The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes

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Received 12 October 1999

Abstract Bv8, a protein from skin secretions of *Bombina variegata*, reacts with receptors present in mammalian brain and intestine (Mollay et al. (1999) Eur. J. Pharmacol. 374, 189–196). As deduced from cloned cDNAs, the murine and human Bv8 homologues have identical amino-terminal sequences and also contain 10 cysteines. From mouse testes, two forms of Bv8 mRNA have been characterized, of which one contains an additional exon which codes for 21 mostly basic amino acids. The mouse Bv8 gene is most active in mid-late pachytene spermatocytes. In mouse testes, Bv8 mRNA can first be detected at the end of the second week post partum.

Key words: Orphan ligand; Spermatogenesis; Hyperalgesia

1. Introduction

Work over the past three decades has demonstrated that amphibian skin produces numerous biologically active peptides [1,2]. Many of these are related to mammalian hormones and/or neurotransmitters. In continuation of our work on the constituents present in skin secretions from two Bombina species [3–5], we have recently isolated a small protein which we termed Bv8 [6]. This name indicates its origin, the skin secretion of *Bombina variegata*, as well as its molecular mass of about 8 kDa. Bv8 is related to protein A, a non-toxic constituent of the venom of the black mamba [7]. Both proteins are distantly related to mammalian co-lipases [8]. Evidence has been presented that the pattern of disulfide bridges is indeed the same in protein A and co-lipase [9].

Pharmacological tests have demonstrated that receptors for Bv8 and protein A are present in mammalian tissues [6]. Both proteins stimulate the contraction of the guinea pig ileum at nanomolar concentrations. In addition, a few minutes after injection into the brain of rats, the animals develop hyperalgesia as assessed by the tail flick and paw pressure test. These observations suggest that homologues of Bv8 also exist in mammals.

In this communication, we present the structure of Bv8 precursors from mouse brain and testes and from human testes as deduced from cloned cDNAs. In addition, we present evidence for the cell and stage-specific expression of the Bv8 gene in meiotic germ cells.

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2. Materials and methods

2.1. mRNA isolation, reverse-transcriptase PCR and isolation of cDNA clones

Isolation of RNA, synthesis of cDNA and RACE were carried out as described earlier [6]. RACE-PCR amplifications were performed using sense (CCG AGA CAG GGG GTG GCA GCT G) and antisense primers (CTT GCG ACA AGG ACT CTC AGT GCG G) as derived from the sequence of a 126 bp exon isolated from mouse embryonic stem cells [10] which is very similar to the region of nucleotides 142 to 267 of the cDNA clone encoding Bv8 from Bombina skin. λgt [11] cDNA libraries from mouse (Stratagene) and human testis (Clontech) were screened using standard procedures. Nucleotide sequences were deposited in the GenBank data bank.

For the analysis of alternatively spliced transcripts, primers were designed which bind sequences on three different exons: exon2-forward: AGC TGC CAC CCC CTG ACT CG, exon3-forward: CAA ATG GAA GGC AGG AAA GAA G and exon4-reverse: TTC CGG GCC AAG CAA ATA AAC C. RT-PCR was performed using 'Ready To Start' reagents from Amersham-Pharmacia Biotech.

2.2. Northern blots and in situ hybridization

Twenty μg of total RNA from different mouse tissues as well as from spermatocytes, spermatids, Sertoli and Leydig cells were used for Northern analysis. As probes, the radiolabeled mouse brain or testis cDNA were hybridized overnight under high stringency conditions as described [6].

Mouse brain Bv8 cDNA subcloned into plasmid Bluescript KS II was linearized and antisense and sense probes labeled with [^{35}S]UTP were synthesized using T3 or T7 RNA polymerase (Promega, Madison, WI, USA). The in situ hybridization protocol has been described previously [12].

2.3. Cell preparation

Germ cells form testis of CD1 mice were separated as described before [12]. The cell fractions composed of 85–90% of mid-late pachytene spermatocytes and round spermatids were collected. Total RNA was prepared and used for Northern blotting. Sertoli cells were isolated as described by Risbridger et al. [13]. In order to remove contaminating germ cells, hypotonic treatment was performed at day 3 of culture [14].

3. Results

3.1. Isolation of cDNAs encoding mammalian Bv8 homologues

From total RNA isolated from mouse brain, a Bv8 cDNA could be amplified using the 3′-and 5′-RACE protocols. Starting from the first ATG codon, this cDNA contains an open reading frame potentially encoding a polypeptide of 86 amino acids. Of these, the first 26 have the typical features of a signal peptide. The following sequence of 47 residues is closely related to the corresponding part of frog Bv8 (62% identity).

