BIOLOGY OF REPRODUCTION **70,** 319–328 (2004)
Published online before print 15 October 2003.
DOI 10.1095/biolreprod.103.022459

Identification of a Member of a New RNase A Family Specifically Secreted by Epididymal Caput Epithelium[1]

Sandrine Castella,[3] Sophie Fouchécourt,[3] Ana Paula Teixeira-Gomes,[4] Joëlle Vinh,[5] Maya Belghazi,[6] Françoise Dacheux,[3] and Jean-Louis Dacheux[2,3]

Equipe "Gamète Mâle et Fertilité" UMR INRA-CNRS 6073,[3] PRC, INRA, 37 380 Nouzilly, France
Atelier d'Electrophorèse et de Microséquençage de Protéines,[4] INRA, 37 380 Nouzilly, France
Laboratoire de Neurobiologie,[5] ESPCI, 75 231 Paris, France
Service de Spectrométrie de Masse pour la Protéomique,[6] 37 380 Nouzilly, France

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

ABSTRACT

In this study, we purified the first member of a new ribonuclease (RNase) A family from fluid of the proximal caput of the boar epididymis. This protein, named "Train A," is the most abundant compound secreted in the anterior part of the boar epididymis. After 2D electrophoresis, it is characterized by more than 10 isoforms ranging in size from 26 to 33 kDa and pI from 5 to 8.5. Several tryptic peptides were N-terminal sequenced, and an antiserum against one of these peptides was obtained. The protein was immunolocalized in the epididymal epithelium of the proximal caput, especially in the Golgi zone and the apical cytoplasm of the principal cells. In the lumen, spermatozoa were negative but droplets of reaction product were observed within the lumen. Full lengths of Train A cDNA were obtained from a λgt11 boar caput epididymis library and sequenced. The deduced protein is composed of 213 amino acids, including a 23-amino acid peptide signal and a potential N-glycosylation site. The mRNA of this protein has been retrieved and partially sequenced in the bull, horse, and ram, and homologous cDNA is found in databanks for the rat, mouse, and human. All the sequences are highly conserved between species. This protein and its mRNA are male-specific and exclusively expressed in the proximal caput of the epididymis, the only site where they have been found. Train A presents an RNase A family motif in its sequence. The RNase A family is a group of several short proteins (20–14 kDa) with greater and lesser degrees of ribonucleolytic activity and with supposed different roles in vivo. However, the presence of a long-conserved N-terminal specific sequence and the absence of RNase catalytic site for Train A indicate that Train A protein is a member of a new family of RNase A.

epididymis, gamete biology, male reproductive tract, sperm maturation

INTRODUCTION

Mammalian spermatozoa acquire their capacity to fertilize an oocyte in the epididymis by multiple and sequential interactions with proteins present in the luminal fluid. This milieu surrounding the spermatozoa is certainly one of the most complex fluids found in any exocrine gland. This complexity results from two particularities, i.e., continuous and progressive changes in its composition throughout the excurrent male duct and the presence of components in unusual concentrations, some of them not found in other body fluids. Specificity is maintained not only by active secretion and reabsorption throughout the tract but also by the presence of significant restrictions in the exchanges between the luminal compartment and blood plasma [1].

Almost all of the testicular proteins that enter the epididymis are rapidly absorbed in the first part of the organ. Protein composition of the epididymal fluid is mostly linked to the secretory activity of the epithelium. In the few species analyzed by proteomic approaches, more than 100 epididymal proteins are secreted by the epididymal epithelium throughout the epididymal tubule (boar, [2]; stallion, [3]; rat, [4]). This secretion activity varies according to the regions of the organ, the anterior part being the most active. Among the proteins secreted, only a few of them (less than 20) represent 90% of the total secretory activity of the epididymis [2]. Most of these major secreted proteins have been identified, and they show characteristics specific to the epididymis such as unusually secreted proteins or proteins with abnormally high concentrations in the epididymal fluid compared with other fluids.

The in vivo function of most of these proteins is uncertain and often not related to their activity found in other tissues (i.e., prostaglandin D_2 synthase, glycosidases, angiotensin converting enzyme, etc.). Whatever the exact role of these secretions, the specialization of each part of the epididymis (caput, corpus, and cauda) has considerable significance for the sequential acquisition of mature sperm properties. In most species, spermatozoa are able to fertilize only in the distal corpus, the caput and corpus being key regions for posttesticular sperm transformations that induce spermatozoon maturation.

In all the species studied, the anterior region is the most active in protein secretion. In the boar, the epithelium of this region secretes 80% of all the luminal proteins secreted by the organ [2]. Most of the major proteins secreted in the epididymis of this species have been identified except for one previously called "Train A." This compound, specific to the proximal caput, represents 89% of the protein secretion of this region and 34% of all the proteins secreted by the organ [2, 5].

The aims of this study are the identification of this unknown compound by protein purification, sequencing, and immunotechnology. The species and tissue specificity of this protein and its messengers were analyzed.

We present here a new protein that is only expressed in

[1]Supported by grants from the Institut National de la Recherche Agronomique (INRA, France) and from the Région Centre (France).
[2]Correspondence: Jean-Louis Dacheux, UMR INRA-CNRS 6073, Nouzilly, France 37380. FAX: 33 2 47 42 77 43; e-mail: jdacheux@tours.inra.fr

Received: 20 August 2003.
First decision: 5 September 2003.
Accepted: 23 September 2003.
© 2004 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. http://www.biolreprod.org

the epididymis. This new compound is identified as the first member of a new RNase A family and was found in the five species studied: boar, ram, stallion, mouse, and rat.

MATERIALS AND METHODS

Chemicals

Dulbecco Modified Eagle medium without methionine and cysteine (DMEM−); x-ray films (Kodak X-OMAT-XAR5, Eastman Kodak, Rochester, NY); keyhole limpet hemocyanin (KLH); *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS); CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate); and goat anti-rabbit IgG coupled to horseradish peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO). [^{35}S]Methionine was purchased from NEN (Les Ulis, France); [α-^{32}P]dCTP was purchased from Amersham (Les Ulis, France); acrylamide (30% acrylamide, 0.8% *N,N*-methylenebisacrylamide) was purchased from Roth (Karlsruhe, Germany); ampholytes pH 2–11 (Servalytes) were from Serva (Heidelberg, Germany); and ampholytes pH 3–10 (Ampholytes) and Coomassie brilliant blue (Phastgel Blue R) were from Pharmacia (Saclay, France). *N*-glycosidase F, O-glycosidase, and trypsin sequencing grade were purchased from Roche (Meylan, France); neuraminidase were purchased from Genzyme Corp. (Cambridge, MA); and acid phosphatase was from Sigma. All other chemicals were of molecular biology grade and were purchased from Sigma.

Animals and Organ Sampling

Six or eight normal adult animals of each species (boars, rams, stallions, rats, and mice) were used in this study. Epididymides, testes, and different tissues from several species were obtained after surgical removal from freshly killed animals at the local or a commercial slaughterhouse. The epididymis was divided into 10 regions (Fig. 1; 0–9) as previously described for the boar [2], ram [6], and stallion [3], and into three zones (caput, corpus, and cauda) for rat and mouse tissue extraction. For Northern blot analysis, epididymal and testicular tissues and other male and female tissues were collected, frozen in liquid nitrogen, and kept at −70°C until analysis. For the protein analysis, luminal fluids from the different epididymal zones were obtained by perfusion as previously described [7]. Testicular fluid was carefully collected by directly puncturing the rete testis. Spermatozoa were separated from the fluids by centrifugation (1500 × *g* for 15 min at 4°C). The fluids were removed and centrifuged again (15 000 × *g* for 10 min) and used directly or stored at −20°C until use.

In Vitro Secretion of [^{35}S]Methionine-Cysteine-Labeled Proteins from Isolated Tubules

In the boar, in vitro secretion of ^{35}S methionine-cysteine-labeled proteins was estimated from all epididymal regions (isolated tubules) and from testis and vasa efferentia samples (minced tissues) as previously described [3]. Briefly, one closed end tubule for each epididymal region was incubated in a mixture of ^{35}S methionine and ^{35}S cysteine in DMEM solution. At the end of the incubation period (5 h at 32°C), the medium was separated from the tissues, and the lumen fluid of each tubule was collected by microperfusion. All the incubation media were centrifuged (16 000 × *g* for 10 min) and the supernatants were used directly or stored at −20°C.

