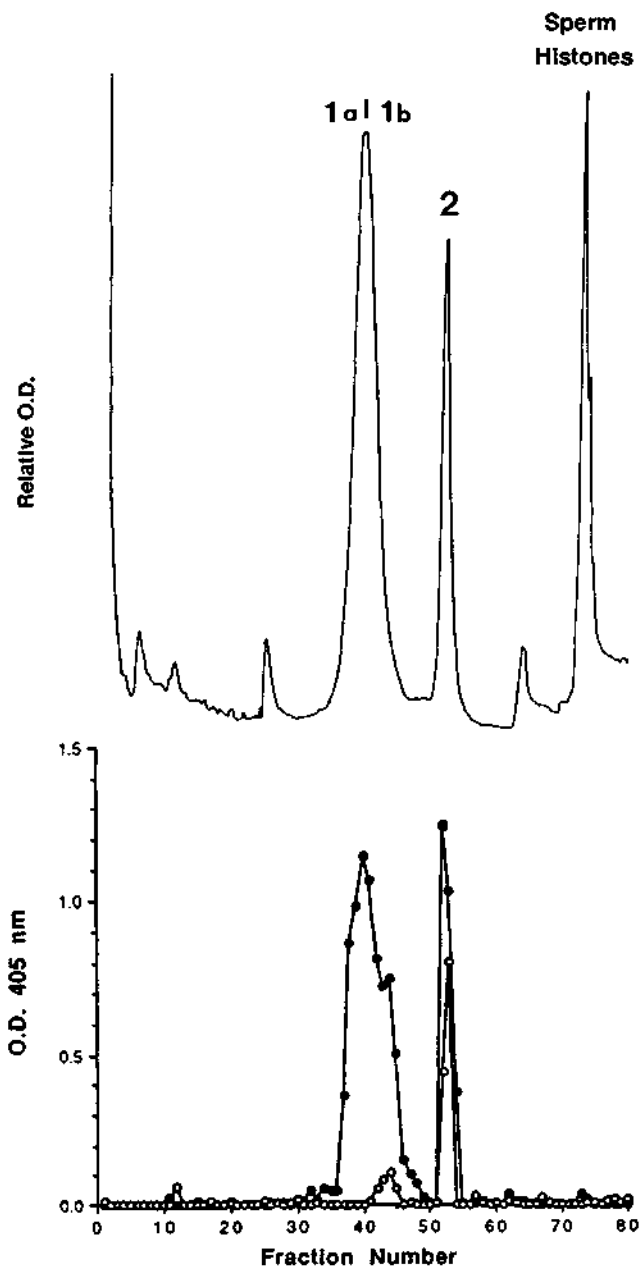

Fig. 4. Immunogram of rat protamine. **Top:** HPLC chromatogram of rat nuclear proteins. The chromatogram was generated as described in the methods section. **Bottom:** ELISA analysis of each HPLC fraction with Mab Hup1N (filled circles) and with Hup2b (empty circles). Peak fractions were analyzed following electrophoresis and immunoblotting as described in Figure 5.

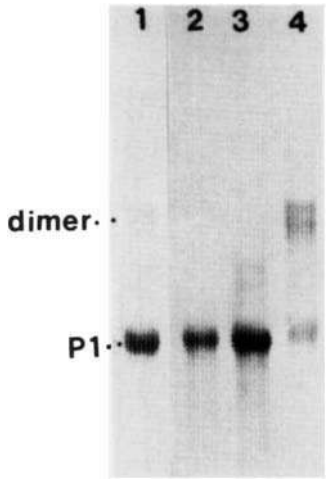

Fig. 5. Acid-urea gel-electrophoretic analysis of the proteins from peaks 1a, 1b, and 2 detected by HPLC in Figure 4. **Lane 1,** unfractionated rat protamine; **lane 2,** peak 1a; **lane 3,** peak 1b; **lane 4,** peak 2. All lanes were stained with amido Schwartz as described in the text.

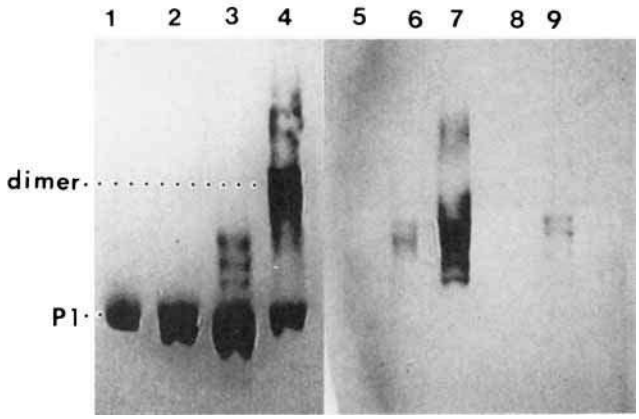

Fig. 6. Immunoblot experiments. Following acid-urea electrophoretic separation and transfer to filters, the lanes were probed with Hup1N and Hup2b as indicated. **Lane 1,** unfractionated rat protamine; **lane 2,** peak 1a; **lane 3,** peak 1b, **lane 4,** peak 2; **lane 5,** peak 1a; **lane 6,** peak 1b; **lane 7,** peak 2; **lane 8,** blank; **lane 9,** whole rat protamine. Lanes 1–4 probed with Hup1N; lanes 5–9 probed with Hup2b.

tected when crude rat protamine was analyzed with Hup2b (Fig. 6, lane 9).

