# Research article

# Identification of the gene encoding Brain Cell Membrane Protein I (BCMPI), a putative four-transmembrane protein distantly related to the Peripheral Myelin Protein 22 / Epithelial Membrane Proteins and the Claudins

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## **Abstract**

Background: A partial cDNA clone from dog thyroid presenting a very significant similarity with an uncharacterized mouse EST sequence was isolated fortuitously. We report here the identification of the complete mRNA and of the gene, the product of which was termed "brain cell membrane protein I" (BCMPI).

Results: The 4 kb-long mRNA sequence exhibited an open-reading frame of only 543 b followed by a 3.2 kb-long 3' untranslated region containing several AUUUA instability motifs. Analysis of the encoded protein sequence identified the presence of four putative transmembrane domains. Similarity searches in protein domain databases identified partial sequence conservations with peripheral myelin protein 22 (PMP22)/ epithelial membrane proteins (EMPs) and Claudins, defining the encoded protein as representative of the existence of a novel subclass in this protein family.

Northern-blot analysis of the expression of the corresponding mRNA in adult dog tissues revealed the presence of a huge amount of the 4 kb transcript in the brain. An EGFP-BCMPI fusion protein expressed in transfected COS-7 cells exhibited a membranous localization as expected. The sequences encoding BCMPI were assigned to chromosome X in dog, man and rat using radiation hybrid panels and were partly localized in the currently available human genome sequence.

Conclusions: We have identified the existence in several mammalian species of a gene encoding a putative four-transmembrane protein, BCMPI, wich defines a novel subclass in this family of proteins. In dog at least, the corresponding mRNA is highly present in brain cells. The chromosomal localization of the gene in man makes of it a likely candidate gene for X-linked mental retardation.

### **Background**

We recently developed a screening procedure for the selection of sequences encoding proteins targeted to the cell nucleus. Our method relies on the expression in transfected cells of enhanced green fluorescent protein (EGFP) fusion proteins from cDNA library constructs [1]. The selected clones encode EGFP fusion proteins that accumulate in the cell nucleus. Many of them were shown to harbor cDNA sequences corresponding to nuclear proteins that were translated in frame with the EGFP coding sequence. However, in nearly half of the selected clones the production of a fusion protein able to accumulate in the nucleus was shown to result from out of frame translation of the cDNA sequence fused to the EGFP coding region. On the average indeed, only one out of three cDNAs was positionned in frame with the EGFP coding sequence in the starting library. It was not expected that functional nuclear localization sequences would be generated at random (i.e. by out of frame translation of cDNA sequences) as often as was observed.

One clone, called "C60", that was isolated in this approach exhibited a significant DNA sequence similarity with a mouse EST sequence present in the EMBL/Gen-Bank database (clone MNCb-0941, accession #: AU035837) [1]. No open reading frame (ORF) had been identified in this sequence yet, but the comparison of our dog sequence with the one from mouse identified a putative ORF on the basis that in the 385 bp-long region of similarity most of the differences occurred at the third position of base triplets in frame with a starting ATG codon. However, both sequences diverged before the stop codon was reached. Assuming that this was the correct reading frame, the cDNA portion in our EGFP fusion construct was translated out of frame (frame +2). This out of frame translation generated a 201aa-long sequence presenting several neighbouring clusters of arginine residues, which somehow resembled basic type nuclear localization signals. Although it could explain why this cDNA was isolated in the screening, it did not allow us to conclude whether the protein normally encoded by the cDNA is a nuclear protein or not. To further characterize the protein encoded by the cloned sequences we decided to isolate a complete copy of the corresponding mRNA.

# Results and Discussion Identification of the complete dog BCMPI mRNA

The random primed cDNA insert harbored by clone C60 [1] was used as probe to screen a dog thyroid oligo-dT primed cDNA library in  $\lambda$  ZAPII phage vector [2]. Sixty positive clones were obtained out of the 500,000 cDNA clones screened. The longest insert (from clone C60-1) had a size of 4 kb and was entirely sequenced. Compared to the sequence of the insert of clone C60, this cDNA ex-

hibited a 2 bp extension in 5' and a 2,944 bp extension in 3'. The 3' poly-A tail was preceded by a correctly placed AATAAA motif (fig. 1). The longest ORF corresponded to the putative ORF identified previously by comparing the sequence from clone C60 with that of the mouse EST present in the database (see background section). It extended over 543 bp (181 aa), from position 193 to 735 in the cDNA sequence. The translation initiator codon was located in a suitable sequence context according to Kozak's rule [3]. As in the interval an updated homologous mouse sequence had been deposited in the database (clone MNCb-0941, EMBL/GenBank acc. #: AB041540), the comparison of both sequences revealed that the coding region was entirely conserved in dog and mouse (fig. 2).