Gel Electrophoresis, Fluorography, and Western Blotting

Gel and sample preparation and methods for isoelectric focusing have previously been described [2]. SDS 6%–16% polyacrylamide gel gradients were used for protein separation. For detection of radioactive proteins, the gels were exposed on preflashed x-ray film for several days at −80°C or on a PhosphoImaging screen (Storm; Molecular Dynamics, Paris, France). For immunodetection, the proteins from acrylamide gels were electrotransferred (0.8 mA/cm^2 for 2 h) by a semidry technique on 0.2-μm nitrocellulose membrane. The membranes were blocked overnight with TBS supplemented with 0.5% (w/v) Tween 20 (TBST) and 5% (w/v) dry skimmed milk on a rocking platform. The primary antibody was added at 1:5000 dilution in TBST and the blots were incubated under mild agitation for 1 h 30 min at 37°C. Blots were then washed with the same buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted at 1:5000 in TBST for 30 min at 37°C. After several washings, the peroxidase was revealed by chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus; NEN, Boston, MA) according to the manufacturer's instructions.

Purification of Train A, Amino Acid Sequencing, and Antibody Production

Train A was purified from porcine epididymal fluid of region 0. The fluid was first separated on Superdex 75 (Pharmacia HR 75) in 20 mM Tris-HCl/200 mM NaCl (pH 7.4), and each fraction was analyzed by SDS-PAGE. The fractions containing the Train A were pooled and applied on a reverse-phase column Fast Performance Liquid Chromotography system (Source 5 RPC, Pharmacia). Elution was performed with a linear gradient of 2%–70% acetonitrile in 0.1% trifluoroacetic acid (TFA). The fractions containing Train A (between 70% and 75% of acetonitrile) were pooled, dried under air-vacuum, rehydrated, and separated on 2D gel-electrophoresis. The spots corresponding to Train A were cut after transfer to PVDF membrane or directly from acrylamide gels (zinc-imidazole staining [8]) and then used for digestion with trypsin. The amino acid sequences from the different peptides were obtained directly after separation on a reverse-phase HPLC system by Edmann technique or by mass spectrometry (nanoLC/Q-TOF, Micromass, Manchester, UK). A total of 19 partial amino acid sequences of the protein were obtained.

The fragment of the protein corresponding to 13 amino acids (AQAFSQSYPNYLR) and to the domain conserved between all the species (EDEVGGNKMLRA) were used to provide specific antibodies. These peptides were synthesized with a cysteine residue added at the C-terminal (Eurogentec, Seraing, Belgium) and then conjugated with KLH as protein carrier as previously described [7, 9]. Each peptide-KLH conjugate was used to produce polyclonal antibodies in two rabbits whose preimmune serum was negative against epididymal fluid proteins. Titration of antibodies against Train A was determined by ELISA assay with the peptide as the antigen.

N- and O-Linked Deglycosylation

N-linked deglycosylation was performed using *N*-glycosidase F (Roche) according to the manufacturer's instructions. The fluid of region 0 or the epididymal medium collected in region 0 after in vitro biosynthesis were incubated with different concentrations of *N*-glycosidase for 18 h at 37°C in 20 mM sodium phosphate pH 7.4. Epididymal proteins (50 μg) from region 0 were incubated in the presence of 2 U of *N*-glycosidase or 1 mU of O-glycosidase for 18 h at 37°C in 20 and 50 mM sodium phosphate, pH 7.4, respectively. Control samples were incubated in the same conditions without *N*-glycosidase. The deglycosylated proteins were then analyzed by SDS-PAGE (1D and 2D) and Western blotting.

Polymerase Chain Reaction with Degenerated Primers

Polymerase chain reaction (PCR; 94°C for 30 sec, 37°C for 1 min, 55°C for 1.5 min for 50 cycles, final extension at 72°C for 5 min) with caput epididymal cDNA of region 0 as template and 30 pmol of each degenerate oligonucleotide primer [forward: 5′-GA(A/G)GA(A/G)(A/T)(G/C)NGA-(C/T)(A/C)A-3′; reverse: 5′-A(A/G)(A/G)TA(A/G)TTNGG(A/G)TA-3′; 1.2 μM each] based on the sequences of two tryptic peptides (LEESDQ and YPNYL, respectively, Table 1) produced a 185-base pair (bp) product that was cloned and sequenced. This insert was used as a probe in the next step.

RNA Extraction

Total RNA was prepared from 200 mg of frozen tissue according to the isothiocyanate guanidinium technique described by Chomczynski and Sacchi [10] for the boar, stallion, and ram, and by RNAble (Eurobio, Les Ulis, France) for mice and rats. RNA was extracted from the testes, efferent ducts, and various epididymal regions. For each total RNA sample, 10 μg was separated by electrophoresis in 1% agarose formaldehyde gel. RNA was then transferred to a nylon membrane (Hybond N$^+$; Amersham) by capillary blotting in 10-strength Saline-Sodium Citrate buffer (SSC) (1.5 M NaCl, 0.15 M trisodium citrate, pH 7) and fixed for 30 sec under UV light. The membrane was stored at −20°C until prehybridization.

DNA Probe and Northern Blot Hybridization

We obtained specific DNA probes for Train A mRNA by reverse transcription-polymerase chain reaction (RT-PCR) using 2 μg total RNA extracted from region 0 (proximal caput) as described above. RT was performed by Avian Moloney Virus reverse transcriptase (Promega, Charbonnières, France) with oligo(dT) primers (Promega). The specific reverse-transcript Train A cDNA was then amplified by DNA polymerase (Goldstar; Eurogentec) with 30 pmol of a pair of specific primers (reverse

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

primer: 5′-GCTCTGAGCATCTTGTTTCCTCC-3′; forward primer: 5′-GAGGAAAGTGATCAGCTACTGAGTGAG-3′) corresponding to 185 bp (bases 160–344) of Train A cDNA. PCR was performed for 40 cycles (94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min) and a final elongation step at 72°C for 5 min. The DNA obtained was then labeled with [^{32}P]dCTP using Megaprime from Amersham. Hybridization was performed using [^{32}P]-labeled probes as described by Thomas [11].

The membrane was prehybridized in six-strength SSC, five-strength Denhardt solution, 0.5% SDS, 100 μg/ml herring sperm DNA, and 50% formamide for 2 h at 42°C. Hybridization with the labeled probe was performed in the same solution but without Denhardt solution for one night at 42°C. The membrane was washed in double-strength SSC, 0.1% SDS. The Train A transcript was visualized by exposure on a Phospho-Imaging screen (Storm; Molecular Dynamics).

The same step was performed for the RNase transcript of human and mouse tissues with specific primers (mice forward 5′-GGAGGATCA-GCCACTGA-3′, mice reverse 5′-CCAGTTGTCTCTTGTCA-3′, human forward 5′-AGCTGAATCTGGTGCAGA-3′, human reverse 5′-GTCGG GAGCTTTTGTGA-3′).

Construction of Boar Epididymal Region 0 cDNA Library

The cDNA expression library was constructed in λgt11 phage from region 0 of the boar epididymis poly(A) RNA using a cDNA library synthesis kit (Time Saver, Pharmacia) according to the manufacturer's instructions. Briefly, total RNA was extracted from region 0 of the boar epididymis. The poly(A) fraction was purified by oligo(dT)-cellulose column. Double-stranded cDNA was synthesized with 5 μg of poly(A) RNA template, ligated to *Eco*RI/*Not*I-digested λgt11 and subjected to the in vitro packaging Reaction (Ready-to-Go, Pharmacia). The packaged library was plated on *Escherichia coli* Y1090 and amplified as a plate lysate on agar plates at 37°C for 12 h.

Hybridization of Train A-Expressing cDNA Clones

The cDNA clones encoding Train A were identified by hybridization with the specific probe used for Northern blotting. The nitrocellulose filter was in contact for 90 sec with Petri dishes; removed; and soaked in 0.5 M NaOH and 1.5 M NaCl for 2 min, in 0.5 M Tris-HCl pH8 and 1.5 M NaCl for 5 min, and finally in SSC 2× for 5 min. cDNA was fixed at 80°C for 2 h and conserved at room temperature in a vacuum-air dried container until prehybridization. Prehybridization and hybridization were performed as for Northern blotting with 5× Denhardt solution for hybridization to the labeled probe. Positive clones were detected after exposure on the PhosphoImaging screen (Storm; Molecular Dynamics).