To search for tissues where this mRNA might be present in higher abundance than in brain, Northern blots were performed (see Fig. 3). As shown below, a strong signal was obtained with RNA from testis. With the cDNA amplified

PII: S0014-5793(99)01473-8

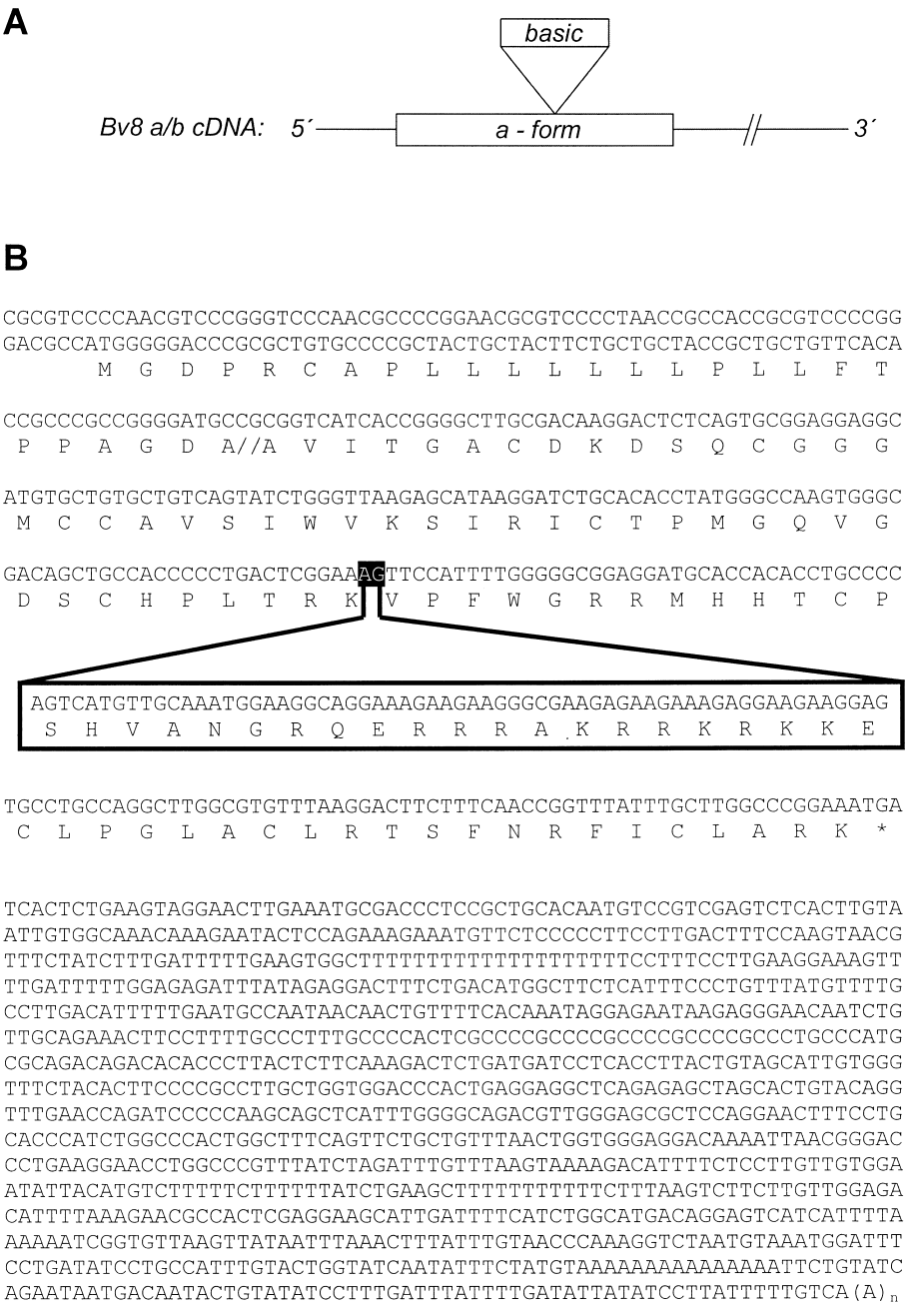

Fig. 1. cDNA and deduced protein sequences of mouse Bv8. A: Schematic drawing of Bv8-a and -b. B: Full length cDNA sequence of Bv8-a and -b (in box) upper lane together with the translation product. The alternatively used exon junctions are highlighted, the signal sequence cleavage site is indicated by a double slash and the stop codon is marked (*).