The 3.2 kb-long sequence located in 3' of the TAG codon (3' UTR) in the dog cDNA was distinctly AT-rich and contained 9 ATTTA motifs. These characteristics have been implicated in the rapid decay and restricted translation of mRNA molecules [4,5,6]. This 3' UTR was shorter in the mouse (1.3 kb) but several portions of it exhibited a remarkably high sequence conservation when compared with the dog sequence (fig. 2). Especially, the AT-rich character and the occurence of multiple ATTTA motifs were preserved. A search in the database also identified a human sequence (DKFZp564E153, EMBL/ GenBank acc. #: ALO49257) presenting a very high degree of sequence conservation over 2.5 kb with the 3' part of our dog cDNA (fig. 2). The coding region of the mRNA was not contained in this human sequence and the observation of such an extended conservation of DNA sequence between UTRs from different species was unexpected. During the preparation of this manuscript, a completed human sequence appeared in the database (DKFZp761J17121, EMBL/GenBank acc. #:AL136550). The coding region was entirely conserved between dog, mouse and man, and most of the ATTTA motifs present in the dog sequence were also preserved in man (fig. 2). It may suggest that BCMP1 mRNA is indeed subjected to tight post-transcriptional controls. However, whether the presence of these sequences really confers instability to the mRNA and restricts its translation remains to be determined experimentally.

A number of EST sequences from various species which were clearly homologous to dog BCMP1 could be retrieved from the database by BLAST searches. They indicated that the BCMP1 gene must also exist in the rat (e.g. acc. # BG381247), beef (e.g. acc. # AW352911), pig (e.g. acc. # BF704530) and in the fish Gillichthys mirabilis (acc. # AF266205), in addition to the already cited dog, mouse and man.