The positive clones were isolated and conserved in SM buffer (NaCl 100 mM, $MgSO_4$-$7H_2O$ 8 mM, Tris-HCl 50 mM pH 7.5) at 42°C for 2 h. Twenty microliters of the supernatant was boiled for 10 min at 95°C, and cDNA was amplified by λ forward primer (5′-GGTGGCGACGA-CTCCTGGAGCCCG-3′) and λ reverse primer (5′-TTGACACCAGAC-CAACTGGTAATG-3′) in the Goldstar system (Eurogentec; 40 cycles: 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min, and a final extension of 10 min at 72°C). The fragment was purified from low melting point agarose, cloned into pCR2.1 T/A cloning vector (Invitrogen), and transformed into Top 10 F′ *E. coli* to generate pCR2.1-Train A plasmid; it was sequenced with forward and reverse M13 primers.

Sequencing Analysis of cDNA Clones

The cDNA inserts were sequenced on an ABI Prism 377 autosequencer according to the dideoxychain terminator method. The cDNA sequence encoding Train A and deduced amino acid sequence were analyzed by GenBank/EMBO databank using the BLAST program.

RT-PCR in Different Species and in Different Tissues

A cDNA fragment corresponding to Train A was amplified and cloned in the ram, stallion, and bull. For the stallion, the fragment was amplified with the primer corresponding to human homologous Train A (see above, *DNA Probe and Northern Blot Hybridization*). For both the ram and bull, the amplification was performed with the primer corresponding to boar Train A. The PCR steps were the same as for the boar (20 cycles of PCR). For each species, amplification of a 350 bp-product of cyclophilin was performed as a standard by forward primer (5′-TAACCCCACCGTCTT CTT-3′) and reverse primer (5′-TGCCATCCAACCACTCAG-3′) in the Goldstar system (Eurogentec; 20 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 5 min at 72°C). For rats and mice, the fragment was amplified with the primer corresponding to murine ribonuclease (see above, *DNA Probe and Northern Blot Hybridization*).

RT was performed by Avian Moloney Virus reverse transcriptase (Promega, Charbonnières, France) with oligo(dT) primers (Promega) in different tissues of the boar. The specific RT Train A cDNA was then amplified by DNA polymerase (Goldstar; Eurogentec) with 30 pmol of specific primers (see *DNA Probe and Northern Blot Hybridization*) corresponding to 185 bp (bases 160–344) of Train A cDNA. PCR was performed for 20 cycles (94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min) with a final elongation step at 72°C for 5 min.

Immunohistochemical Staining

Boar epididymides were removed from freshly killed animals and fixed immediately by immersion in Bouin fixative. Small pieces of tissue from each epididymal segment (Fig. 1) were excised and fixed in Bouin solution for 24 h. Tissues were then transferred to 70% ethanol, which was changed several times, followed by standard embedding procedures including dehydration in alcohols, clearing in toluene, and final paraffin embedding.

For immunolocalization of Train A, sections (5–6 μm) of paraffin-embedded tissues were floated on 48°C water drops and deposited on slides precoated with 3-aminopropyltriethoxy-silane (Sigma). Sections were deparaffinized in toluene and rehydrated in ethanol with increasing concentrations of water. Some sections were immersed in 0.01 M sodium citrate solution and boiled for 5–10 min in a microwave oven and then cooled and rinsed in PBS.

Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 min, and the nonspecific binding sites were blocked with 10% normal goat serum (NGS) for 1 h. Immunostaining was performed using the avidin-biotin-peroxidase complex technique (Vectastain Elite ABC kit, Vector Laboratories). Primary and secondary antibodies were diluted in a solution of PBS containing 10% NGS. Tissue sections were exposed to anti-Train A antibody (diluted 1/1000 to 1/8000) overnight at 4°C. After several rinses in PBS, the sections were incubated with secondary antibody (anti-rabbit IgG biotinylated) for 1 h at room temperature following incubation in ABC reagent. The peroxidase activity was revealed by a solution of diaminobenzidine (DAB) in 0.05 M Tris buffer containing H_2O_2.

Staining specificity was checked by incubation of sections with preimmune rabbit serum. The sections were counterstained with hematoxylin and then dehydrated, rinsed in toluene, and mounted in DePeX.

RESULTS

Purification and Characterization of Train A

Train A is a major polymorphic protein present only in the fluid of the proximal caput of the boar epididymis (Fig. 2A). It is the most abundant protein synthesized in the anterior caput and is composed of at least 10 isoforms ranging in size from 26 to 33 kDa and pI from 5 to 8.5 (Fig. 2A). Purification was achieved from porcine epididymal fluid sampling in region 0. The protein was collected by gel filtration separation through a Sephadex 75 column (Fig. 2, B and C) in the 25–35 kDa fraction and eluted from a reverse phase column with 72% acetonitile-TFA (Fig. 2, D and E). After these two liquid chromatography steps, final purification was achieved by protein separation by a 2D gel electrophoresis on which Train A represented more than 90% (Fig. 2F).

The amino acid sequences for the major spots representing this protein were all N-amino terminal blocked. N-terminal sequences from tryptic peptides were obtained by Edmann technique and by de novo sequencing with the mass spectrometry technique (Table 1). For all the internal partial sequences, no homology was found in the various databanks accessible at the time when the results were obtained.

The polyclonal antibody raised against an internal peptide (Table 1, peptide 3) specifically recognized the protein on Western blotting and detected its presence only in the luminal fluid of the anterior region (Fig. 3A). The immunodetection of Train A in the luminal fluid coincided with

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

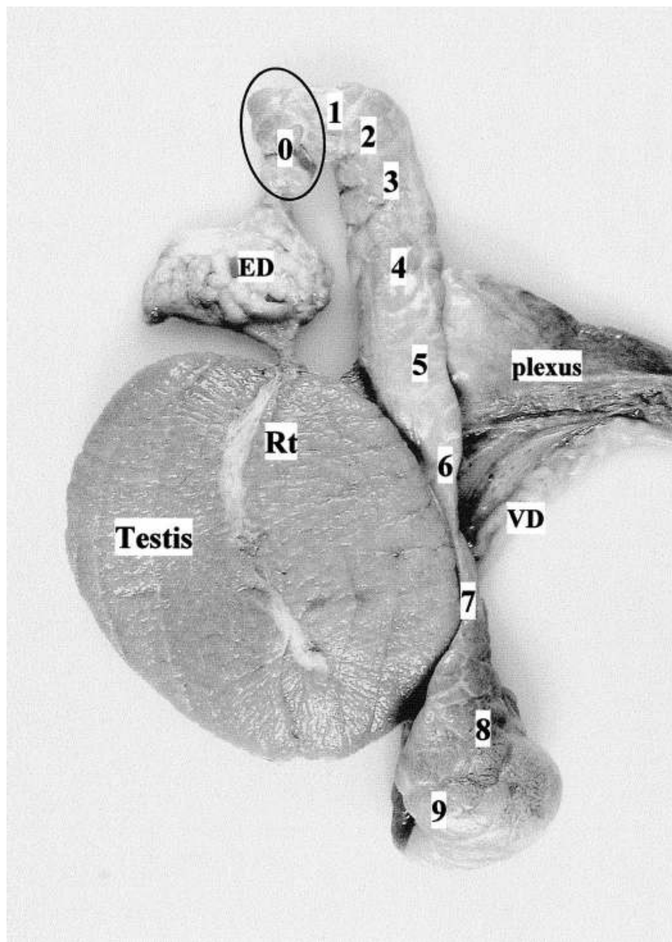

FIG. 1. Various regions of the boar epididymis from which tissue and fluid samples were collected. The epididymis was subdivided into 10 regions (0–9): 0–1, proximal caput; 2, middle caput; 3–4, distal caput; 5–7, corpus; and 8–9, cauda. The circled region represents the localization of Train A secretion. The size of the epididymis was about 15 cm length. ED, Efferent duct; VD, deferent duct; Rt, rete testis; plexus, pampiniform plexus.

the localization of its secretion: i.e., more intense in region 0 than in region 1 (Fig. 3B). All the isoforms immunodetected in these regions were found to be the same as those secreted in the lumen (Fig. 4B).

Incubation of the native protein in the presence of *N*-glycanase reduced the number of isoforms, and only three major isoforms still remained (Fig. 4, A–C), with a 5-kDa shift in their molecular mass and a pI around 6 (Fig. 4A). Phosphatase, neuraminidase, and O-glycanase had no effect on the number and characteristics of the different isoforms (data not shown).

Molecular Cloning and Sequence Analysis of cDNAs of Train A Protein

A cDNA probe for Train A was obtained from RT-PCR of mRNA of the proximal caput and degenerated primers deduced from several peptide sequences. A band of 185 bp was amplified and sequenced with two of these primers (see *Materials and Methods*). Full-length cDNA was obtained from the cDNA library of the boar proximal caput epididymis in the λgt11 expression vector. From this library, 4% of the clones were positive after hybridization with the 185 bp radio-labeled probe. The longest insert found corresponded to a sequence of 811 bp. This sequence contained an opening reading frame of 642 bp from a start codon and a polyadenylation signal after the stop codon, showing that this sequence represented the full-length cDNA of Train A (Fig. 5; EMBL access number AJ430467).

