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Alternative splicing and tissue specific expression of the 5′ truncated bCCE 1 variant bCCE 1Δ_{514}

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Abstract In many non-excitable as well as electrically excitable cells, depletion of intracellular Ca^{2+} stores after stimulation of G protein coupled receptors or receptor tyrosine kinases is followed by Ca^{2+} entry across the plasma membrane, a mechanism referred to as capacitative calcium entry (CCE) [Putney, J.W., Cell Calcium 11 (1990) 611–624; Fasolato, C. et al., Trends Pharmacol Sci. 15 (1994) 77–83]. Recently, we reported that bCCE 1, a homologue of the *Drosophila* protein *trp*, exhibits the characteristics of CCE channels [Philipp, S. et al., EMBO J. 15 (1996) 6166–6171]. In this study, we report the cloning of a 5′ truncated splice variant (bCCE 1Δ_{514}) of the full-length bCCE 1. The bCCE 1Δ_{514} cDNA encodes a protein of 486 amino acids with the ATG triplet encoding M^{514} of bCCE 1 as translation initiation codon and, therefore, comprises two putative transmembrane segments corresponding to the predicted transmembrane segments 5 and 6 of bCCE 1. bCCE 1Δ_{514} transcripts appear to be specifically expressed in the adrenal gland and genome analysis reveals an alternative splice site within an exon of the CCE 1 gene leading to the formation of bCCE 1Δ_{514}.

Key words: Capacitative calcium entry; bCCE 1 splice variant; Adrenal gland; cDNA cloning; RNA blot hybridization; Genome analysis

1. Introduction

Formation of the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) after activation of G protein coupled membrane receptors or receptor tyrosine kinases by hormones, neurotransmitters or growth factors leads to depletion of intracellular calcium stores. Store depletion is followed by Ca^{2+} entry through Ca^{2+}-selective Ca^{2+} release activated channels as well as non-selective cation channels. This mechanism has been described as capacitative calcium entry (CCE) [1,2] and might be involved in various cellular events such as steroidogenesis in adrenal gland [3], migration of nerve growth cones [4] or cell proliferation [5].

In search for molecular correlates for this capacitative mechanism, the *Drosophila* genes *trp* [6] and *trpl* [7] have been suggested to function as calcium entry channels (for a review see [8]). Recently, we cloned the mammalian *trp* homologues bCCE 1 and CCE 2 which both exhibit the features of CCE channels [9,10]. Like *trp* and *trpl*, bCCE 1 and CCE 2 as well as full-length cDNAs cloned from other mammalian *trp*-related genes (hTrp1 and hTrp 3) show a common topology of six to eight putative transmembrane segments [9–14]. Splice variants were reported in the 5′ untranslated region of the bCCE 1 gene [9] as well as in the 5′ coding region of the human and mouse Trp 1 gene [11,12,14,15].

Here we report the cloning of bCCE 1Δ_{514} from adrenal gland, a 5′ truncated splice variant of the full-length *trp* homologue bCCE 1 which we originally cloned from retina and adrenal gland [9]. bCCE 1Δ_{514} comprises two putative transmembrane segments corresponding to the predicted transmembrane segments 5 and 6 of bCCE 1. We show that bCCE 1Δ_{514} transcripts appear to be specifically expressed in the adrenal gland and give evidence that its formation results from an alternative splicing mechanism as concluded from genome analysis of the corresponding region of the mouse and bovine CCE 1 gene.

2. Materials and methods

2.1. cDNA library screening

An oligo(dT) primed cDNA library was constructed using 5 μg of poly(A)$^+$ RNA from bovine adrenal gland. Screening of $\sim 5\times10^5$ clones using a ^{32}P-labelled cDNA probe covering nucleotides 713–1882 from bCCE 1 (probe 2 in Fig. 1 and [9]) yielded NN44, a full-length clone encoding the bCCE 1 protein of 981 amino acids, and five clones (NN40, NN1, NN43, NN21 and NN20) encoding a truncated splice variant of bCCE 1, bCCE 1Δ_{514}. In addition, partial clones covering regions between transmembrane domain 6 and the 3′ end of the bCCE 1 cDNA were identified. All sequences obtained were sequenced on both strands using either ^{35}S-labelled dATP or infrared dye labelled primers and an automated laser fluorescence DNA sequencer (LI-COR).