100		
12	CTG SGG GGG AAG STC CGC GGC GGC GTC GTC GCT GCT GCT GCT G	95
97	SEG CTT COC TOC AGA SGA SKY CGS SCT COC SSC TITY SCG SAC COS CCT	.144
3.85	SEC SM. CAC SCC ACC SOC OEL SEE SCE COL OSC ASC SEC SCC DOL USE SEC.	7.05
1	Met Ala Ser Ala Gly Ser Gly Her Glu Glu Val Arm Val Ser Val Leu	10
222	ATE SCT TOS SEE AGE AGE SEE AGE SAG SAG STE CSC STG TOS STE CTG	249
17	The Peo Leu Lyo Leu Val Gly Leu Val Cys Tie Phe Leu Ale Leu Cys	32
2.01	ACE COD CTG AND CTG STE GEG CTG GTG TGC ATC TTG CTG GGG GTG TGG	200
33	Leu Asp Leu Gly Ale Val Leu Ser Fro Ale Trp Val Thr Ale Asp His	9.5
209	CTG GAC CTG GGG GCC GTG CTG AND CCG GCC TGG GTC ACG GCC GAC CAC	336
122	Cin for for the Law Say Law Sen Cir Say Con Are Low Don Als Say Law	- 61
337	Gin Tyr Tyr Let Ser Let Trp Gin Ser Cyn Arg Lyw Pro Ala Ser Let CAS TAC TAC CTS TCC CTS TGG HAS TOC TWO CGS ASA CCC SCC AGO TYC	364
95		0.0
365	AMP THE TYP HIS CYS GIV SET THE LAW SET SET AMP TYP GIR THE ALE SHE ATC TOS CAS DEC SAS TOS ASS CYC AGO AGO GAT TOS DAS ANT GUT	422
423	The Let Ale Let Let Cay Gly Gly Ale Ale Ile Ile Let Ile Ale Phe ACT CTG GCT TEA CTC TTG GGC GHT GCT GCC ATC ATT CTT ATT GCA TTG	900
491	Leu Val Sly Leu Jle Ser Ile Cys Val Sly Ser Arg Arg Arg The Tyr CYG GYG GGT THS ARY TOT ATC TOC GTG GGA TOT CGA AGG CGC TTC TAC	312
323	Arg Fro Yal Als Val Ber les Ftm Ale Ale Val Val Leo Sin Val Cys AGA CCT STY GIT STC ATS CTT TTT SCA SCA STY STY TTA CAG STY TSC	376
	AGA CUT STY GUT STC ATS CTT TTT SCA SCA STY GTY TYA CAG STY TSC	-110
329	der Leu Val Les Tyr Fro fie Lyr Ste lie (Lu Thr Val Der Leu Lyn	144
573	ASC CTS STC CTF THC CCA ATC AND TWO ATT SAN ACT STE ASS TTG ANA	424
393	Ile Tyr Ris Glo Fee Am Trp Gly Dyr Gly Leu Ala Trp Gly Ale Thr	160
625	AFT TAC CAT GAO THE AAC THE DEF TAT DOS CTE DEE THE DOT DEA ACT	4.77
363	lie He fer FAe Sly Siy Ala Lie Leo Tyr Cym Leo Ren Fan Lye Asn	276
673	ATA TTY TICE TYP SEG SHT OCC AND UNY TAY TOU CITE AAC CUT AND AAC	120
377	The Sin Amp Tye Tye ***	
322	THE SAA SAC THE THE THE AND CAR DAS TOT CAS ATT THE RAW ACK ACC	768
769 817	AAC DAT DOA ANA ANA GUA TTA CTO CTO TAT CTT TTO TAA CTC ACT CTT TIC TAA AAC AND TOT GUA GTA TOK AGG AGT TTE CTC AGT TGA TTT AND	704
805	THE TAN AND ANY THE SON ONE AND AND AND AND THE SON ONE AND AND	912
913	THY AGG CAT C'US CAC AAA WYA AGA CAG C'YG AYT AGA CAY AGG CAA AYG	260
961	CTG CAA ACT TOT AAT ATG TIT ATG TOTA TIT TIT TIT AGA AGT GAA AGG	1903
2009	STC THT ATG ACA SCA ACC ATT STO AGA TAC TAS TTO GAA TGG GAA ATG GCT GAA TGT GCT AST SGC TGC AGE SGA SCA GGT USC ATA AGA AAC ATT	1006
1105	TAG AND THE CIT THE TOT GAT AND SAY TOO ACT GOT ARA EAG THE TTA	1157
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1201	THE AGT EAS CITY YOU AND GUT GAD CITY COL THE AGE NOT TAN THE GAD	12 9 6
32.69	YEC TOA GITE TAA TOO GITE AAA GOV APA TAG ATT ICIA CCT TCA AGA ATA	229%
1297	OTC TIT TTO ANY SEG AND CAN THE TIT DOC ANT CAN CAT CTC OUT SEG ANA DOG STC AND ACT TOO ACE CAT STO CITY THE ACT AND TOO TITE GAS	1302
1345	ANA GGA ETC ANE ACT TOG AGA CAT GTG CTT TTU ATT ATG TGG TTA GAA AFT GTG CCF CAG GCG TAT CTA GAA TGA GGA CAA TTG AGA GTU TGT ACT	1392
1481	THE ECT SOS GEN ACT AND AND AND THE GEN THE GIT THE GITC CIVA	1153
1400	ANT AGE OFF TAX AND ATT TAX DIA AGA TTA GOS CAX ANA ATT HTG CON	1524
13.57	ATO TAA CAA GCT TAA ATA TTT TOK DAG AAG GTG AAC DAT TAG TAT ADA	1564
2505	THE TAG TAK ACT ACT COS TAG CCC TAT ACA THE AFT THE CTA TTA CAC	1632
1633	ATA AAA STA ACA TYT SCY CTA STS AAG TGA TTY GRA CAC TAA CCY TST ACA CST TAA GAS SCY TAG ATT GST ACT CAC ACT TAC TYA CTG TAC AAA	1729
2729	AGY ACA THE ACT TTA AND CTT TTO CTC TCT OTT CTC TAC TOT TTC ACT	1776
1777	DEA AND TYT TAX THE ANT TOT ANA ERA EAR AND TYE CAX THE TOT TAX	1824
3820	CET HAN ANT TON TWO TTO TTO YOU AND CAC YAT AND TON AGE AND AND AND	1872
2473	AGC AAT TWO ART GAT CAT TTT GTV AAA AAT TCA GTW AAA TAG AAG GCT	1929
1949	SCT TIT TIT TIC AAG TAC CIT AAA TST CIT TAT TAA AAA ATA AAA GAA TRG TGA TAG ATT TRT AGA AAT GIT TITA ATG TCT CAG TRG AGG AGT AGA	2016
3015	AET CAY THE TEA TEA CUT HOT CUT CITY AND THE ACT HAT BOD OTA ACT	224.4
2065	GAT TTO TOO ACK DAA TTA TOR YOU ADT TAT OFT ACK OWA ATC ACT TOT	2512
2113	THE CTO TTC ANA THE AND HAN WEN TON ATE WIR GOD ANY AND TIT HICK	2160
2209	ACT AAY CTC AGA CTG CAA ACT TOT OUT AAY CCT OFF THC TAT ACA AAY TTG GAT AGT TEX HEY GTA AAC ATK TET TEA TAT CAA HOA TAC AGT TCT	2208
3257	ART ATA AAR GTC ATA AAT AAT TAG TOT TOT TAA CAR AGG CAC TAA AAG	2304
2305	AGT ATT CCT GGT TTT SGC CCT TTT SCA SAA AGA AGC ATT SGA AAA ATC	2352
2353	ARY TAN AND ATR CTY AND TYC TAY TRY ATY GOA ARA TOO GTY AND AGO	2400
2402	AND ACT ACA TOR NEW STA STA TIT AND MAT THE UTT TAK CTC CCA GAA CAN ANT TOT GCT TEC AND ACH TOT TAK NAW CTN TET GCC AWA ACA ACA GAA	2496
2497	ANT TICT GOT TTC AND ATM TOT TAT MAN ATM TTT GOT ANA ACA ACA GAA ANA ANA AYO TAT TTT ATT ATT CAN ATM CTT AND ANA ANA ANA ANA COD	2161
2545	TOO TAT OUT OUS ACT TIT ONE TAS SAY CAC AAA STA AAT AND AAS AAC	2592
2593	CAS SCT GAR TOT THE TIT AGO ART GAO AGA CAR ARG THA TER ART GOR	2640
2000	TOO TAG AGA GAR ACC TIT ATG ARA STA TAY GAT TOT CTO TAG AAT CRO ARG ARA CTA TOG STG ARG TIT TOO AGA ARA ATA TOR STA TTA TAY ART	2736
2727	ARE ARA CTA TOG UTG RAG TTT TCC RGR RAR ATE TOR GTA TTA TRT ART CCT TTG RAG CRA TTT TCT TGR GRA REA STA RAR TCT SGG CCA GRC TGT	2284
2392	CTT SCA OTT AND TOG OTA THE TOR SHO CAS CAT ONE ANA TAC GAT ADD	2932
2033	THE THE AYA GOD ARY TOO GIT ACT AAC CAC TOT COA THE THA GAN ARE	2800
2929	CTC TTY AYT COT SAA TAA TOS AAS AAC AST ACT TTO STO CTO ACA TOA	2928
2977	AND AGO CAC TIT TOT TAG TOO YAG TIG AGG ATG CAG TOT GOT GIT AGA COA GIA COA CAT CITO GAG TOT TIT CAA GIT TID ATT CITO TOT CAA TAT	3024
3000	ORC AGO STE GAR ART OAS ANT ATH TYN TAU GCT ARE GCA SAC ATO TIT	3072
3073	SCA STA CAC TSA ANA STA GAT THE THE EAT AND THY THE COT CHE COL	2110
3121	ANT WAR GAN COO TRY STE STE TOT CTA COA TOA CTE REA TTE GAR ANT ATT TOA TOA CER ACO RAC TOR STE DRA CAT WAR AND THE THE TOE TOE	3116
3217	SAA ATA TUB TAT UST ETA TUA AAA TAA ACA UST SAA ATT TOS TOA TOE SAA ATA TUB TAT UST ETA TUA AAA TAA ACA UST SAA TIT TOA UST ACA	3216
2265	TTA THE CTT THE CHE TTY AND THY HET THA THE TYP LET AGA AND AAA	2212
3313	ACT STE AAC TAY AAA TOU ATT STE DET CTE ASA TAY ATA CAT STA CAU	3360
3361	ACA TAO AGE ATA AAA TAC TEA GER GOG ETA OTT ATT TOG ATT TEE TEE ACA ACT ATT TAG ETT TET GTA AGE TEA ACA TOT AAA TET TAA AGA CAA	3456
2457	ATA TAG AGA GAD CTA TET GTT TER AAT ATA AAT GAT ATA TAT GGA TTA	3304
3505	GCA TOT ACC TOT ATA TTA TYA AAC ATO CAA TGA ACT GAC TOG TAA GTG	3557
2203	APS TOT ART TOT ATS SOT ARC ART STA ATT TAT TOR SAC TST ATT TOT	3600
3601	STA CAS ASC ASC SCA CAC TAX DIT KTS DIT OTS THE DIT OT THE TAX THO	3666
3697	CTA AAA CTS TSC CTA SAA ATT TCA TCT STC TTA ASA AAT AAA ATT ATA CTT CAT SCT STT SET SET ATA STT TCT CTA CTS CTS TTC TAT ATT TAT	2244
3745	THE THE THA HER THE GAC AND THE ACT ACT THA HER THA ATT CAN GOT	3292
3353	ART CTG GTT ATT TIT TIT AND AUT OUT LING GOD GOA ACC TOT TIC YOU	3840
3911		
	CTC CAS THE TTY TEA STY TOO AND TYC ACA ATC AND TCT TCA TTY GAT	3449
2557	CAT THE THY THA ATT THE AND THE AGA ATT AND THE TOA THE EAT CAT THE THE AGE THA CAT ATE AND THA TOE ATE THE REA ANA TAA ANA THA RIGHT TOE THE CAT THE ANA ANA ANA ANA ANA ANA ANA ANA	3656