The deduced protein contained 213 amino acids, with a 23-amino acid putative signal peptide, leading to a mature protein of 189 amino-acids with a theoretical mass of 21.1 kDa and pI of 5.98. All of the partial amino acid sequences previously obtained in the study were retrieved in this deduced amino acid sequence of Train A.

There was a conserved domain of pyrimidine-specific endonucleases between amino acids 93 to 213, representing 30%–33% of homology with the RNase A protein family and characterized by the presence of eight conserved cysteines (Fig. 6).

Four putative proteins in the databanks presented a high homology with this whole amino acid sequence of Train A: 81.1% identity with a putative human gene (LOC338879, XM_292225); 76.5% with a rat putative gene (LOC305840, XM_223962); and 81.9% with mouse mRNA (493047 4F22Rik, AK015573; Fig. 7).

Immunohistochemical Detection of Train A in the Epididymal Epithelium

Immunolocalization of Train A was performed in the different regions throughout the epididymis. Expression of this protein was strikingly limited to the principal cells of the proximal caput (regions 0 and 1; Fig. 8, A–C). In these cells, intense staining was localized in the supranuclear cytoplasm, probably corresponding to the Golgi zone (Fig. 8C) where the saccules and vacuoles are particularly well developed [12, 13]. A conspicuous reaction was also detected in the apical cytoplasm, with some accumulation and concentration near the plasma membrane varying according to the cells, and very high in some cells (Fig. 8C). A slight and homogeneous reaction was also present throughout the cytoplasm of the cells, especially after treatment of sections in a microwave oven (not shown). The microvilli of the principal cells appeared negative (Fig. 8, A–C), but in some tubules droplets of reaction product (such as globules and vesicles) were observed within the lumen or close to the epithelium, especially near the microvilli (between or at the apex; Fig. 8B). In the lumen, these droplets presented heterogeneity in their staining: some appeared reactive and others less reactive or negative (Fig. 8B). Spermatozoa present in the lumen were not stained. All the other epididymal regions appeared completely negative (Fig. 8D), without any evidence of this specific secreted protein in the lumen of the duct. Use of normal rabbit serum or omitting the primary antibody from sections showed no reaction over the epithelium, lumen, or intertubular space of the epididymis.

Tissue and Species Specificity of Train A

In the boar, Train A was immunodetected only in the fluid of the proximal caput (regions 0–1) and secreted in vitro by the isolated tubules of the same region; the other parts of the genital tract were negative (Fig. 3, A and B). Train A mRNA was detected only in the proximal caput of the boar (with higher expression in region 0) by Northern blotting (Fig. 3C) or RT-PCR after 20 cycles (Fig. 3D). No mRNA was detected in other tissues studied by Northern blot analyses, even after long exposure (Fig. 9A) or after

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

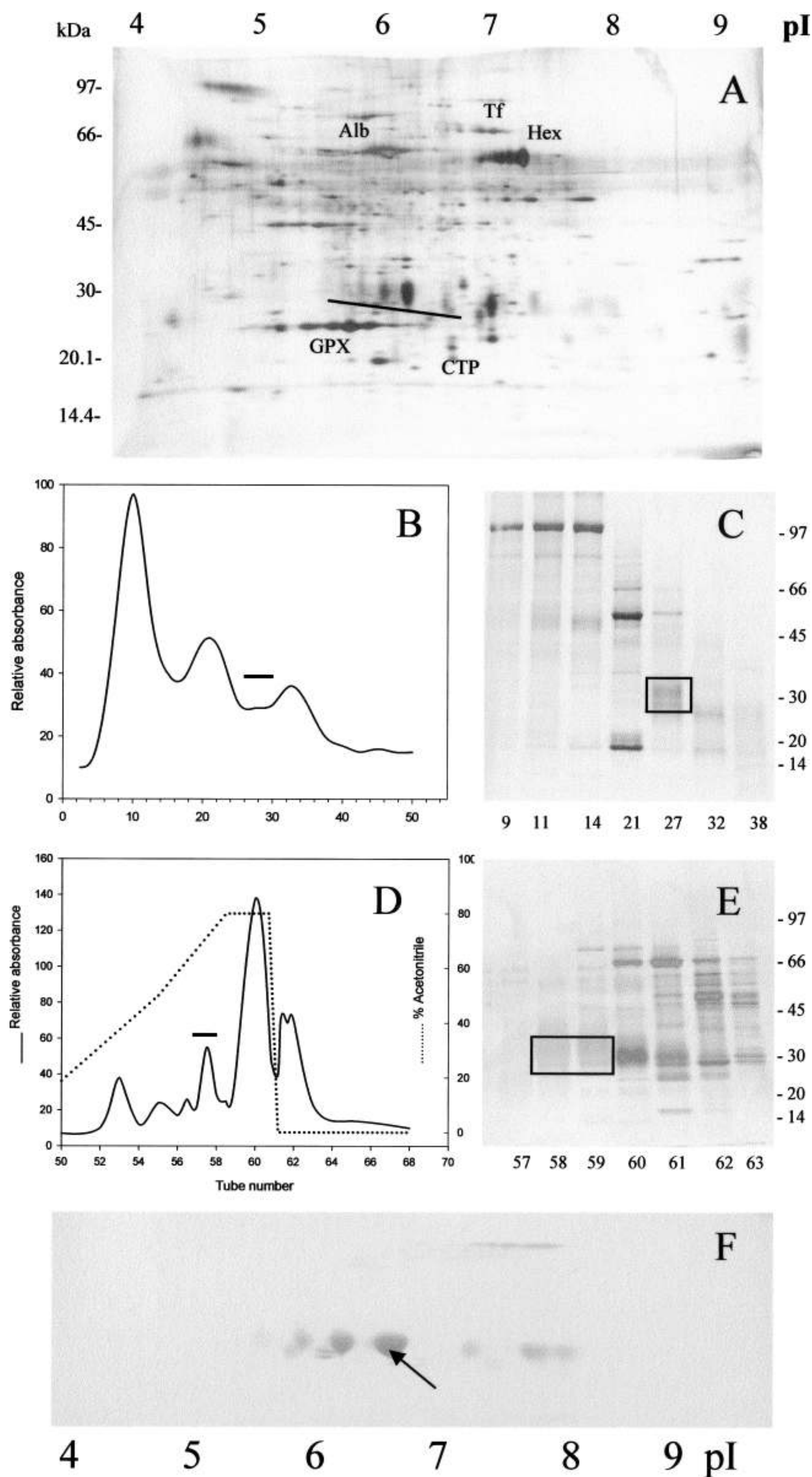

FIG. 2. Purification of isoforms of Train A. **A**) Silver-staining of the luminal proteins from region 0 of the boar separated by 2D gel electrophoresis. Train A is underlined. Molecular masses are indicated on the left and pI values at the top. Profiles of the proteins eluted through gel filtration Superdex 75 (**B**) then separated (for fractions 25/29) by reverse-phase chromatography (**D**). The protein content in the fraction was analyzed by 1D SDS-PAGE (**C** and **E**) at each purification step. Molecular masses are indicated on the right, and Train A is indicated by a square. The fractions containing Train A were separated on 2D SDS-PAGE (**F**), and the largest spot was cut to be sequenced (arrow). Tf, Transferrin; GPX, glutathione peroxidase; Hex, hexosaminidase; CTP, cholesterol transfer protein.

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

20 cycles of RT-PCR, which is in contrast to the abundance of Train A mRNA in the epididymal caput (Fig. 9).

In other species, (ram, stallion, rat, and mouse), Train A mRNA was detected by Northern blotting (Fig. 10A) and by 20 cycles of RT-PCR only in the proximal caput of the epididymis (Fig. 9). Bands of 185 and 500 bp were obtained by RT-PCR in the ram with boar primers, the stallion with human primers, and in the mouse with the mouse primers, respectively. One size of messenger was observed on Northern blotting for all species, except for the horse where an additional larger size band was present.

Train A sequences were obtained by RT-PCR for the stallion, ram, and bull (incomplete at the 3′ end; Fig. 11). Homology between Train A proteins was very high. Similarity with porcine protein was 75% for bovine (162 amino acid overlap), 72% for ovine (162 amino acid overlap), and 72% for equine (165 amino acid overlap; Fig. 11). Conserved motifs could be detected in the N-terminal part of

TABLE 1. List of internal sequences of Train A obtained after digestion by trypsin and mass spectrometry or Edmann sequencing.

