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Buck (*Capra hircus*) genes encode new members of the spermadhesin family

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ABSTRACT

Spermadhesins are the major proteins of the boar seminal plasma and form a group of polypeptides probably involved in different steps of reproduction. In previous work, a member of the spermadhesin family from buck (*Capra hircus*) seminal plasma, named BSFP, was isolated and characterized by mass spectrometry and the N-terminal was sequenced. This work aimed to found and characterize the BSFP gene and detect its expression along the male genital tract using real-time PCR and electrophoresis. The cDNAs of seminal vesicle, testis, epididymis, bulbourethral gland and ductus deferens were prepared from a mature buck. Following 3′ and 5′-end amplifications using seminal vesicle cDNA, we cloned and sequenced 4 highly similar (97 to 98%) nucleotide sequences encoding spermadhesins, named Bodhesin-1, -2, -3 and -4. All deduced amino acid sequences presented the CUB domain signature and were 49 to 52% similar to boar AWN. The Bodhesin-2 is identical to the BSFP N-terminal. After real-time PCR we noticed specific amplifications for all Bodhesins in seminal vesicle, testis, epididymis and bulbourethral gland, with the exception of Bodhesin-2 in epididymis. The amplicons presented melting temperature and size of approximately 78°C and 130 bp, respectively. Bodhesin expression is significantly higher in seminal vesicle when compared to the other tissues. The present work confirm that goat is the fourth mammalian species, together with pig, cattle and horse, in which spermadhesin molecules have been found. To our knowledge, this is the first report of buck spermadhesins genes using molecular cloning and expression profile.

Running head: Buck spermadhesin genes

Key Words: spermadhesin; gene; seminal vesicle; buck; Capra hircus; bodhesin

INTRODUCTION

The seminal plasma, the fluid in which mammalian spermatozoa are suspended in semen, is a complex mixture of secretions originated from epididymis and accessory glands (Solís et al., 1998). The seminal plasma protein composition varies from species to species. These components have important effects on sperm function influencing fertilizing ability of spermatozoa and exert effects on the female reproductive physiology (Tedeschi et al., 2000). Among these proteins, the bulk of seminal plasma proteins belongs to a group of lectin-like proteins, named spermadhesins.

Spermadhesins are a group of polypeptides of 12-16 kDa found in seminal plasma and peripherally associated with the sperm surface (Töpfer-Petersen et al., 1998) able to interact to some sugar-containing receptors on cell surfaces. The capacity of binding to sugar moiety is a characteristic biological activity of lectins and lectin-like proteins. All animal lectins present in their amino acid sequence a carbohydrate-recognition domain (CRD). However, spermadhesins differ structurally from the majority of lectins. They show a distinct domain named CUB (Bork and Berckmann, 1993), a widespread 110-amino acid module, which was called after three proteins where it was first identified (complement subcomponents - C1r/C1s, embryonic sea urchin protein - Uegf and bone morphogenetic protein 1 - Bmp1). This domain consists of a sandwich made up of two sheets, each containing four anti-parallel strands and one parallel strand (Romão et al., 1997; Romero et al., 1997; Varela et al., 1997).

Spermadhesins were already described in boar, stallion, bull and buck. Boar presents five spermadhesins termed AQN-1, AQN-3, AWN, PSP-I and PSP-II (Calvete et al., 1995; Varela et al., 1997). Homologous to AWN, another spermadhesin was described in horse, called HSP-7, which becomes attached to the sperm surface at the time of ejaculation

(Reinert et al., 1997). In addition, the bovine spermadhesins, aSFP (Wempe et al., 1992) and Z13 (Tedeschi et al., 2000), seems to be growth factors with effects on ovarian cells. Recently, we reported the N-terminal of a member of the spermadhesin family from buck seminal plasma, named BSFP (buck seminal fluid protein). This protein was homologous to AWN, AQN and HSP-7 (Teixeira et al., 2002, 2006).

Only after the above proteins had been described, some spermadhesin cDNAs were cloned (Wempe et al., 1992; Kwok et al., 1993; Ekhlasi-Hundrieser et al., 2002). However, buck spermadhesin gene is still unknown. The objective of this work was to clone and characterize this gene and detect its expression along the male genital tract.

MATERIAL AND METHODS

Animal tissues and total RNA isolation

Distinct male reproductive tissues such as seminal vesicle, testis, epididymis, bulbourethral gland and ductus deferens were excised from a single sexual mature buck (*Capra hircus*) of an undefined breed. The animal was anesthetized and sacrificed according to the guidelines of animal care (Van Zutphen and Balls, 1997). The tissues were accessed by surgical procedure following their anatomy location (Ashdown and Done, 2003). After collection, the tissues were kept under liquid nitrogen until total RNA isolation. In order to identify and characterize the cDNA encoding buck spermadhesin we performed total and mRNA isolation of seminal vesicle. In addition, for gene expression analysis in real-time PCR we used only total RNA of all tissues described above.