2.2. Northern blot analysis

Total RNA from bovine adrenal gland, atrium, ventricle, rabbit heart and Ltk$^-$ cells was isolated by the guanidinium thiocyanate method, and poly(A)$^+$ RNA was separated by oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA was fractionated by gel electrophoresis (1.2% agarose and 1.5% formaldehyde), transferred to nylon membranes (Hybond-N, Amersham) and hybridized with either a PCR amplification product corresponding to nucleotides −162 to −22 of bCCE 1Δ_{514} (probe 1) or a cDNA fragment containing nucleotides 713–1882 from bCCE 1 (probe 2). The probes were labelled by random priming using [α-^{32}P]dCTP. Hybridization conditions used were as described [16]. As a control of the integrity and amount of the transferred RNA all membranes were stripped and rehybridized to a 239 bp PCR fragment from the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

2.3. Polymerase chain reactions

The PCR reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, dGTP, dATP, dTTP, dCTP, 0.2 mM each, 25 pmol of forward and reverse primer and 0.5 U Taq polymerase (Boehringer Mannheim). Control reactions devoid of cDNA template were included in each PCR amplification experiment.

Probe 1 used for Northern blot analysis was generated using primer 14 (5′-ATC CGT CAA GCC CAG GCC-3′) and primer 15 (5′-CTC GTC CTT ATA ATT CCT TCG-3′) corresponding to nucleotides −22 to −39 and −162 to −142 of bCCE 1Δ_{514}, respectively, and 10

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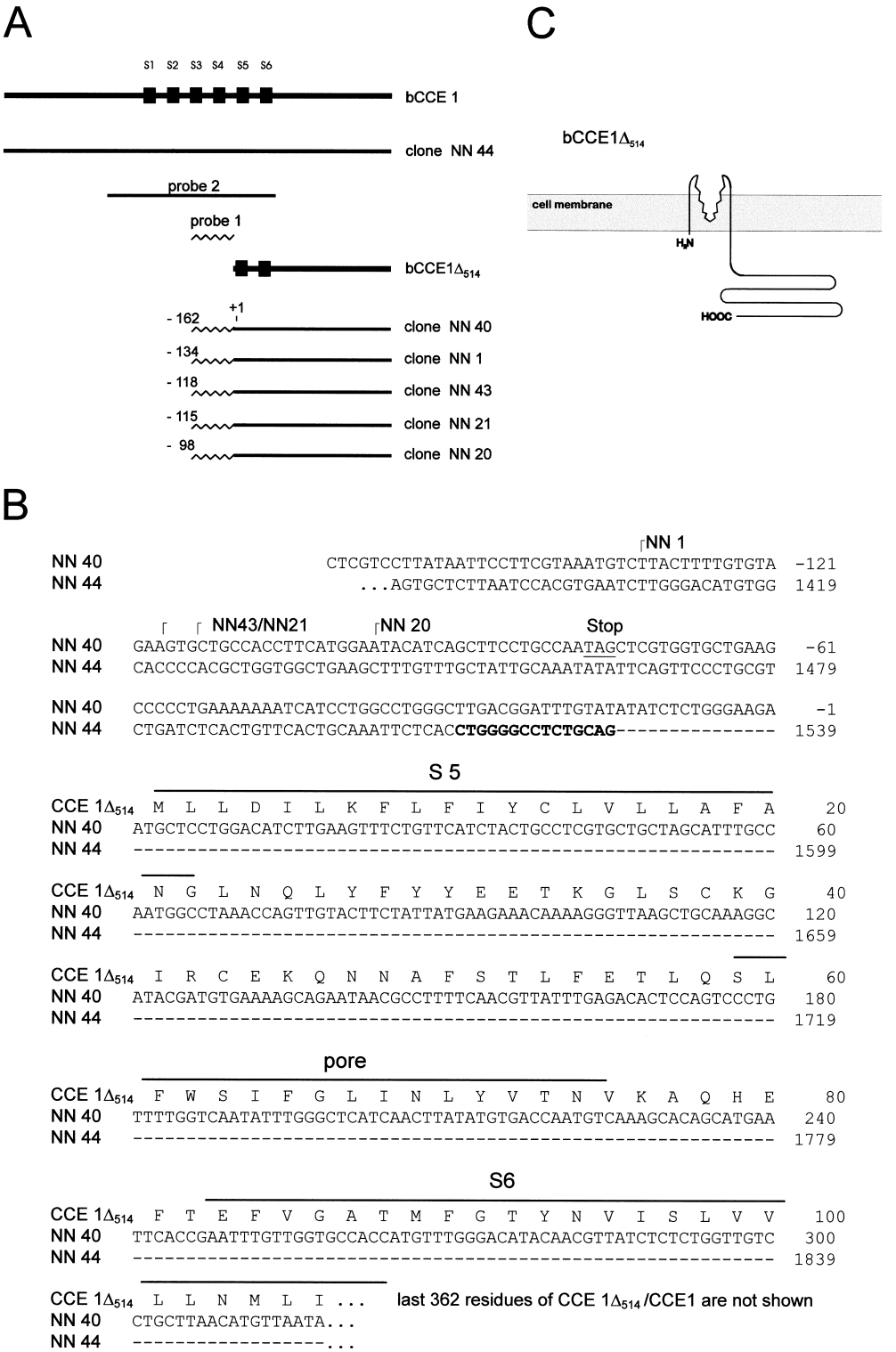