Sequence number	Amino acid sequence
Seq1*	A_{32}VLEESDQLLSEFQ
Seq2	T_{64}METL
Seq3	**A_{96}QAFSQSYPNYLR**
Seq4	S_{151}PPVACELK
Seq5	R_{168}PFDLTFXK
Seq6	L_{177}SKPGQVTPXCNYVT
Seq7	H_{197}ILIS

* The theorical mass of Seq1 corresponding to the predicted N-terminal tryptic peptide of the protein was 3096, but those obtained by mass spectrometry was 2884 Da.

the protein such as xE_{88}DEVGGxxMLRAx (Fig. 11). However, an antiserum obtained from 12 amino acids from this conserved sequence (Fig. 5, peptide 2) detected Train A protein only in the boar epididymis and not in the other species.

DISCUSSION

Train A was previously shown in the boar to be synthesized and secreted as a major protein, representing 34% of the total secretion of the organ and 89% of the proximal caput [1, 2]. In the present study, this compound was identified after microsequencing several internal peptides of the protein and cloning its complementary DNA.

The protein was purified and immunodetected with a specific antiserum and found exclusively in the proximal caput of the epididymis, especially in the Golgi zone and in the apical cytoplasm of the principal cells.

Train A protein was composed of 213 amino acids, including a peptide signal of 23 amino acids and a theoretical mass of 21.1 kDa and pI of 5.98. In the epididymis, Train A secreted as polymorphic isoforms (over 10) ranging from 26 to 33 kDa and pI from 5 to 8.5, which was thought to represent numerous posttranslational modifications.

Neuraminidase had no effect on the polymorphism, suggesting that the protein is not sialylated. One potential O-glycosylation site was predicted in S_{177}, but incubation with O-glycanase had no effect on the isoforms. After treatment with N-glycanase, the apparent molecular mass decreased to around 50% of the initial mass, and the number of isoforms was reduced to three major spots with pI around the calculated pI.

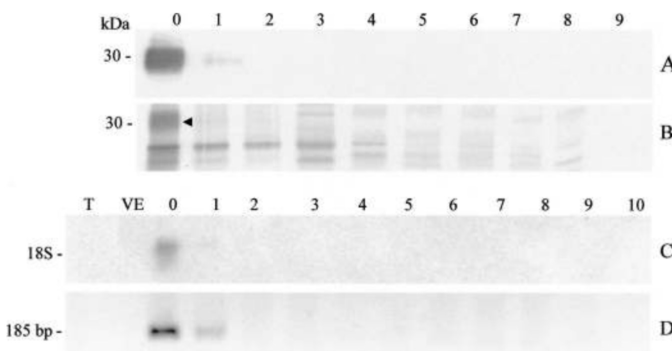

FIG. 3. Localization of protein and messengers of Train A in the boar epididymis. **A**) Immunodetection by Western blot in the luminal fluids from regions 0–9 separated by 1D electrophoresis. **B**) In vitro fluorography of [35S]methionine-labeled epididymal proteins secreted in isolated tubules (Train A indicated by arrowhead). **C**) Detection of Train A mRNAs by Northern blot with a specific probe obtained by RT-PCR. **D**) RT-PCR (20 cycles) with specific Train A primers. T, Testis; VE, efferent ducts.

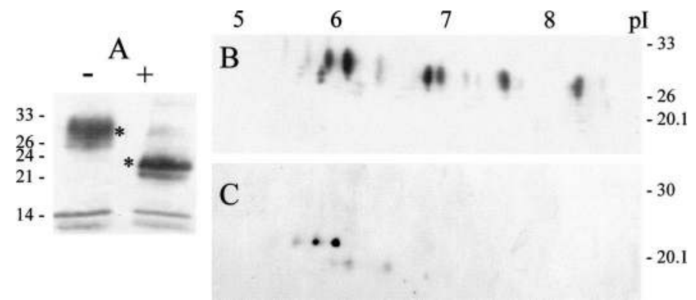

FIG. 4. Action of N-glycanase on Train A secreted in the fluid of region 0 of the boar epididymis. **A**) Western blot after 1D electrophoresis of fluid of region 0 of the epididymis (−), and after action of N-glycanase (+). Train A was detected with a specific antibody against internal peptide 1 of Train A. **B**) Isoforms of Train A detected with the same antibody on Western blot after 2D electrophoresis in normal fluid of region 0 and (**C**) after N-deglycosylation.

Five other sites of posttranslational modifications are potentially present on this sequence: two protein kinase C phosphorylation sites, amino acid positions T_{56}KK and S_{166}AR, two casein kinase II phosphorylation sites, positions S_{84}EDE and S_{201}CND, and one N-myristoylation site in position G_{26}LQMAA. Phosphatases had no effect on the characteristics of the isoforms, suggesting that phosphorylation had no effect on the polymorphism of the protein or that the protein was not phosphorylated. We have no evi-

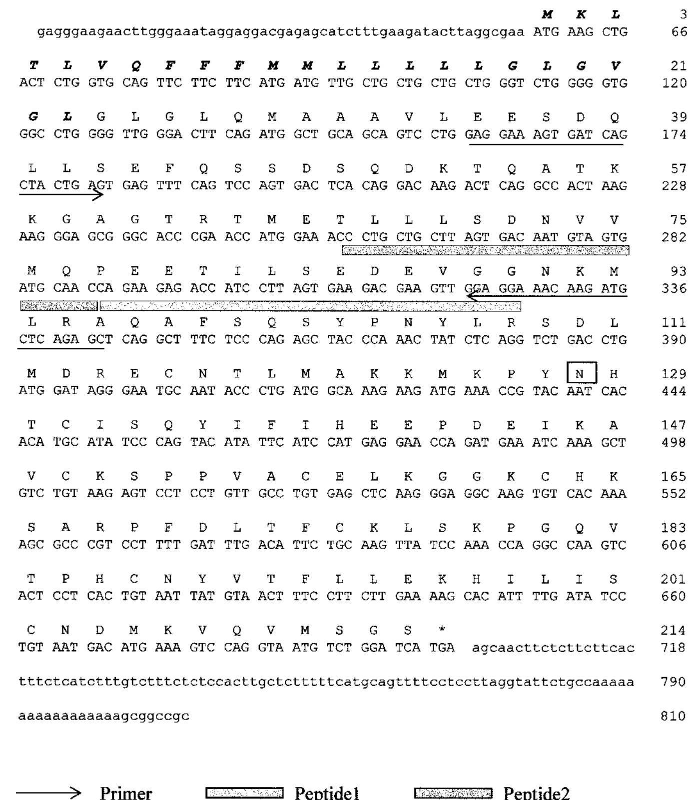

FIG. 5. Nucleotide and deduced amino acid sequences of Train A. The Train A open reading frame contains 639 bp between ATG (58–60) and TGA (697–699) coding for a 213-amino acid protein. The predicted amino acid sequence of Train A contains a signal peptide (amino acids 1–23) represented in italics. Sequences corresponding to the specific primers are underlined. Potential *N*-glycosylation site is boxed. Peptides used for production of antibodies are represented by shaded bars.