from mouse brain we then screened cDNA libraries from mouse and from human testis. From the former, a clone was isolated containing an insert of 1429 bp, excluding the poly(A) tail. It contains an open reading frame encoding a polypeptide of 121 amino acids (Fig. 1). In addition, a shorter version was cloned by PCR using primers specific for the sequences covering the start and stop codons of Bv8. The long form termed Bv8-b (basic) contains an insert of 21 amino acids (see Fig. 1B), of which 11 are lysines or arginines. As these occur in two clusters, it seems likely that some proteolytic cleavage takes place as these molecules cross the secretory pathway. The shorter variant was termed Bv8-a as it resembles the amphibian Bv8 more closely. A closely related cDNA was isolated from a human testes cDNA library.

A comparison of the amino acid sequences of the mouse, human, and *Bombina* Bv8 precursors is shown in Fig. 2. The mouse and human precursors are very similar, both with respect to their signal peptide and the mature region. In particular, they all contain 10 cysteines with similar spacing and frequently also identical neighboring residues. The most striking feature is the highly conserved N-terminal sequence AVITGACDKDS(V)QCG. This suggests that this part is crucially important for the biological function of these polypeptides.

3.2. Tissue distribution of Bv8 gene expression

Northern blots with RNA from different mouse tissues yielded a strong signal in testis, while a faint band was present in all the others (see Fig. 3A). Therefore, RNA from various tissues was used as a template for RT-PCR in the presence of exon-specific primers. Bv8-a is expressed by all tissues tested, whereas Bv8-b mRNA only could be detected unambiguously in testis (Fig. 3B). This indicates alternative splicing of the *Bv8* transcript in this organ.

We also tested the expression of the Bv8 gene during mouse testicular development. The Bv8 transcript could barely be detected at the end of the second week, while a strong signal was observed from 3 weeks onwards (Fig. 3C). The Bv8 mRNA could not be detected in testis of mice with the Tfm mutation.

3.3. Characterization of Bv8 expressing cells in testis

We used in situ hybridization to investigate the localization of the Bv8 mRNA in testis. In adult mouse testis, a strong signal was observed in the tubules (Fig. 4A and C) in a region which roughly corresponds to the area occupied by meiotic cells. A closer analysis revealed that Bv8 mRNA was localized in mid-late pachytene spermatocytes at the stages VII, VIII and IX of the seminiferous epithelial cycle (Fig. 4C). No specific signal was present in the interstitium.

The expression of Bv8 mRNA in mouse testis was also investigated using highly purified populations of both germ and somatic cells. The 1.8 kb Bv8 transcript is present in pachytene spermatocytes, whereas expression is low in round spermatids (Fig. 5). Sertoli cells as well as Leydig cells (data not shown) did not yield a signal. This result is in good agreement with the in situ data.

4. Discussion

I.c.v. injection of Bv8 from frog skin or protein A from the venom of the black mamba causes hyperalgesia [6]. We therefore searched for mRNAs encoding mammalian homologues of Bv8 in brain. From mouse brain, a partially spliced cDNA could be isolated. We know from partial sequences of the mouse *Bv8* gene that the last 418 bases of this cDNA are derived from an intron (unpublished experiments). cDNAs containing the complete coding information for Bv8 could be isolated from mouse and human testis. The size of these mRNAs closely corresponds to the 1.8 kb signal seen on Northern blots. From mouse testis, two variants were characterized which code for a longer form termed Bv8-b and a shorter one, Bv8-a. The former contains an insert of 21 amino acids. The two clusters of lysine/arginine residues present in this insert are potential cleavage sites for furin or other prohormone convertases [15], in particular for the testis-specific PC4 enzyme [16]. This would yield a two-chain form of Bv8 with possibly different biological activities. On the Northern blot with testis mRNA, a second weaker signal was present at about 0.8 kb which may code for yet another variant of Bv8.

Small amounts of Bv8 mRNA are present in all other tissues tested. However, with RT-PCR only the mRNA encoding the shorter Bv8-a variant could be detected.