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) following company descriptions. Briefly, the frozen tissues were ground into powder and added to the Trizol reagent. After samples homogenization, we proceeded performing phase

separation and RNA precipitation using chloroform and isopropyl alcohol, respectively. Total RNA pellets were washed with 70% ethanol and dissolved in RNase-free water.

Seminal vesicle mRNA isolation

Poly(A)-rich RNA was obtained from total RNA using mRNA purification kit (Invitrogen, Carlsbad, USA) containing affinity chromatograph columns of oligo(dT) cellulose. The yield and quality of total and mRNA were determined spectrophotometrically using 260 nm and 260/280 nm ratio, respectively. Following seminal vesicle mRNA isolation we proceeded with 3´and 5´-end amplification.

3´-end amplification of spermadhesin cDNAs

The first-stand cDNA was synthesized through reverse transcription polymerase chain reaction (RT-PCR) using a 3´-adaptor-Oligo(dT)18 (Clontech, Mountain View, USA), 0.6 µg of mRNA and Moloney Murine Leukemia Virus Reverse Transcriptase – (M-MLV RT) purchased from Promega (Madison, USA). The 3´-rapid amplification of cDNA end (3´-RACE) was performed through a 30 cycles polymerase chain reaction (PCR). We used a specific sense primer, termed SMD-SE (Table 1), designed based on a homologous region of boar and stallion spermadhesin nucleotide and amino acid sequences, as well as buck spermadhesin N-terminal sequence (Teixeira et al., 2002). The antisense primer was complementary to QT named Q0 purchased from Clontech (Mountain View, USA). The products were analyzed on a 1% agarose gel, once stained with ethidium bromide.

cDNA cloning

Subcloning was made with the pGEM-T Easy Vector System (Promega, Madison, USA). *E. coli* (JM109, Promega, Madison, USA) transformation with subcloning products

was performed by calcium chloride method. Briefly, cells were concentrated by centrifugation and resuspended in a solution containing calcium chloride. Competent cells were mixed with the plasmid DNA and then heat shocked. The cells were grown in a nonselective medium and plated on ampycilin-containing medium. Plasmid extraction and purification was done following manufacture instructions of the GFX Micro Plasmid Prep Kit (GE Healthcare, Piscataway, USA). Purified plasmids were quantified spectrophotometrically.

cDNA sequencing and sequence analysis

The candidate clones were sequenced using the insert-flanking sense primers M13F or T7 and antisense primers M13R or SP6 purchased at Clontech (Mountain View, USA). Nucleotide sequence was performed using Dye terminator chemistry (DYEnamic ET Dye Terminator kit) on MegaBACE 750 DNA Analysis System (GE Healthcare, Piscataway, USA).

Each clone was sequenced twice for both sense and antisense insert-flanking primers and a consensus sequence was obtained after FredFrap analysis. Database searches for related genes in local versions of nucleotide sequence were conducted using BLAST (Altschul et al., 1990) at NCBI (http://www.ncbi.nlm.nih.gov).

5´-end amplification of spermadhesin cDNAs

One specific antisence primer, named SMD-AS, for the 3´-end coding region of buck spermadhesin cDNA where synthetized (Table 1) for 5´-RACE. Synthesis and amplification of cDNA end was performed by 5´-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 according to manufactur´s protocols (Invitrogen, Carlsbad, EUA) using 5μg of seminal vesicle RNAm. The PCR reaction was conducted using the same

specific antisence primer described above (SMD-AS) and an anchor primer supplied by the kit. The products were analyzed on a 1% agarose gel, once stained with ethidium bromide. Cloning and sequence analysis of spermadhesin 5′-end cDNA followed the same protocols described above.

Similarity search and sequence comparison

The 3′ and 5′-end sequences were alligned and their homology relationship was performed using ClustalW (Higgins et al., 1994) available at EBI (http://www.ebi.ac.uk), setted with default parameters.

The predicted amino acid sequence of buck spermadhesin cDNAs were compared against protein sequence data bank at NCBI using BLASTP (Altschul et al., 1997). Some sequences were chosen as the best score after alignment and they were used to identify conserved residues in homologous sequences. Mammalian spermadhesins sequences from *Sus Scrofa* (AAB21990, P26322, S39434), *Equus caballus* (P80720) and *Bos taurus* (AAA30745) were aligned using ClustalW (Higgins et al., 1994).