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

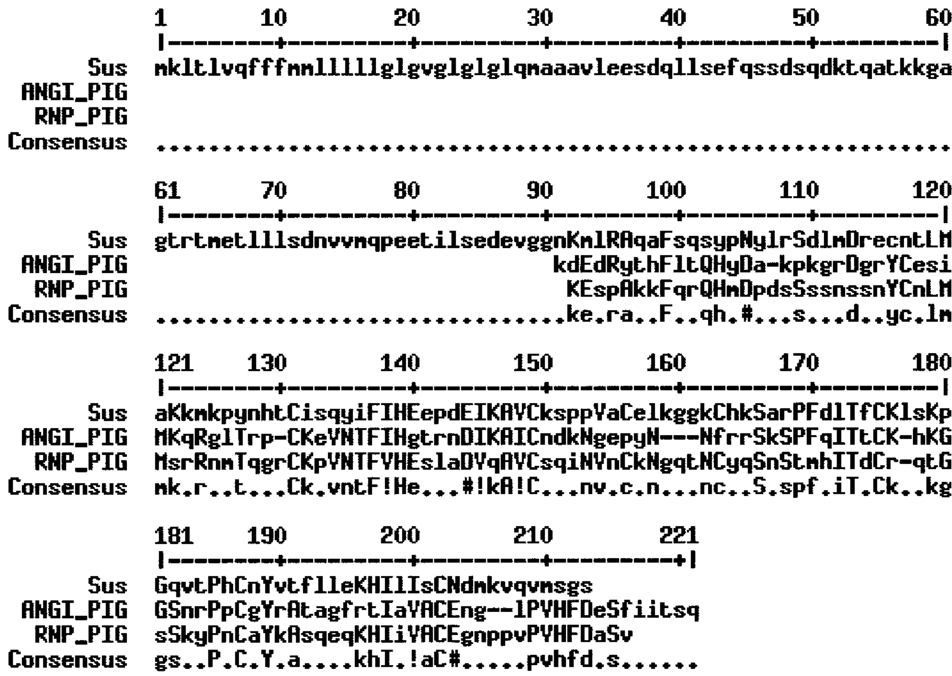

FIG. 6. Sequence alignments between porcine Train A (Sus, AJ430467) and porcine pancreatic ribonuclease (RNP_PIG, P00671) and angiogenin (ANGI_PIG, P31346). Homology with RNase A family begins at Arg_{95}. Alignment was performed with MultiAlign software [39].

dence of the presence of myristoylation, but this potential modification could be related to Edmann reaction blockage of the N-terminal site found for this protein, the myristoylation site being at the N-terminal position after release of the peptide signal. Furthermore, the measured mass of the N-terminal tryptic peptide was lower than the theoretical one, suggesting either the presence of a posttranslational modification or a longer length of the predicted signal peptide. Therefore, this high degree of polymorphism and the presence of isoforms with basic pI is probably linked mainly to the presence of a long sugar residue chain at the single potential *N*-glycosylation site present on Asn_{128}.

In the pig, several ESTs corresponding to this protein were recently sequenced from a cDNA multitissue library, including the epididymis [14]. The mRNA or the gene of Train A were retrieved in bovines, equines, ovines, rats, and mice. The nucleotide sequences of Train A were highly homologous between species. However, differences in the length of the messenger were noticeable between species. For example, in mice the 3′ untranslated nucleotide sequence of mRNA was longer than in other known mRNA of this protein.

Train A was highly conserved between species, with several specific motifs such as sequence xE_{85}DEVGGxx MLRAxxxxQSx similar for each species. The single potential site of N-glycosylation corresponding to Asn_{128} in the pig was conserved except in the human sequence, which showed no glycosylation site at all. An additional potential N-glycosylation site was present in rodents.

All the sequences obtained from the different species showed conserved RNase domain in the second half part. This homology between Train A proteins and ribonuclease A was principally linked to the presence of the eight conserved cysteines characteristic of this super family. The ribonuclease A family is a diverse group of secretory proteins that share distinct structural features and enzymatic activity [15]. Several members of this group have developed specific biochemical activities and physiologic functions. The presence of eight conserved cysteines, the conservation of Asp_{121} and the catalytic H-K-H triad (His_{12}-Lys_{41}-His_{119} in the bovine pancreatic ribonuclease numeration) are signature motifs of this superfamily. Several groups of RNase A family have been identified in various species. This family is most fully documented in humans and is composed of eight different RNases: RNase A (RNase 1 [16]), two eosinophil RNases (RNases 2 [17] and 3

FIG. 7. Alignment of amino acid sequences for Train A from pig (AJ430467) and predicted rat (XM_223962), mouse (AK015573), and human protein (XM_292225).

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

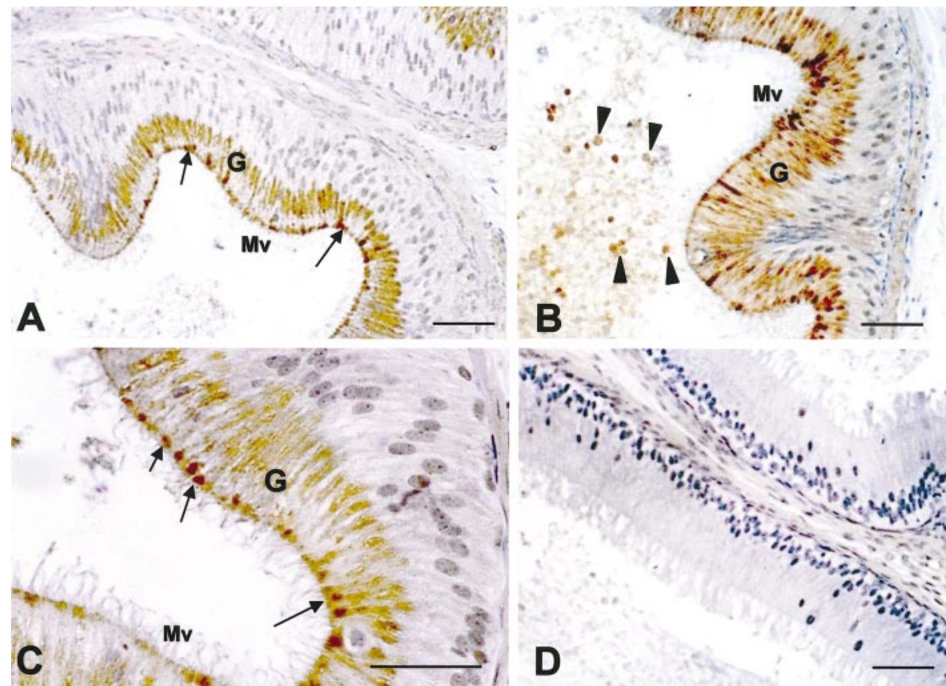

FIG. 8. Immunocytochemical localization of Train A in the proximal caput—zone 0 (**B** and **C**) and zone 1 (**A**)—and in the midcaput, zone 2 (**D**) of the boar epididymis. Note the intense immunostaining in the supranuclear cytoplasm probably corresponding to the Golgi zone (G), and a conspicuous reaction in the apical region near the plasma membrane (arrows), varying according to the cells and even very high in some cells. The microvilli of the principal cells appeared negative (Mv) but in some tubules, droplets of reaction product (such as globules or vesicles) were present near the microvilli or within the lumen (arrowheads). Note the heterogeneity of their immunostaining. All the other epididymal regions appeared completely negative (**D**) without any presence of this specific protein secreted in the lumen of the duct. Bar = 50 μm.

[18]), RNase 4 [19], RNase 5 or angiogenin [20], RNase 6 detected in neutrophils and monocytes [21], RNase 7 present on the skin surface [22], and RNase 8 isolated from human plasma [23].

However, homology of Train A from different species with the proteins of the different groups of this superfamily is no more than 25%, whatever the species. In Train A sequences, the RNase A consensus pattern C-K-x(2)-N-T-F in position C_{131} and the H-K-H triad (expected positions: 103, 132, and 211 in the pig) are not conserved.

With the presence of an additional sequence before the RNase motif, Train A represents the largest sequence of the RNase A family. The sequence characteristics and specificity for the other members of this family indicate that this protein represents a new group of proteins in the RNase superfamily.

Among all the tissues and species analyzed, the messengers of this protein were preferentially expressed in the proximal part of the epididymis. The high percentage of Train A clones (4%) found in the boar caput epididymis cDNA library confirmed the intense expression of the gene in this tissue. Furthermore, several ESTs from this messenger were also found in a pig normalized multitissue cDNA library, including epididymal tissue [14]. These messengers seemed to be preferentially expressed in the epididymis since they were not detected by PCR technique in other tissues, including male and female genital tissues. However, cDNA similar to this messenger has been found in the adult male mouse testis (AK015573.1, cDNA RIKEN full-length enriched library [24]), although this mRNA could not be detected in this organ in the present study either by Northern blot or by RT-PCR. Three murine ESTs from different cDNA libraries were also found: one from a spontaneous tumor, metastatic to a mammary tumor (EST: BF451140.1);

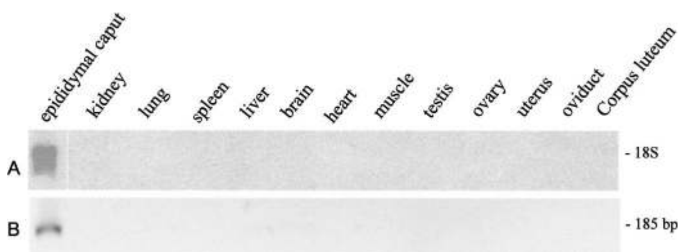

FIG. 9. Detection of Train A mRNA with the specific probe obtained by RT-PCR in total RNA from the epididymis (region 0) and various tissues of the pig (male and female) by Northern blot (**A**) and by RT-PCR after 20 cycles (**B**).