In testis, the expression of the Bv8 gene is regulated in a temporal and spatial manner during spermatogenesis. The in situ hybridization data indicate that Bv8 is expressed predom-

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A) MGDPRCAPLLLLLLLLP:LLFTPPAGDA AVITGACDKDSQCG
B)       CAPLLLLLLLPPLLLTPRAGDA AVITGACDKDSQCG
C)          MKCFAQIVVLLLVIAFSHG AVITGACDKDVQCG
                                 ********** ***

GGMCCAVSIWVKSIRICTPMGQVGDSCHPLTRKSHVANGRQERR
GGMCCAVSIWVKSIRICTPMGKLGDSCHPLTRKNNFGNGRQERR
SGTCCAASAWSRNIRFCIPLGNSGEDCHPASHK..........
 * *** * *   ** *** *  *  ***   *

RAKRRKRKKEVPFWGRRMHHTCPCLPGLACLRTSFNRFICLARK
KRKRSKRKKEVPFFGRRMHHTCPCLPGLACLRTSFNRFICLAQK
..........VPYDGKRLSSLCPCKSGLTCSKSG.EKFKCS
          **  * *    ***  ** *       * *
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Fig. 2. Sequence of Bv8 from different species. A: Mouse Bv8, complete precursor assembled as shown in Fig. 1. B: Partial sequence of the human Bv8 precursor deduced from a cloned cDNA. C: Bv8 precursor from skin of *Bombina variegata* [6]. The mature frog protein starts with the sequence AVIT.... Bv8-b proteins contain an insert of 21 residues, which is marked by dots in the Bombina sequence. Identities between the three proteins are marked (*).

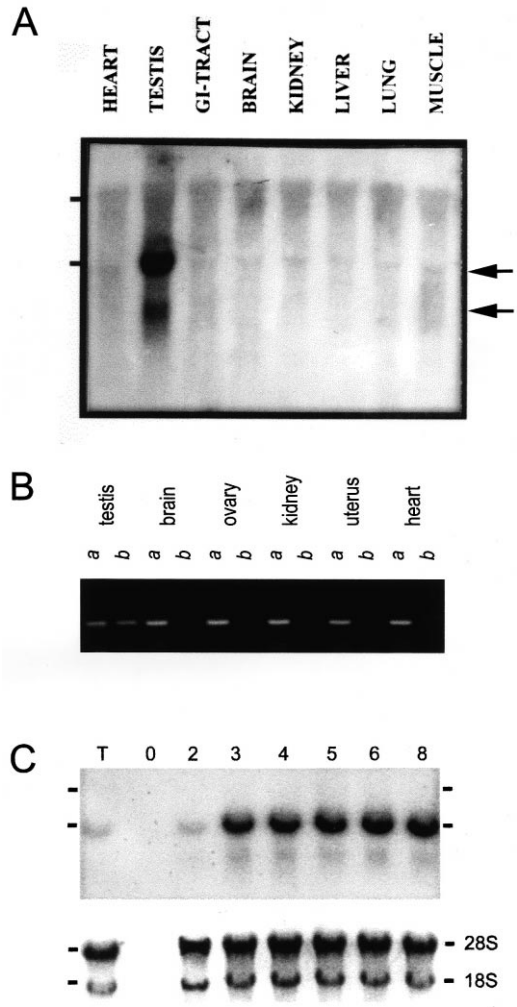

Fig. 3. Northern blot experiments. A: RNA from different mouse tissues probed with the cloned Bv8 cDNA from mouse brain. Exposure time: 2 days. B: RT-PCR using specific primers for Bv8-a or -b and mRNA from various tissues as indicated. C: RNA from mouse testis 2, 3, 4, 5, 6 and 8 weeks after birth. T, testis from mice with the Tfm mutation; 0, control lane without RNA. Exposure time: 3 days. Lower panel: 28S and 18S RNA from same blot visualized with ethidium bromide.