Gene expression analysis using real-time PCR

First strand cDNA synthesis was performed for all collected tissues using 1.2 µg of total RNA, 500ng/µl of 3'-adaptor-Oligo(dT)$_{18}$ (Promega, Madison-WI, USA) and 200 units of Superscript II (Invitrogen Lifetechnology, Carlsbad-CA, USA) in a total volume of 25 µl. The distincts tissues cDNA samples were diluted yielding cDNA synthesized from 48 to 0.48 ng of total RNA per µl. Specific primers (Table 1) were disigned based on buck spermadhesin cDNAs sequences: one sense (BDH-SE) and three antisense primers (BDH-AS1, BDH-AS2 and BDH-AS3). In order to verify the possible amplifications of buck

spermadhesins genes, we tested each antisense BDH primer with the common sense primer BDH-SE.

Amplification of buck spermadhesin cDNA was carried out in a Rotor-Gene 3000 operated with Rotor Gene software version 6.0.19 (Corbett Research, Mortlake, Australia). Each reation consisted of: 7.2 ng cDNA, 500 nM of primers, 10 µl of SYBR Green PCR Core Reagent (Applied Biosystems, Foster City, USA), in a reaction volume of 20 µl. Amplification conditions were as follows: 95°C (30 sec), 55°C (30 sec), 72°C (20 sec) in 45 repetitions. Specificity of each reaction was ascertained after completion of the amplification protocol. This was achieved by performing the melt procedure (55-90°C; 1°C/5 sec). Comparisons of messenger RNA levels among Bodhesins-1, -2 and -3 were done using formula described by Dussault and Pouliot (2006), where Bodhesin-1 was set as the reference gene.

RESULTS

Synthesis, amplification and sequencing of cDNAs

Reverse-transcription coupled to polymerase chain reaction (RT-PCR), using a Qt-adaptor protocol, allowed the isolation of full length cDNA molecules (Fig. 1A). Using primers Q0 and SMD-SE, we could detect an apparent single band of approximately 700 base pairs (bp) in seminal vesicle (Fig. 1B).

After 3 and 5´-RACE amplifications, buck spermadhesin cDNAs were isolated by screening 73 recombinant bacterial clones by PCR using insert-flanking primers M13R and M13F. Analysis of nucleotide sequences from 39 recombinant clones revealed four different cDNAs (Fig. 2) that could encode four spermadhesins named Bodhesin-1 (Bdh-1), Bodhesin-2 (Bdh-2), Bodhesin-3 (Bdh-3), and Bodhesin-4 (Bdh-4). Noteworthy, Bdh-4 cDNA was

sequenced after 5´-RACE proceddings. Bdh-1, -2 and -3 cloned contained inserted cDNAs ranging from 606 to 608 bp. In addition, Bhd-4 clone presented a fragment of 477 bp.

Based on all four cDNAs, Bodhesin mRNAs comprise a 405 nucleotide open reading frame (bases 70 to 474) including the stop codon (Fig. 2). Particularly, all four cDNAs were terminated by two consecutive stop-codons (TAGTGA). The 5´-untranslated region (5´-UTR) is approximately 69 bp long (Table 2) followed by a signal peptide from bases 70 to 132. The 3´-untranslated region (3´-UTR) is approximately 278 bp long and contains one polyadenylation signal (AATAAA) that is only 13 bp upstream of the poly(A) tail.

Sequence comparison

The encoded proteins deduced from Bdh-4 cDNA presented 134 amino acid residues including a 21 aminoacid long sinal peptide. This signal peptide comprises a sequence identity ranging from 80 to 90% at amino acid level compared to other spermadhesins (Fig. 3A).

The analysis of the primary structure of all four deduced mature Bodhesins showed a conserved region that predicts a single CUB domain (Fig. 3B), a typical feature of the spermadhesin family members (Varela et al., 1997). The similarities among Bodhesin isoforms 1, 2, 3 and 4 ranged from 97 to 98%, calculated by ClustalW with default parameters. Bdh-2 and Bdh-4 presented a His13 instead of Leu13 when compared among the other Bodhesins. Additionally, Bdh-2 and Bdh-4 showed a Ser112 or Thr112, respectively. However, Bdh-1 and -3 presented a glutamine at this position. Furthermore, Bdh-3 showed a lysine residue in substitution of an arginine in Bdh-1, Bdh-2 and Bdh-4 at position 72. Finally, Bdh-1 presented a leucine at position 73 while the other Bodhesins had a

phenylalanine. Other nucleotide differences were seen among the Bodhesin cDNAs, but they do not encode distinct amino acid residues or they are positioned in the 3´-UTR.