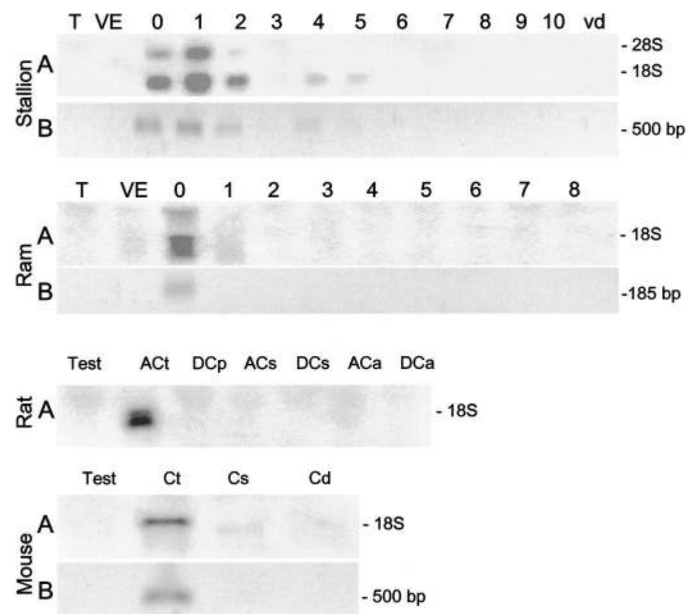

FIG. 10. Detection of Train A mRNA in different species (stallion, ram, mouse, and rat) in total RNA by Northern blot (**A**) and by RT-PCR after 20 cycles (**B**) from testis (T, Test); efferent ducts (VE); and from various epididymal regions, which are numbered as 0–10 for stallion and ram according to [3, 6]. Identified in rat as ACt, anterior caput; DCp, distal caput; ACs, anterior corpus; DCs, distal corpus; ACa, anterior cauda; DCa, distal cauda. Identified in mouse as Ct, caput; Cs, corpus; Cd, cauda.

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

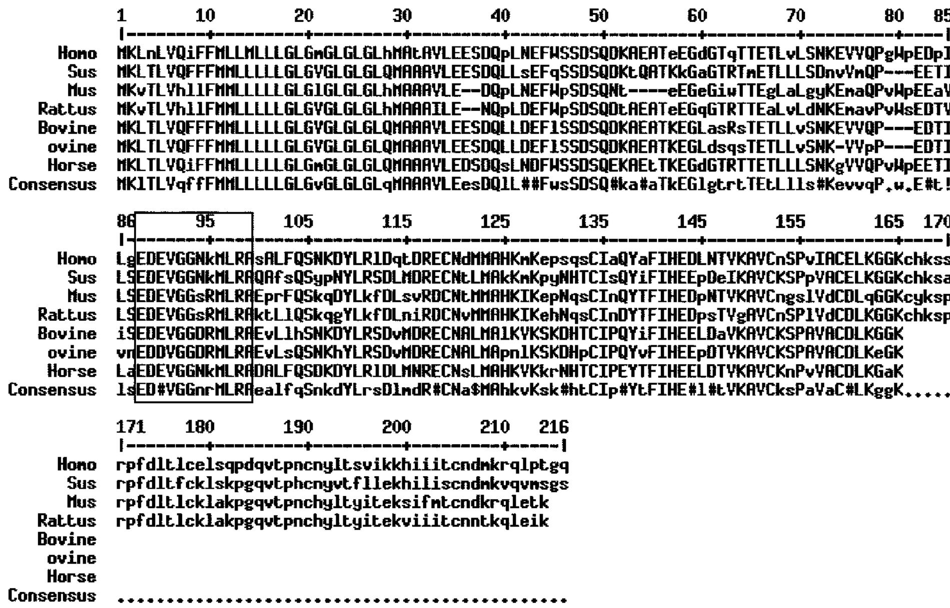

FIG. 11. Homology for Train A protein between boar, ram, stallion, bull, rat, murine, and human predicted proteins. Peptide 3 was extracted from the sequences in the box in order to obtain an antiserum. The sequences for equine, bovine, and ovine have been deposited in EMBL with accession number, respectively, AJ580632, AJ580633, AJ580634.

one from a 16-day-old embryo head (EST: BB362165.1); and one from a 3-day-old neonate thymus (EST: BB243486.1). Detection of these ESTs indicated that these messengers could be transitorily expressed in other specific tissues or during anomalous cell development.

The Train A gene was localized in a single copy in the same cluster of the RNase superfamily genes: in region 14q11.2 in the human, in 14C1 in the mouse, and in 15p14 in the rat. According to rodent genomic sequences, the Train A gene probably constituted two exons such as in the mouse: exon 1 of 35 bp and exon 2 of 1486 bp separated by an intron of 1487 bp, the exon 1 being into the untranslated region of the mRNA (http://www.ensembl.org; Ensembl gene: ENSMUSG00000021872; Fig. 12). This gene structure agrees with that of the RNase gene family that is composed of two exons separated by an intron in the 5′ untranslated region, the entire coding sequence residing on exon 2 [25, 26]. However, two sizes of message were observed in the horse. The exact structure of the gene Train A remains to be clarified.

Using immunocytochemical techniques we have also defined the localization of this new RNase in the principal cells of the boar proximal caput. The localization of the protein throughout the classical pathway of protein secretion (i.e., endoplasmic reticulum [ER], Golgi zone, presumably apical vacuoles concentrated near the plasma membrane) is in agreement with the presence in the protein structure of a conventional signal peptide and one potential N-glycosylation site. These results suggest merocrine secretion. However, ultrastructural immunogold studies are necessary to clarify several points observed in this study: 1) the absence of diffuse immunoreactive protein accumulating and sticking around the microvilli, in contrast with other epididymal proteins secreted by the conventional route [13, 27, 28]; 2) the presence of RNase-containing droplets in the lumen; and 3) the close proximity of some luminal droplets to the microvilli of the principal cells. These results suggest an apocrine-like secretory mechanism. A nonclassical protein secretion via vesicles (aposomes) has been described in the epididymis of various species (monkeys [29, 30], cats [31, 32], bulls [33], rats [34], mice [35], and humans [36]) and referred to as "apocrine secretion" [37, 38]. It has been suggested that this alternative extrusion mechanism seems to be specific for certain proteins, mainly membrane-associated proteins and proteins synthesized within the cytoplasm without a signal peptide. However, several studies have shown that the apical blebs may contain a variety of organelles, such as dispersed ER elements, free ribosomes, and vesicles of various sizes. It has recently been hypothesized that in the rodent epididymis [38], vesicles derived from the trans-Golgi network and containing glycosidases could be delivered into the lumen after fragmentation of the apical blebs in the lumen. The mechanism of the secretion of Train A is intriguing, and further ultrastructural investigations are nec-

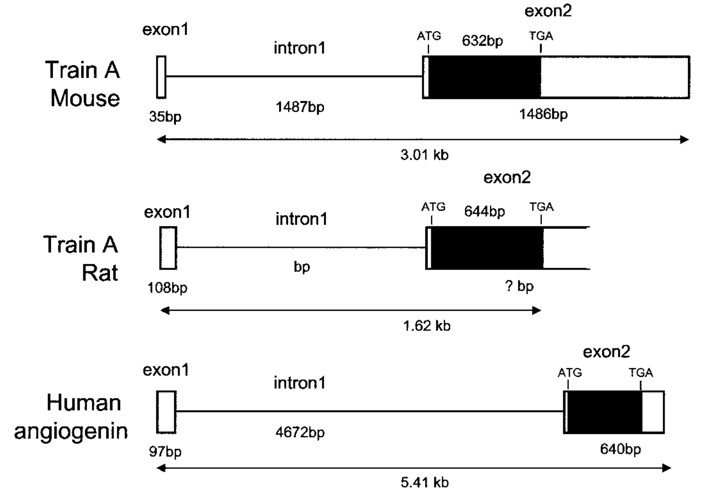

FIG. 12. Schematic of the genomic structure of the murine and rat Train A and human angiogenin genes. These representations were obtained for murine transcript AK015573, rat mRNA XM_223962, and human angiogenin P03950. The gene information was given by Ensembl Genome Browser (available at http://www.ensembl.org) with mouse product of Ensembl gene ENSMUSG00000021872, rat product of EST gene ENSRNOESTG00000016310, and human angiogenin ENS00000169422. Translated sequence regions are shown in black. In rat, the sequence of mRNA XM_223962 was published only for the translated region, which started at a wrong methionine. According to the homology sequences obtained in our results among species (Fig. 11), the 5′ region of the mRNA was completed according to mouse sequence, and rat exon 1 was considered as a 5′ untranslated region of the mRNA.