DISCUSSION

We have generated a new monoclonal antibody that recognizes P1 from a large number of divergent species. The epitope recognized by Hup1N has yet to be determined, but our preliminary studies using synthetic peptides as antigen (unpublished observations) suggest that the antibody recognizes an epitope localized to the amino portion of P1. This is a highly conserved region of the molecule and would explain the cross-reactivities observed. The failure of Hup1N to bind rabbit protamine is also consistent with an amino-terminal localization of the epitope for Hup1N, since rabbit P1 has substituted a valine for arginine at amino acid position 1 (i.e., in the highly conserved amino hexapeptide sequence of P1). Similar peptide studies suggest that the epitope for Hup2b is localized to the amino half of the P2 molecule.

Coupling HPLC separation with immunochemical detection represents a powerful method for analyzing protamines. Antibodies to the mature protein will detect protein precursors as well as the fully processed protein. We estimate that immunochemical detection is 100–1000 times more sensitive than detection with absorbancy. This estimate is based on the ability to detect proteins with the antibodies at dilutions that show no absorbancy. In addition to an increased sensitivity, the specific recognition properties of the antibody converts the HPLC chromatogram into a multidimensional data set. Fractionation of rat nuclear proteins by HPLC followed by an ELISA analysis of the fractions with Hup1N clearly demonstrates that rat P1 is distributed among HPLC peaks 1 and 2. The ELISA results obtained with Hup1N are consistent with our observations following electrophoretic and immunoblot analyses of the HPLC fractions. Peak 1 appears to be mostly P1. The trailing half of peak 1 contains at least 3 more slowly migrating electrophoretic bands, in addition to P1. These more slowly migrating proteins may be the proteins detected on the immunoblots of peak 1b probed with Hup1N, indicating that they are different forms of P1. The reduction in electrophoretic mobility of these proteins is too great to simply represent different phosphorylated forms of P1. Rat P1 has been shown to have a maximum of 4 phosphorylation sites, and when fully phosphorylated only a small reduction in electrophoretic mobility was reported (Marushige and Marushige, 1975, 1978; Kistler et al., 1976). It is not known if these slower migrating P1 proteins represent ADP-ribosylated forms of P1.

The data presented here also suggests that P1 is present in HPLC peak 2 and that it exists in at least 2 forms. (i) The more slowly migrating forms detected in peak 2 on the immunoblots probed with Hup1N may represent non-disulfide cross-linked P1. A similar protein has been isolated from bull sperm (Ashmore et al., 1991). In addition, two non-disulfide linked proteins have recently been reported by Mazrimas et al. (1986) from an HPLC peak comparable to peak 2 reported here. These authors demonstrated that the nature of the cross-linking varied between the 2 nondisulfide dimers. (ii) Monomeric P1 also was detected in HPLC peak 2 and probably represents degradation of the non-disulfide cross-linked P1. Ashmore et al. (1991) have demonstrated that the non-disulfide cross-linked protein autodegrades in the presence of acid into monomeric P1.

No immunoreactivity, using Hup2b as the probe, was observed in HPLC peak 1a, and only a few extremely

faint bands of unknown origin were observed when peak 1b was probed. These proteins in peak 1b that were immunoreactive with Hup2b most likely are responsible for the weak reactivity observed in the ELISA when fractions from the trailing edge of peak 1 were probed with Hup2b. The lack of a strong reactivity with Hup2b across the peak 1 region of the chromatogram was expected, since peak 1 was thought to contain only P1. On the other hand, mature, fully processed P2 (in species that express the P2 gene and properly process the P2 precursor protein) normally elutes from the column just ahead of the P1 peak in other *Rattus* species (Balhorn et al., 1990). The complete lack of immunoreactive material in HPLC fractions 1–40 argues strongly that rat sperm do not contain fully processed P2. In contrast, peak 2 clearly contains proteins that are immunoreactive with Hup2b. Immunoblots of the proteins present in these fractions following electrophoresis through acid-urea gels show that numerous proteins were recognized by Hup2b. The bulk of the Hup2b-reactive proteins have an electrophoretic mobility that is intermediate between the P2 precursors observed in mouse and hamster (Balhorn, 1989) sperm and mature P2 from these species. The mobility of some of the rat proteins recognized by Hup2b are similar to the incompletely processed precursors HPS1 and HPS2 present in human sperm (Sautiere et al., 1988; Gusse et al., 1986) and 2 similar proteins isolated from stallion sperm (Pirhonen et al., 1989). Two other minor proteins have mobilities similar to the intact and partially processed mouse and hamster proteins. No proteins were detected with Hup2b in chromatographic peak 2 that had electrophoretic mobilities expected of a mature rat P2 protein. Thus it would appear that the low level of expression observed for the rat P2 gene (Bunick et al., 1990) results in the synthesis of a small amount of the rat P2 that is only partially processed.

In summary, using our procedure rat P1 was found as a dimer and in a higher order complex. The data presented in this communication support the conclusion that mature P2 is not present in rat sperm. However, the data also demonstrate that a partially processed form of P2 is present at reduced levels. These conclusions are consistent with previous studies (Bunick et al., 1990; Tanhauser and Hecht, 1989) that have established the presence of a P2 gene and a reduced level of P2 messenger RNA in rat.

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