Comparison of the predicted amino acid sequence of the mature Bdh-4 against the protein sequence data bank at NCBI revealed similarities with other spermadhesins. The sequences with the highest similarities (36 to 46%) were aligned and used to identify conserved residues in homologous sequences (Fig. 3B). The highest identity was seen with boar AWN (46%). However, when we compared the whole amino acid sequence, the similarity increased to 52% with AWN and AQN-1. The predicted amino acid sequences of Bdh-1, -2 and -3 shared 50%, 49% and 51% similarity with AWN, respectively.

Expression of Bodhesin genes in the buck genital tract

After PCR amplifications of seminal vesicle cDNA (4.8 ng/µl of total RNA), using specific primers for Bdh-1, -2 and -3, we could observe products with the melting temperature (Tm) ranging from 77.96 to 78.14°C (Table 3) and the expected size of approximately 130 bp (Fig. 4). To obtain these products we performed approximately 25 cycles (Table 3). Adapting the formula described by Dussault and Pouliot (2006), the seminal vesicle expression levels of Bdh-2 and -3 mRNAs were 6.71 and 3.76 higher than Bdh-1, respectively.

Using the same set of primers, but different tissues (testis, epididymis, bulbourethral gland), we noticed amplifications for all different Bodhesins, with the exception of Bdh-2 that could not be seen in epididymis. In addition, any amplicons were obtained from ductus deferens. The observed products were approximately 130 bp long (Fig. 4) with an average Tm ranging from 78.31 to 78.53°C (Table 4). However, different from seminal vesicle, we needed a 10 fold increase in the amount total RNA and used over 37 PCR cycles to obtain these products.

DISCUSSION

Previously, our group isolated, purified and described the N-terminal and molecular mass of a protein structurally characterized as the first buck spermadhesin named BSFP (Teixeira et al., 2002). However, we had not yet described the gene that encodes that protein. After 3´-RACE procedure with the specific primer SMD-SE, a product of approximately 700 bp was amplified from the 1st strand cDNA poll of seminal vesicle.

Following 3´ and 5´-end amplifications, we cloned and sequenced four highly similar cDNAs. We were able to verify that the deduced polypetides (Bdh-1, -2, -3 and -4) presented the canonical CUB domain signature (Bork and Berckmann, 1993). In addition, the Bodhesin primary structures were similar to other spermadhesins, especially to boar AWN. Interstingly, the signal peptide of Bdh-4 comprises a highly sequence identity to other spermadhesins. Similar results were described in boar by Ekhlasi-Hundrieser and coworkers (2002) reporting a consensus sequence restricted to spermadhesins. Taking together these infomations, we assume that all Bodhesins are new members of the spermadhesin family. However, it is not know, at this time, if there are any others in the buck reproductive tract.

Among the proteins deduced in this work, Bdh-2 was the most identical to the BSFP N-terminal (Teixeira et al., 2002), indicating that they are probability the same protein. Few differences were observed among the Bodhesin cDNAs and they might be implicated in some divergences in amino acid sequences of all four deduced proteins. Bdh-2 and Bdh-4 showed a thymine at the nucleotide 170 instead of an adenine in Bdh-1 and Bdh-3. This difference encodes the polar amino acid histidine in substitution of the hydrophobic amino acid leucine in the other Bodhesins. The same polar residue was also seen in the N-terminal sequence of BSFP (Teixeira et al., 2002). The sequence consensus of the CUB domain presents a hydrophobic amino acid residue at that same site (Varela et al., 1997). According to Romão

and coworkers (1997), there are hydrophobic core residues that stabilize the β-barrel organization and define the CUB domain signature. We can not assure if these findings would determine a significant difference in the structure of Bdh-2 and -4 when compared to the other spermadhesins. However, apart of the conserved CUB domain, spermadhesin can show structural features that are unique to each protein as observed to aSFP in relation to PSP-I and PSP-II (Romão et al., 1997). These particular differences might have implications in the structure-activity relationship and in gamete recognition during reproductive functions (Calvete et al., 1995; Reinert et al., 1996). Additionally, Bdh-2 and -4 cDNAs displayed a different codon at position 466 determining a semi-conservative substitution of Gln133, found in Bdh-1 and -3, to Ser133 or Thr133, respectevely. This replacement would not affect the CUB domain architecture probably because these amino acid residues are located outside the last β-sheet in the C-terminal. We noticed conserved mutations at nucleotides 347 (Bdh-3) and 349 (Bdh-1) that would exert synonymous substitutions at the amino acid level (93, Arg → Lys and 94, Phe → Leu). Based on proteins with the CUB domain consensus, these positions contain positively charged and hydrophobic residues, respectively (Bork, 1991). Consequently, we presume that these variations would not interfere in the protein folding. Taking all these results together, Bodhesins seem to belong to a multigene family encoding proteins with preserved signature of the CUB domain. Members of the spermadhesin family share similar structure scaffold (Romero et al., 1996; Romero et al., 1997; Romão et al., 1997) which have been attributed to different functions (Töpfer-Petersen et al., 1998). The deduced Bodhesins showed a higher identity to boar AWN and horse HSP-7. In both species, these spermadhesins exhibited ligand-binding abilities to heparin, zona pellucida and spermatozoa (Sanz et al., 1992; Calvete et al., 1993; Dostàlovà et al., 1995; Reinert et al., 1997). Hence, they seem to play a role in sperm-egg interaction at fertilization (Töpfer-Petersen et al., 1998). On the other hand, our group demonstrated that buck spermadhesin did