**Figure I Nucleotide sequence of dog BCMPI cDNA**. The aminoacid sequence encoded by the ORF appears above the corresponding DNA sequence. The underlined sequence corresponds to the insert of the original clone C60 (see text). ATTTA motifs appear in bold and the polyadenylation signal is highlighted in blue.

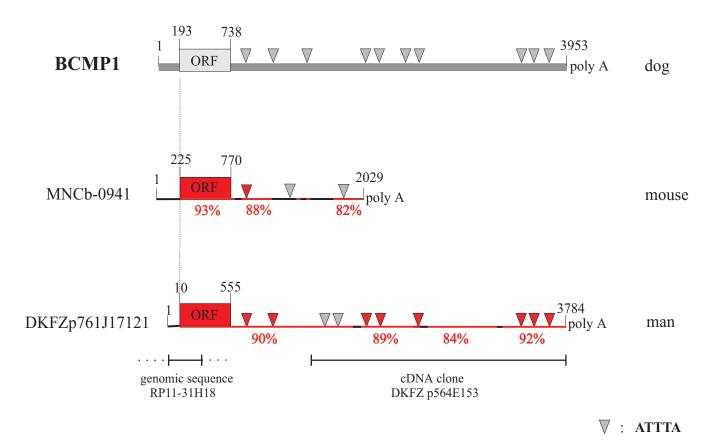


Figure 2
Structure of BCMP1 mRNA. Schematic representation of known sequences from mouse and man that are homologous to dog BCMP1 sequence (short EST sequences were not considered). Coordinates are given for each sequence individually. Highly conserved regions are highlighted in red with indication of the percentage of identity relative to the dog sequence. Inverted triangles symbolize ATTTA motifs. They appear in red when the motif is conserved in the dog sequence.

### Analysis of BCMPI mRNA expression in the dog

Originally, the cDNA had been isolated from a dog thyroid cDNA library. In order to investigate whether the corresponding mRNA was also present in other cell types, a northern blot experiment was performed using poly-A+ RNA preparations from various dog tissues (fig. 3). Huge amounts of the 4 kb transcript were detected in brain cells. The presence of the mRNA was also detectable in most of the other RNA preparations but to lesser extents as compared to that found in brain RNA. The encoded protein is thus expected to be particularly abundant in the brain, unless the peculiar 3' UTR of the mRNA mediates a deep control on its translation (see above).