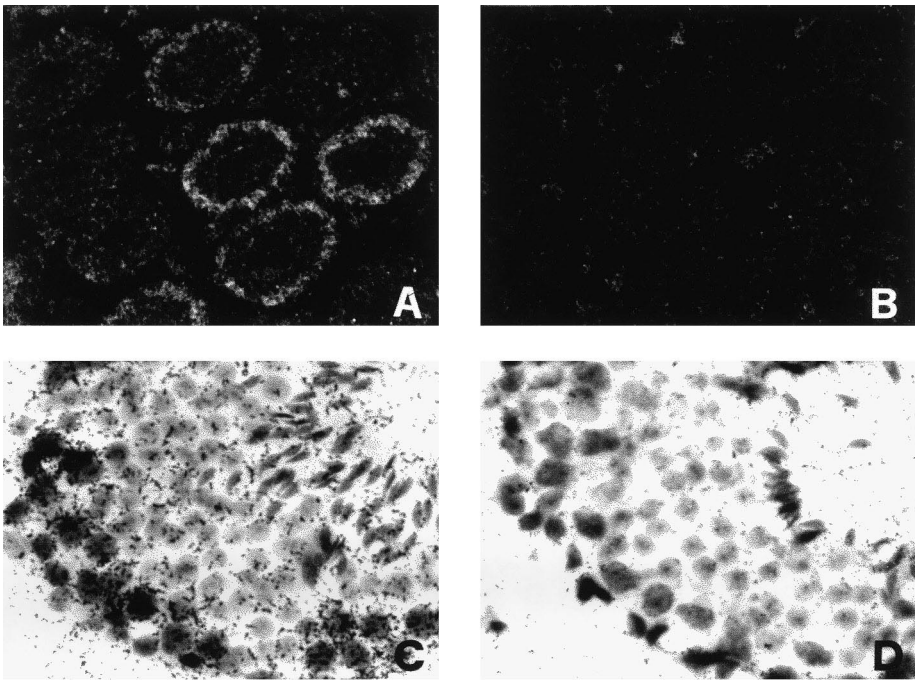

Fig. 4. Darkfield (A and B; 75×magnification) and brightfield (C and D; 470×magnification) photomicrographs of testicular sections after in situ hybridization with an antisense Bv8 RNA probe (A and C) or a sense RNA probe (B and D). Exposure time, 3 weeks. Note the hybridization signal over tubules at particular stages of maturation (A). The higher magnification (C) shows that Bv8 expression was intensely localized in mid-late pachytene spermatocytes at stages VII–IX. No signal above the background level could be detected using the sense probe (B and D).

inantly in mid-late pachytene spermatocytes during the stages VII–IX of the seminiferous epithelium cycle. These data are in agreement with the Northern blot analysis of RNA extracted from pachytene spermatocytes which were highly purified by sedimentation at unit gravity. In addition, in mouse testis, only small amounts of the Bv8 mRNA could be detected at 2 weeks after birth, whereas its level greatly increases at 3 weeks and from then on persists at high level. This all is also in line with the finding that Bv8 mRNA is absent in the testis of mice bearing the Tfm mutation [17]. XY males with the Tfm mutation on their X chromosome do not respond to testosterone and thus differentiation of germ is blocked at early spermatocyte stages.

The expression pattern of Bv8 after birth correlates well with the maturation of mouse testis. In particular, at postnatal day 17 the most advanced germ cells in seminiferous tubules are at the mid-pachytene stage in a first wave of spermatogenesis. By day 22, the meiotic divisions have been completed in some tubules, whereas in the most others late pachytene and diplotene spermatocytes are present. Moreover, we found that homogeneous populations of isolated round spermatids (haploid cells) contain very low levels of Bv8 transcripts. In line with this, in situ mRNA hybridization revealed only low levels of Bv8 transcripts in areas of the tubules where round spermatids are located.

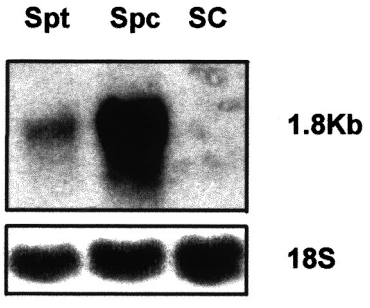

Fig. 5. Northern blot analysis of RNA isolated from various testicular cell types. Spt, round spermatids; Spc, pachytene spermatocytes; SC, Sertoli cells. Exposure time: 12 h. Lower panel: Equivalent loading of the RNA was demonstrated by hybridization of the same membrane with 18S ribosomal RNA probe. Exposure time, 2 h.

At stages VII and VIII, two major events occur: the last DNA duplication prior to the meiotic divisions and spermiation. Bv8 could possibly function as an autocrine or paracrine signaling molecule in these cellular processes. The secretory activity and morphology of Sertoli cells is in fact dependent upon the stage of neighboring germ cells [18]. So it is conceivable that specific factors, like Bv8, that are released from germ cells are important in this context. However, it is also possible that the Bv8 protein is stored in spermatocytes and functions only later in mature spermatozoa.

Our results demonstrate that homologues of frog skin Bv8 and protein A from snake venom exist in mammals. We have started to construct Bv8 knockout mice to investigate the role of this protein in testes and other organs.

Acknowledgements: We thank Mrs. Tiziana Menna (Rome) for germ cell preparations, and Dr. Pietro Melchiorri (Rome) for initiating this collaboration. This work was supported in part by grant P13279 from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung and by grants from the Ministery of Education of Italy.

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