not bind to heparin (Teixeira et al., 2006) and probably do not participate in gamete recognition. However, it remains to be solved if the Bodhesin cDNAs really encode proteins with distinct or reduntant functions.

The expression of the spermadhesins along the male genital tract has not yet been systematically studied. Here the expression patterns of Bodhesins are described by real-time PCR and agarose gel electrophoresis analysis. The small differences among the melting temperature values and the single electrophoretic bands indicated specific amplifications of Bodhesins in seminal vesicle. According to the amount of template used in PCR reaction and the Ct values, Bodhesin expression is significantly and mensurable higher in seminal vesicle when compared to the other tissues. Several works described that seminal vesicle is the main source of spermadhesins, although other reproductive tissues also expresse them (Kwok et al., 1993; Dostàlovà et al, 1994; Sinowatz et al., 1995).

Inspite of the low expression levels in reproductive tissues, other than seminal vesicle, the use of a higher sensible method, such as real-time PCR, permited the detection of Bodhesins in testis, epididymis and bulbouretral gland. This found was supported by Tm values and electrophoretic patterns obtained in these tissues. Supporting our findings, using RT-PCR and immunological approaches, several boar spermahesins were identified in prostate, seminal vesicle, epididymis and testis (Ekhlasi-Hundrieser et al., 2002). Similar to the present work, boar AWN and stallion HSP-7, was also identified in seminal vesicle and epididymis (Reinert et al., 1997; Hoshiba and Sinowatz, 1998; Ekhlasi-Hundrieser et al., 2002). In contrast to AWN, Bodhesin and HSP-7 could be found in testis. Interestingly, our data clearly show the expression of spermadhesins in bulbourethral gland, which was not yet demonstrated to other animals. This indicates that homologous proteins may have variations of the expression pattern in different mammalian species.

The present work confirm that goat is the fourth mammalian species, together with boar, cattle and horse, in which spermadhesin molecules have been found. To our knowledge, this is the first report of buck spermadhesins genes using molecular cloning and expression profile. Among the male sexual glands, seminal vesicle was the major site of Bodhesins procuction. Further experiments will be necessary to express and characterize these proteins in order to improve the knowledge involving goat reproductive mechanisms.

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Fig. 1. (A) Eletrophoretic analysis of RT-PCR synthesized cDNA of seminal vesicle (SV). **(B)** Amplification of Bodhesin cDNA 3´-end using primers Q0 and SMD-SE.

Fig. 2. Nucleotide sequences of cDNAs encoding four spermadhesin isoforms named Bdh-1, Bdh-2, Bdh-3 and Bdh-4. Nucleotides that could translate into variable amino acid residues are enclosed in grey boxes. Underlined letters: regions to specific primers, named SMD-SE and SMD-AS, used for 3´ and 5´-amplifications, respectively. Letters with double underline: sequence encoding signal peptide. Bold letters: sequence encoding Bdh-4 N-terminus. Italic-bold letters: polyadenylation signal.

Fig. 3. Multiple alignment of the amino acid sequences of spermadhesins. **(A)** Alignment of signal peptide of buck, boar and bull spermadhesins. **(B)** Alignment of buck, boar, stallion and bull mature spermadhesins. Those characteristics of amino acid residue that define both the signature (CUB sign - Varela et al., 1997) and the position of the secondary structure elements (CUB pred - Bork and Beckmann, 1993) of the CUB domain are shown using the following codes: a, aromatic (Phe, Tyr, Trp); h, hydrophobic (Leu, Ile, Val, Met, Ala); t, turn-like or polar (Gly, Pro, Asn, Gln, His, Ser, Thr); n, negatively charged (Glu, Asp); p, positively charged (Arg, Lys); β, β-strands (labeled βa-βi). The two disulphide bonds (S – S) between the nearest-neighbor cysteine residues (C) which are conserved in all known spermadhesin molecules and in some CUB domains are shown in grey. Underlined letters: Buck Seminal Fluid Protein (BSFP) N-terminus. The different amino acid residues among bodhesins are shown in boxes.