# Prediction of BCMPI protein structure and subcellular localization of an EGFP-BCMPI fusion protein

The 181 aa-long protein sequence encoded by the mRNA did not present any significant ressemblance with sequences present in protein databases. A search for the presence of protein family signatures (PfamHMM on Expasy server) revealed the occurrence in the novel protein

of sequence motifs ressembling significantly to one of the two motifs specific to the peripheral myelin protein 22 (PMP22) family of proteins and to the motif specific to the claudins (fig. 4). The two identified signatures overlapped partially in the novel protein sequence. PMP22 and the related epithelial membrane proteins (EMPs) [7], as well as the claudins [8], all belong to the superfamily of four-transmembrane domain (4TM) proteins. As could be expected, the search for the existence of putative transmembrane domains in the novel protein (HMMTOP on Expasy server) identified the presence of four of such domains (fig. 5). The protein thus appeared to be a novel member of this large family of proteins, somehow related to PMP22/EMPs and the claudins. As these are integral membrane proteins, and as the mRNA encoding the novel protein was predominantly found in brain, the newly identified protein was termed "brain cell membrane protein 1" (BCMP1).

According to the putative BCMP1 structure, the extracellular loop between TM1 and TM2 would be larger than the intracellular loop between TM2 and TM3 and the ex-

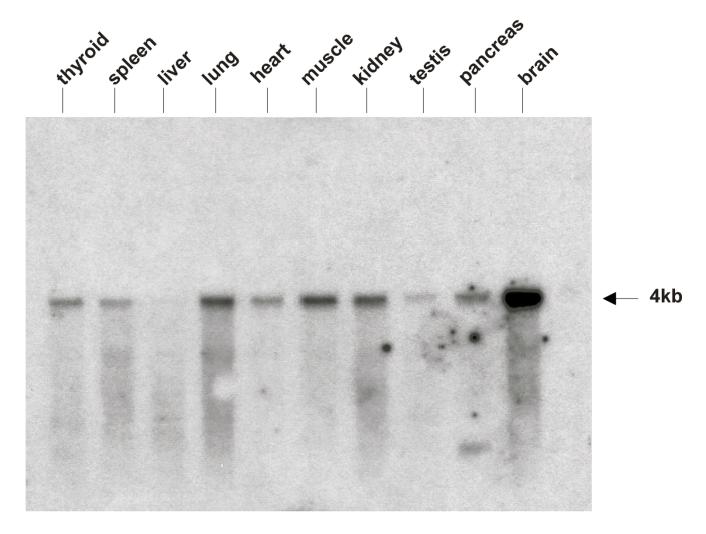


Figure 3
Northern-blot analysis of BCMP1 mRNA expression. PolyA+ mRNA preparations from various dog tissues were probed with the coding region of BCMP1 cDNA. The arrow points to the signal corresponding to the expected 4 kb-long transcript.

tracellular loop between TM3 and TM4, as it was supposed to be also the case in PMP22/EMPs and claudins. However, the intracellular amino-terminal arm proceeding the first transmembrane domain appeared to be much longer in BCMP1 than in its relatives.

In order to refine the classification of BCMP1 within the four-transmembrane domain protein family, a phylogenetic tree was constructed on the basis of the alignment of the available protein sequences related to the PMP22/EMPs and claudins (fig. 6). Dog BCMP1 and its mouse and human orthologs segregated as a distinct subgroup in the tree. Their closest relatives were the recently identified mouse PERP gene product [9] and the protein encoded by the CG6982 gene in drosophila (EMBL/GenBank acc. #: AAF56054). This group of proteins thus

shared primary structure determinants which defined a distinct subclass in the protein family.

In order to assess experimentally the postulated membranous localization of BCMP1, an EGFP-BCMP1 fusion protein was expressed in transiently transfected COS-7 cells and the subcellular localization of the hybrid protein was observed by fluorescence microscopy (fig. 7A and 7B). A fine granular fluorescence was observed all over the surface of cells expressing the EGFP-BCMP1 fusion protein, consistent with a plasma membrane localization of the tagged protein. A stronger fluorescence surrounding the cell nucleus was also observed. It indicated that a significant part of the expressed fusion protein accumulated in the endoplasmic reticulum. The pattern of EGFP fluorescence remained almost un-

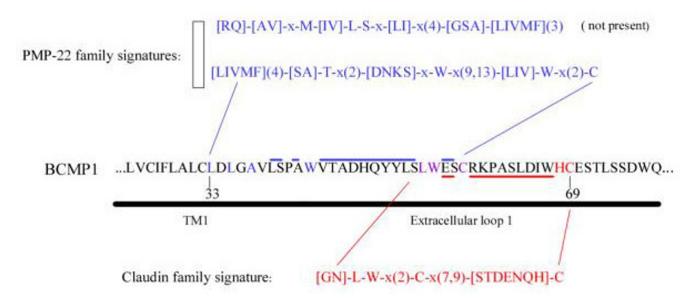


Figure 4 Identification of PMP22 and Claudin family signatures in BCMP1 primary structure. Conserved residues are coloured (blue: PMP22 signature, red: claudin signature, violet: overlapping PMP22 and claudin signatures) and conserved spaces between specific residues are over - or underlined. The part of the predicted BCMP1 structure (see fig. 5) to which the primary sequence corresponds is shown (TM1 = first transmembrane domain).

changed when the cells were permeabilized with saponin in order to stain the nuclear DNA (fig. 7C and 7D). This indicated that the fusion protein was embedded in the membranes and that it was not able to readily diffuse out of the cell.