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022

essary to define the nature of the apical immunoreactive intracellular organelles, the structure of the luminal vesicles, and the role of the RNase-containing globules in the lumen of the duct.

In conclusion, we have identified a new protein that is preferentially expressed by the epididymis and secreted specifically by the epithelial cells from a restrictive anterior region of this organ. This protein is the first member of a new RNase family. The enzymatic function of this compound must be investigated, and the role of this new compound must be established in the processes of posttesticular maturation of sperm.

ACKNOWLEDGMENTS

We thank Prof. R. de Llorens for helpful discussions, Mrs. A. Collet and G. Tsikis for technical assistance, Michèle Peloille for DNA sequencing, E. Venturi for providing the animals, and A. Beguey for photographic work.

REFERENCES

1. Dacheux JL, Gatti JL, Dacheux F. Contribution of epididymal secretory proteins for spermatozoa maturation. Microsc Res Tech 2003; 61: 7–17.
2. Syntin P, Dacheux F, Druart X, Gatti JL, Okamura N, Dacheux JL. Characterization and identification of proteins secreted in the various regions of the adult boar epididymis. Biol Reprod 1996; 55:956–974.
3. Fouchécourt S, Metayer S, Locatelli A, Dacheux F, Dacheux JL. Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. Biol Reprod 2000; 62:1790–1803.
4. Turner TT, Riley TA, Vagnetti M, Flickinger CJ, Caldwell JA, Hunt DF. Postvasectomy alterations in protein synthesis and secretion in the rat caput epididymidis are not repaired after vasovasostomy. J Androl 2000; 21:276–290.
5. Castella S, Dacheux JL. Identification d'une protéine majoritairement sécrétée dans l'épididyme de verrat. Biol Cell 2002; 94:10.
6. Metayer S, Dacheux F, Dacheux JL, Gatti JL. Comparison, characterization, and identification of proteases and protease inhibitors in epididymal fluids of domestic mammals. Matrix metalloproteinases are major fluid gelatinases. Biol Reprod 2002; 66:1219–1229.
7. Fouchécourt S, Dacheux F, Dacheux JL. Glutathione-independent prostaglandin D2 synthase in ram and stallion epididymal fluids: origin and regulation. Biol Reprod 1999; 60:558–566.
8. Rabilloud T, Charmont S. Detection of proteins on two-dimensional electrophoresis gels. In: Rabilloud T (ed.), Proteome research: Two-dimensional gel electrophoresis and identification methods. Paris: Springer; 2000:107–126.
9. Green N, Alexander H, Olson A, Alexander S, Shinnick TM, Sutcliffe JG, Lerner RA. Immunogenic structure of the influenza virus hemagglutinin. Cell 1982; 28:477–487.
10. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–159.
11. Thomas PS. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci U S A 1980; 77:5201–5205.
12. Dacheux F, Dacheux JL. The intracellular pathway of antagglutinin secretion in the boar caput epididymidis as revealed by immunogold labelling. Cell Tissue Res 1987; 249:89–99.
13. Dacheux F, Dacheux JL. Androgenic control of antagglutinin secretion in the boar epididymal epithelium. An immunocytochemical study. Cell Tissue Res 1989; 255:371–378.
14. Bonnet A, Tosser-Klopp G, Benne F, Cabau C, Villeger S, Soares M, Bonaldo F, Hatey F. A pig normalised multi-tissue cDNA library. 7th World Congress on Genetics Applied to Livestock Production; 2002; Montpellier, France.
15. Beintema JJ, Kleineidam RG. The ribonuclease A superfamily: general discussion. Cell Mol Life Sci 1998; 54:825–832.
16. Weickmann JL, Elson M, Glitz DG. Purification and characterization of human pancreatic ribonuclease. Biochemistry 1981; 20:1272–1278.
17. Hamann KJ, Barker RL, Loegering DA, Pease LR, Gleich GJ. Sequence of human eosinophil-derived neurotoxin cDNA: identity of deduced amino acid sequence with human nonsecretory ribonucleases. Gene 1989; 83:161–167.
18. Barker RL, Loegering DA, Ten RM, Hamann KJ, Pease LR, Gleich GJ. Eosinophil cationic protein cDNA. Comparison with other toxic cationic proteins and ribonucleases. J Immunol 1989; 143:952–955.
19. Zhou JH, Ohtaki M, Sakurai M. Sequence of a cDNA encoding chicken stem cell factor. Gene 1993; 127:269–270.
20. Strydom DJ, Fett JW, Lobb RR, Alderman EM, Bethune JL, Riordan JF, Vallee BL. Amino acid sequence of human tumor derived angiogenin. Biochemistry 1985; 24:486–494.
21. Rosenberg HF, Dyer KD. Molecular cloning and characterization of a novel human ribonuclease (RNase k6): increasing diversity in the enlarging ribonuclease gene family. Nucleic Acids Res 1996; 24:3507–3513.
22. Harder J, Schroder JM. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. J Biol Chem 2002; 277: 46779–46784.
23. Zhang J, Dyer KD, Rosenberg HF. RNase 8, a novel RNase A superfamily ribonuclease expressed uniquely in placenta. Nucleic Acids Res 2002; 30:1169–1175.
24. The RIKEN Genome Exploration Research Group Phase II Team and the FANTOM Consortium. Functional annotation of a full-length mouse cDNA collection. Nature 2001; 409:685–690.
25. Kurachi K, Davie EW, Strydom DJ, Riordan JF, Vallee BL. Sequence of the cDNA and gene for angiogenin, a human angiogenesis factor. Biochemistry 1985; 24:5494–5499.
26. Hamann KJ, Ten RM, Loegering DA, Jenkins RB, Heise MT, Schad CR, Pease LR, Gleich GJ, Barker RL. Structure and chromosome localization of the human eosinophil-derived neurotoxin and eosinophil cationic protein genes: evidence for intronless coding sequences in the ribonuclease gene superfamily. Genomics 1990; 7:535–546.
27. Okamura N, Dacheux F, Venien A, Onoe S, Huet JC, Dacheux JL. Localization of a maturation-dependent epididymal sperm surface antigen recognized by a monoclonal antibody raised against a 135-kilodalton protein in porcine epididymal fluid. Biol Reprod 1992; 47: 1040–1052.
28. Dacheux F, Oble S, Venien A, Dacheux JL. Purification and localization of a 27 kDa epididymal glycoprotein of the boar sperm surface. In: Baccetti B (ed.), Comparative spermatology 20 years after. New York: Raven Press; 1992:465–470.
29. Ramos AS Jr, Dym M. Fine structure of the monkey epididymis. Am J Anat 1977; 149:501–531.
30. Cavicchia JC. Fine structure of the monkey epididymis: a correlated thin-section and freeze-cleave study. Cell Tissue Res. 1979; 201:451–458.
31. Arrighi S, Romanello M, Domeneghini C. Ultrastructural study on the epithelium lining ductus epididymis in adult cats (Felis catus). Arch Biol 1986; 97:7–24.
32. Morales A, Cavicchia JC. Release of cytoplasmic apical protrusions from principal cells of the cat epididymis, an electron microscopic study. Tissue Cell 1991; 23:505–513.
33. Agrawal Y, Vanha-Perttula T. Electron microscopic study of the secretion process in bovine reproductive organs. J Androl 1988; 9:307–316.
34. Fornes MW, Barbieri A, Cavicchia JC. Morphological and enzymatic study of membrane-bound vesicles from the lumen of the rat epididymis. Andrologia 1995; 27:1–5.
35. Hermo L, Jacks D. Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Mol Reprod Dev 2002; 63:394–410.
36. Aumuller G, Renneberg H, Schiemann PJ, Wilhelm B, Seitz J, Konrad L, Wennemuth G. The role of apocrine released proteins in the posttesticular regulation of human sperm function. Adv Exp Med Biol 1997; 424:193–219.
37. Aumuller G, Wilhelm B, Seitz J. Apocrine secretion—fact or artifact? Anat Anz 1999; 181:437–446.
38. Hermo L, Robaire B. Epididymal cell types and their functions. In: Robert B, Hinton BT (eds.), The epididymis from molecular to clinical practice. A comprehensive survey of the efferent ducts, the epididymis and the vas deferens. New York: Kluwer Academic/Plenum; 2002:81–102.
39. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 1988; 16:10881–10890.

Downloaded from https://academic.oup.com/biolreprod/article/70/2/319/2712610 by guest on 20 August 2022