Fig. 4. Tissue-specific expression of Bodhesins in the buck reproductive tract. Total RNA was extracted from seminal vesicle (SV), testis (TS), epididymis (EP), bulbourethral gland (BU) and ductus deferens (DD). Following RT-PCR and real-time amplifications with specific primers for Bdh-1, Bdh-2 and Bdh-3 amplification products were separated according to size in 1.8% agarose. In control assays cDNA was omitted (W) or a Bdh cDNA inserted into a plasmid was used as template (C+). A 50 bp ladder (M) was used as lengh standard.

TABLE 1. Specific Primers Used for 3′-RACE, 5′-RACE and Real-time PCR of Bodhesins.

Name	Nucleotide sequence
SMD-SE	5′-TGTGGGGGSGTCCWCAGA-3′
SMD-AS	5′-TCACTAAGCTKYTGGACGC-3′
BDH-SE	5′-TGGACCATCCTCTTGAAGA-3′
BDH-AS1	5′-CTGAACAGAGCCTCCCAA-3′
BDH-AS2	5′-CTGAACAGAACCTCCCAA-3′
BDH-AS3	5′-CTGAACAGAACTTCCCAA-3′

TABLE 2. Spermadhesin cDNAs.

	5´-UTR	ORF	3´-UTR	Encoded protein (aa)	cDNA accession	Reference
Bdh-1	69[a]	405[a]	279	134[a]	DQ204877	This paper
Bdh-2	69[a]	405[a]	278	134[a]	EF157969	This paper
Bdh-3	69[a]	405[a]	277	134[a]	EF157970	This paper
Bdh-4	69	405	-	134	EF157971	This paper
AWN	29	465	217	154	AJ853850	Haase et al., 2005
AQN-1	29/31	399	250/276[b]	132	AJ853854 AJ853855	Haase et al., 2005
aSFP	29[c]	405	252	134	M84603	Wempe et al., 1992
AQN-3	29	414	266	137	AJ853851	Haase et al., 2005

[a] Inferred by comparison to the Bodhesin-4 gene sequence where experimental evidence was available.

[b] Two alternative splice variants of AQN-1 exist.

[c] The transcription start sites of the bovine genes were inferred by comparison of the bovine genomic sequences to the porcine genomic sequences where experimental evidence was avaible.

TABLE 3. Threshold Cycle (Ct) and Melting Temperature (Tm) Values (± SEM) Calculed from Real-time PCR Amplifications with Seminal Vesicle cDNA (Total RNA = 4.8 ng/μL).

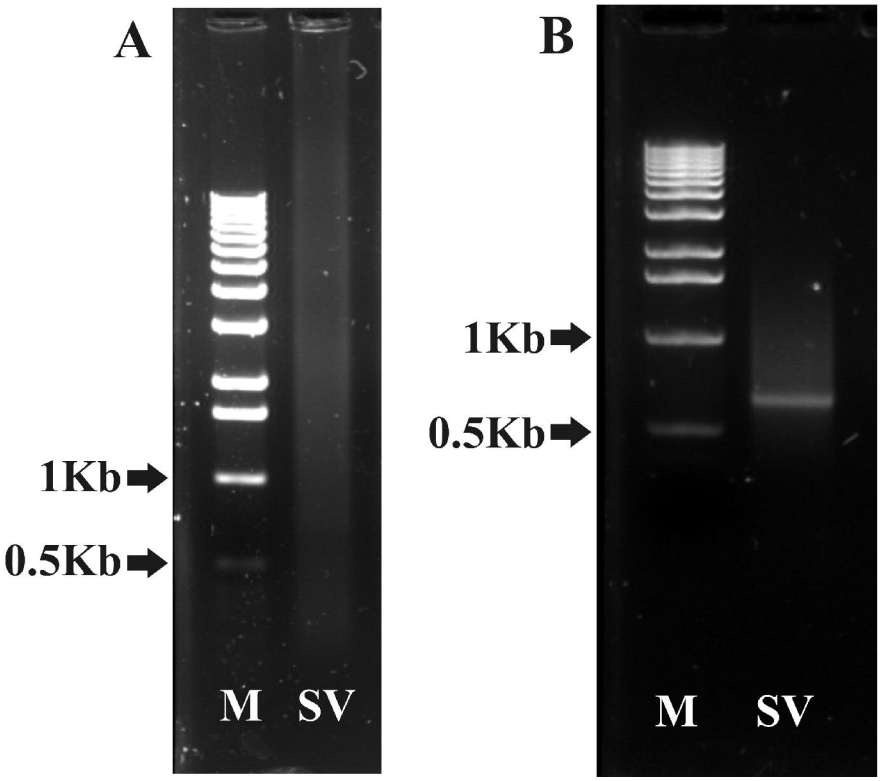
	Bdh-1	Bdh-2	Bdh-3
Ct [a]	27.11 ± 0.07	24.52 ± 0.36	25.17 ± 0.52
Tm (^{o}C) [b]	78.06 ± 0.023	78.14 ± 0.02	77.96 ± 0.05

[a] mean of 2 or 3 experiments. [b] mean of 6 to 9 experiments.