## Localization of the BCMPI gene in dog, man and rat

In dog, the BCMP1 coding sequence was typed in duplicate on the 118 cell lines of the RHDF5000-2 radiation hybrid panel [10] on the latest version of the RH map [11]. The BCMP1 gene was linked to chromosome X close to FH2548 with a Lod score of 11.88. Marker FH2548 is located close to the DMD locus in dog (distance: 4.4 cR<sub>5000</sub>, approx. 500 kb). More informations about dog RH maps can be found at <a href="http://www-recomgen.uni-vrennes1.fr/doggy.html">http://www-recomgen.uni-vrennes1.fr/doggy.html</a>.

The human EST sequence DKFZp564E153 (EMBL/Gen-Bank acc. #: ALO49257) that corresponds to the 3' UTR of dog BCMP1 mRNA had been localized on chromosome X. The corresponding human genomic sequence could not be found by BLAST searches against sequences available in the database. However, by using the coding region of dog BCMP1 a significant match was identified with genomic sequences assigned to human chromosome 8 (clone RP11-31H18, EMBL/GenBank acc. #: ACO41003). The similarity extended from position 1 to 418 in the human cDNA sequence (fig. 2), which corresponded to the amino-terminal part of the protein up to

the first extracellular loop. As an intron was found at this same position in PMP22, EMP-1 and EMP-3 genes, it appeared likely that we had identified the first coding exon of the human BCMP1 gene. PMP22, EMP-1 and EMP-3 genes all contain an additional intron separating the sequences encoding the first transmembrane domain and the first extracellular loop into two exons [7]. This intron is clearly not present in the human BCMP1 gene. In order to clarify the location of the gene in the human genome (chromosome X or chromosome 8?), the GeneBridge-4 WGRH panel was used to map the sequences encoding human BCMP1 using a pair of primers directing the amplification of a 666 bp-long fragment encompassing the entire first coding exon and the exon-intron junction. It revealed that the amplified segment was located on chromosome X, 0.20  $cR_{3000}$  from marker Wl-7096 and 6.51 cR<sub>3000</sub> from marker DXS1214. This location agreed with the previous assignment of the EST sequence DKFZp564E153. It also corresponds to the cytogenetic location Xp11.4. As the DMD gene maps at Xp21.2 in m an, it is thus also close to the BCMP1 gene in this species. The chromosomal localization result revealed unambiguously the existence of a single BCMP1 locus in the human genome. As a consequence, it indicated that the sequences of the genomic clone RP11-31H18 had been inappropriately assigned to chromosome 8 instead of chromosome X in the database.

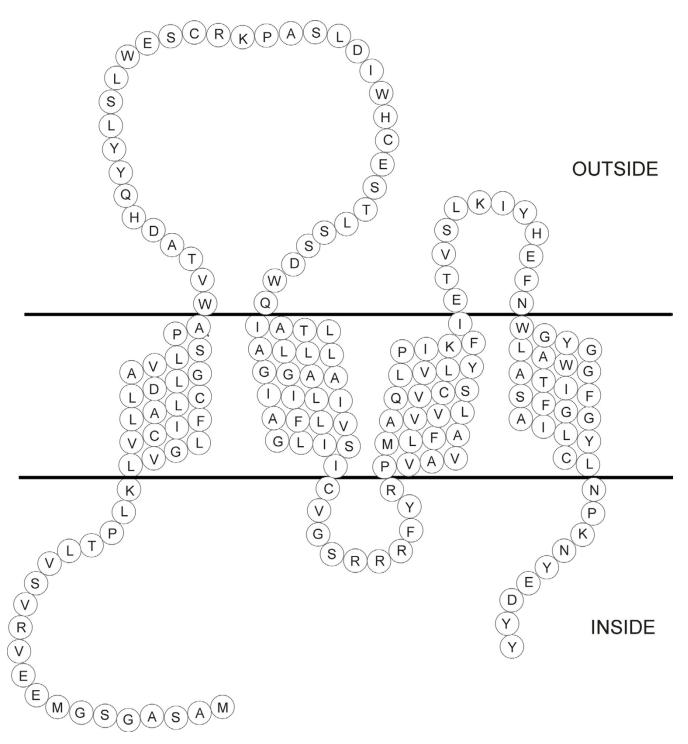


Figure 5
Predicted structure of BCMPI in the plasma membrane. The structure was drawn on the basis of the predictions obtained from HMMTOP on the Expasy server.