TABLE 4. Threshold Cycle (Ct) and Melting Temperature (Tm) Mean Values (± SEM) Calculed from Real-time PCR Amplifications with Seminal Vesicle (SV), Testis (TS), Epididymis (EP) and Bulbourethral Gland (BU) cDNA.

	SV	TS	EP	BU
Ct	25.73 ± 0.45 [a]	> 37 [b]	> 38 [b]	> 41 [b]
Tm (°C)	78.04 ± 0.03 [c]	78.31 ± 0.07 [a]	78.50 ± 0.10 [b]	78.53 ± 0.10 [b]
Total RNA (ng/µL)	4.8	48	48	48

[a] mean of 8 or 9 experiments. [b] mean of 2 or 3 experiments. [c] mean of 24 experiments.



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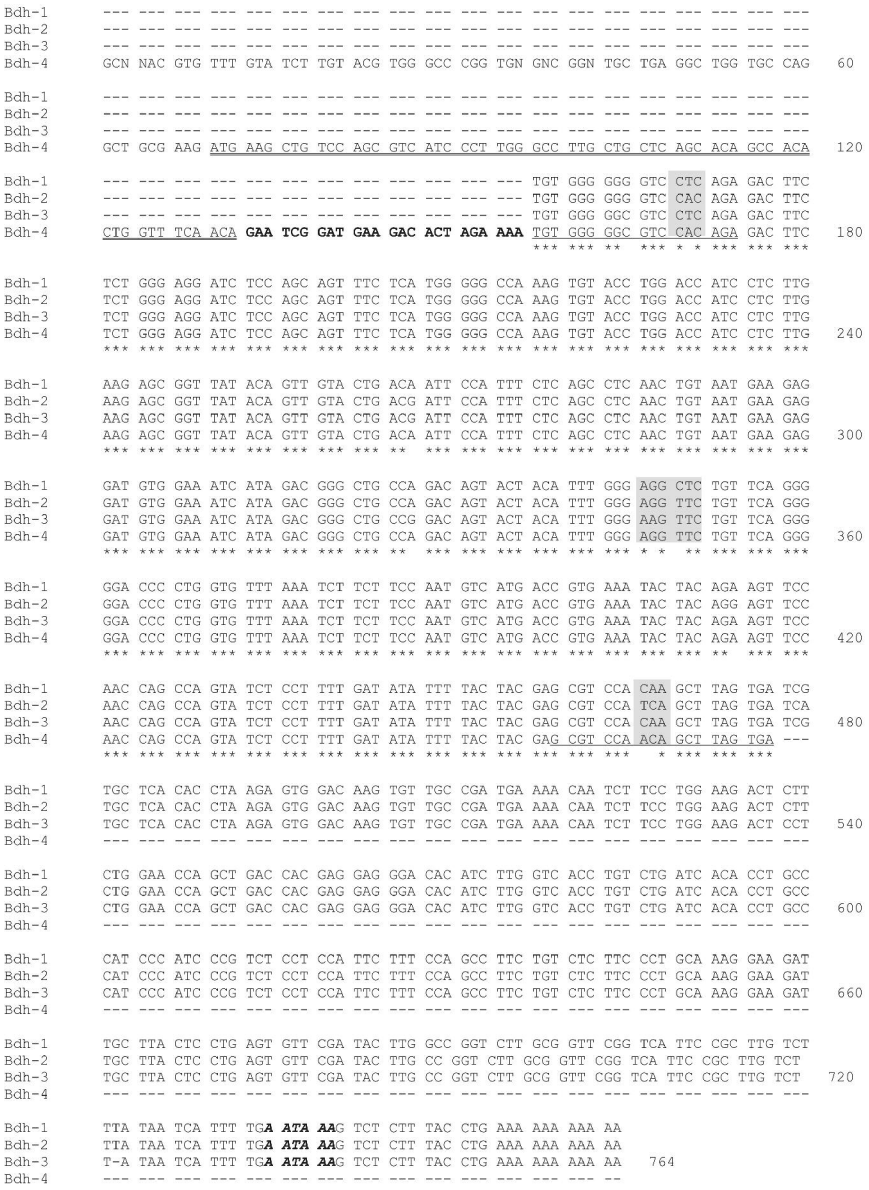

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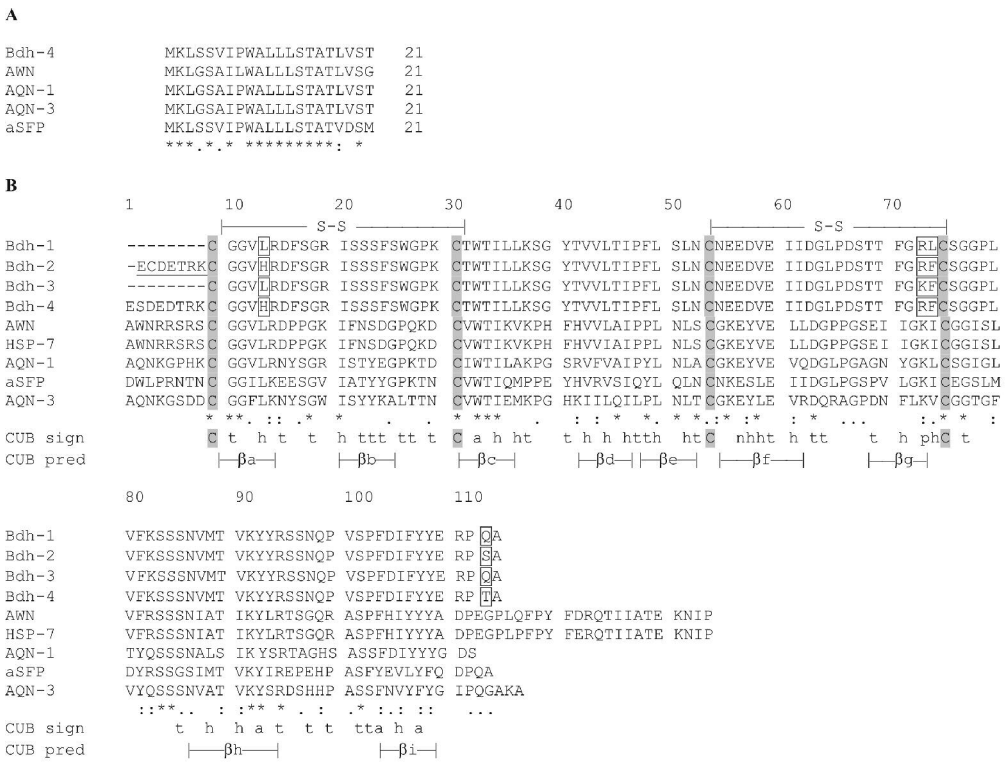

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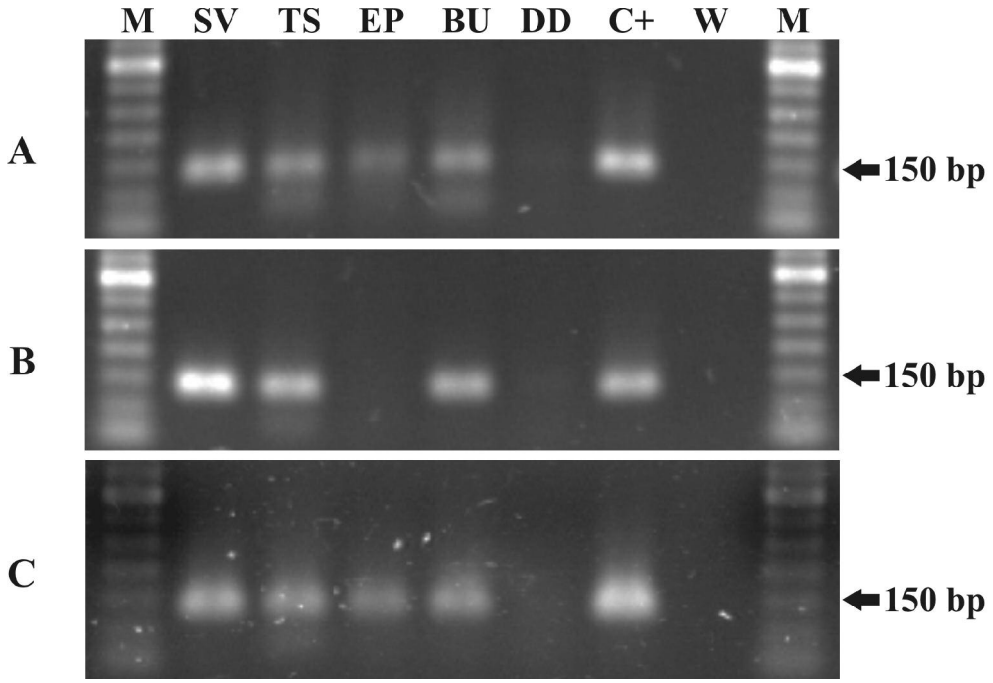

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