In the annotated human genome sequence available on the Ensembl server, the first coding exon of the human BCMP1 gene (gene ID:ENSG0000101959) is present in the chromosome X sequence (the sequences of clone RP11-31H18 have now been properly reassigned to chromosome X; see ContigView on Ensembl server). Part of the coding region of human BCMP1 and the whole 3'

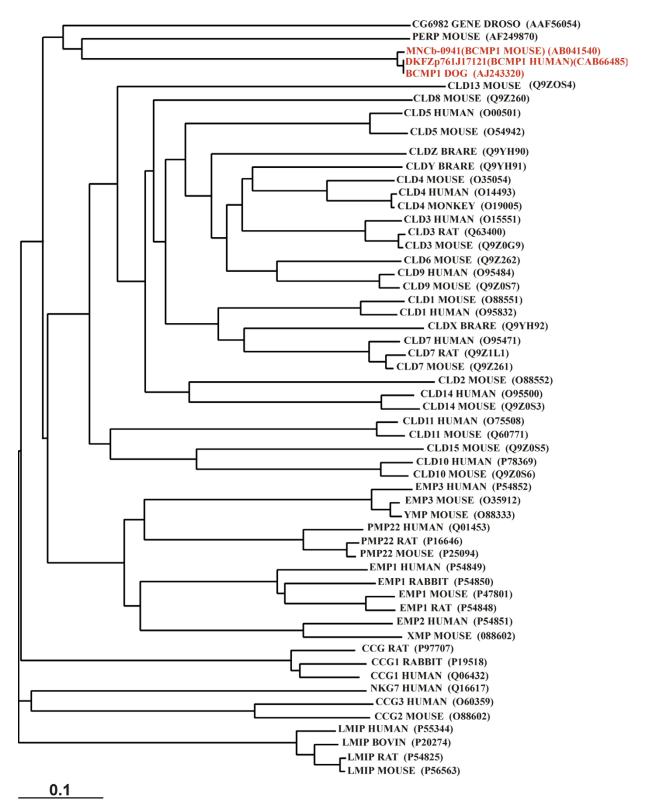


Figure 6
Phylogenetic tree of four-transmembrane proteins related to PMP22/EMPs and the claudins. See materials and methods section for the description of the tools used for the construction of the tree. Database accession numbers of individual sequences are given in parentheses. Dog BCMPI and its mouse and human orthologs appear in red.

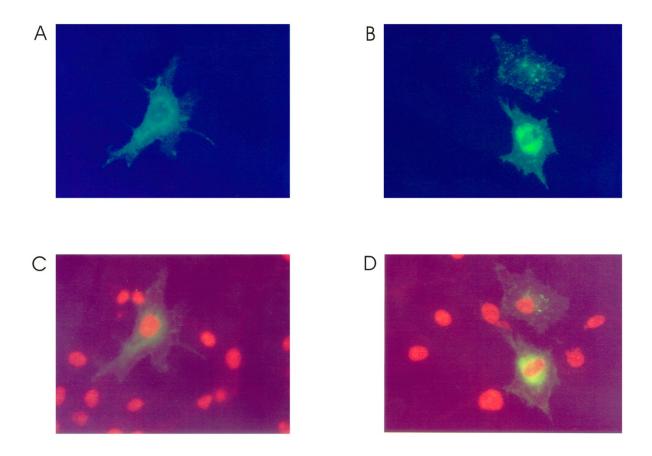


Figure 7
Subcellular localization of the EGFP-BCMPI fusion protein. The fusion protein was expressed in COS-7 cells. Parts A and B: observation of EGFP fluorescence in the perinuclear region and at the cell membrane. Parts C and D: cells were permeabilized and the nuclear DNA was stained with ethidium bromide in order to visualize the cell nucleus.

UTR region corresponding to DKFZp564E153, are still missing in the currently available human genome sequence.

Using primers derived from the rat EST236642 (EMBL/GenBank acc. # AI408352), which is 91% identical to the segment 2028-1434 of the mouse brain cDNA MNCb-0941, itself similar to dog BCMP1 cDNA, the rat gene (symbol: *Bcmp1*) was assigned to the chromosome X, between DXRat67 and DXRat28, at 497.9 cR along the MCW map (LOD score: 9.0; the local map is:DXRat67-29.9 CR - *Bcmp1* - 0.4 cR - DXRat28). The marker DXRat67 co-localizes with the gene *Dmd* [12], itself cytogenetically assigned to Xq22 [13]. The rat genes *Bcmp1* and *Dmd* are thus closely linked, as was already observed in dog and man.

#### Conclusions

We have described here the identification of the gene encoding a novel protein, called Brain Cell Membrane Protein 1 (BCMP1), which belongs to the large family of four-transmembrane proteins and appears to be highly expressed in the brain. The gene seems to be conserved on chromosome X within mammals, in close association with the DMD locus in man, rat and dog at least. The encoded BCMP1 protein shares significant ressemblances with both PMP22/EMPs [7] and the claudins [8], but exhibits distinct features, notably a predicted intracellular amino-terminal extension, which distinguishes it from the other known members of the family.

PMP22/EMPs are integral membrane proteins that seems to be implicated in various cellular processes, such as cellular differentiation, control of proliferation, and apoptosis [7]. PMP22 has been shown to play a critical role in peripheral nerves, where it is involved in the as-

and

sembly of peripheral nerve myelin and in the regulation of proliferation and differentiation of Schwann cells. The claudins also constitute integral membrane proteins which are localized exclusively at tight junctions [8]. Claudin-1, -2 and -3 have been shown to present calcium-independent cell-adhesion activity [14].

Alterations in the PMP22 gene are responsible for hereditary motor and sensory neuropathies in human and rodents, known as Charcot-Marie-Tooth type 1A (CMT1A) disease and Trembler (Tr) mouse respectively [7]. Individuals presenting nonsyndromic recessive deafness (autosomal recessive deafness DFNB29) were recently shown to harbor mutations in the gene encoding claudin-14 [15]. The Xp11.4 region of the human genome which comprises the BCMP1 gene has been linked to several forms of syndromic X-linked mental retardation, such as MRXS-2, -4, -6 and -10, and to a number of nonsyndromic MRX cases [16]. The TM4SF2 gene which apparently encodes another member of the superfamily of four transmembrane proteins, a tetraspanin [17] more distantly related to BCMP1 than are PMP22/EMPs and the claudins, is located very close to the BCMP1 gene in man. Mutations in the TM4SF2 gene and gene inactivation resulting from chromosomal translocation have been shown to be involved in several cases of X-linked mental retardation [18]. Whether the BCMP1 gene is also involved in such genetic disorders and what is the function of the encoded protein thus constitute the obvious questions which will support our future investigations.

# Materials and methods

### **DNA** constructions

Standard DNA manipulations were conducted according to published procedures [19]. The full length BCMP1 cDNA clone was obtained by screening a dog thyroid cDNA library in λ ZAPII phage vector [2] using the original clone C60 [1] as probe. The DNA sequences corresponding to the cDNA insert in clone C60 were amplified by PCR using primers complementary to the sequences flanking the insert the construct, in 5'CAGATCTCGACCCACGCG3' and <sup>5</sup>TACCTGCGGCCGCGATAT<sup>3</sup> respectively, and were labeled with digoxigenin (DIG labeling and detection kit, Boehringer Mannheim). Hybridization, washing and signal detection were performed as recommended by the supplier of the labeling system. The cloned DNA was sequenced on both strands using the Big Dye Terminator methodology and a model 377 DNA sequencer (Applied Biosystems). The construct encoding the EGFP-BCMP1 fusion protein was obtained by inserting a PCR fragment corresponding to the BCMP1 ORF between the EcoRI and BamHI sites in the pEGFP-C1 vector (Clontech). The following primers were used to amplify these sequences from the DNA clone of

C60:5'TTCGAATTCGGCGGGCAGCGGC3'
5'TGTGGATCCTAGTAGTAGTCTTC3'.

### RNA analysis

Northern blot analysis was performed on  $4 \mu g$  of polyA+mRNA from various dog tissues. Acridine orange staining of the gel confirmed that each lane contained identical amounts of RNA. A  $^{32}$ P-labeled PCR fragment corresponding to the BCMP1 ORF was used as probe (see above for preparation of the DNA fragment). Hybridization and washes were conducted in standard conditions in the presence of 50 % formamide [19].

### Cell transfection

Transfection of COS-7 cells was performed using the DEAE-dextran method [20]. About 200 ng of a crude plasmid DNA preparation was engaged per dish (diameter: 3 cm). The subcellular localization of EGFP fluorescence was observed 48 hours after transfection using an Eclipse TE300 inverted microscope (Nikon) equiped with NB-2A and NG-2A filter blocks. The transfected cells were permeabilized using saponin (0.075% final concentration) and nuclear DNA was stained with ethidium bromide (1  $\mu$ g/ml final concentration) in order to visualize the cell nucleus.

### **Chromosomal localization**

Dog BCMP-1 could be readily typed on the dog x hamster radiation hybrid panel RHDF5000-2 composed of 118 cell lines from panel RHDF5000 [10]. The following pair of primers, 5'TCTGGAGTGAACTAATGGGCTAA3' and <sup>5</sup>GCAGTCTGAGATTAGTGGCAAA<sup>3</sup> generated a PCR product of 137 bp on dog genomic DNA. PCR results were scored in terms of present, absent or ambiguous in the 118 hybrid cell lines. The typing data were incorporated into the latest radiation hybrid map [11], using the Multimap package [21]. The GeneBridge 4 human x hamster radiation hybrid panel DNA (Research Genetics Inc.) was screened by PCR using the following primers: <sup>5</sup>GGCAGCGGCATCCAGGAA<sup>3</sup> <sup>5</sup>TGGGGAAGACCAACAGAGAACC<sup>3</sup>. The PCR results were analyzed according to the prescription of the supplier of the panel DNA.

The panel of rat x hamster radiation cell hybrids [12] was typed by PCR with the following primers: 5'-AACTGT-GAATACCAATCTAAGT-3' and 5'-GTTTTTCATTAT-GCAGTTACAG-3'. The mapping results were obtained from the rat radiation hybrid map server at the Medical College Wisconsin [(http://rgd.mcw.edu/RH-MAPSERVER/)].

### Bioinformatics

Sequences comparisons were performed using the BLAST tool ([http://www.nc-

bi.nlm.nih.gov/BLAST]). Protein sequences alignments and phylogenetic tree were constructed using Clustal X (Ver. 1.8) and TreeView (Ver. 1.6.1) respectively. Structural predictions based on protein sequences were obtained using programs available on the Expasy server ([http://www.expasy.ch]). Localization of the BCMP1 gene in the human draft genome sequence was achieved using data and tools available on the Ensembl server ([http://www.ensembl.org]).

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