Fig. 1. Clones obtained after screening a cDNA library from bovine adrenal gland (A), nucleotide and deduced amino acid sequence of the bCCE 1 splicing region (B) and model of secondary structure of bCCE $1\Delta_{514}$. A: The bCCE 1 full-length clone NN44 and the five 5′ truncated splice variants encoding the bCCE $1\Delta_{514}$ protein are shown. Predicted transmembrane domains 1–6 are indicated as S1–S6. The 5′ nucleotides of the five bCCE $1\Delta_{514}$ cDNA clones (NN40, NN1, NN43, NN21, NN20) are indicated corresponding to nucleotide numbers given in B. Positions of probes 1 and 2 used for Northern blot analysis and library screening are shown. B: Nucleotide and deduced amino acid sequence of the 5′ truncated splice variant bCCE$1\Delta_{514}$ aligned to the bCCE 1 nucleotide sequence in the splicing region. Identities are given as -. Nulceotides 5′ of nt 1525 of NN44 matching the consensus sequence of a intron-exon junction are in bold letters and a stop codon in frame of the triplet encoding the initiation methionine of bCCE $1\Delta_{514}$ is underlined. C: Membrane topology of bCCE $1\Delta_{514}$. Start methionine of bCCE $1\Delta_{514}$ corresponds to M^{514} of bCCE 1 which is the first amino acid residue of the transmembrane domain 5 of bCCE 1 according to hydropathy analysis [5].

ng of clone NN40 cDNA as template. 30 cycles (94°C, 1 min; 58°C, 1 min; 72°C, 1.5 min) were performed with a programmable thermocycler. An amplification product of the expected size (147 bp) was amplified.

Tissue distribution of bCCE $1\Delta_{514}$ was examined using the bCCE $1\Delta_{514}$ specific sense primer 37 (5′-TTG TGT AGA AGT GCT GCC ACC-3′) and the bCCE 1/bCCE $1\Delta_{514}$ common primer 38 (5′-CAA CTG GTT TAG GCC ATT GGC-3′) corresponding to nucleotides −127 to −107 and 78–58 of bCCE $1\Delta_{514}$. As a template we used cDNA prepared by oligo(dT)-primed reverse transcription of poly-$(A)^+$ RNA isolated from the bovine tissues indicated. Twenty-five cycles (94°C, 1 min; 62°C, 1 min; 72°C, 1 min) were performed and one tenth of the PCR mixture was electrophoresed on a 7% polyacrylamide gel. As a positive control we amplified a PCR product using GAPDH specific primers (sense 5′-CTG GTC ACC AGG GCT GCT T-3′, antisense 5′-CGA CGT ACT CAG CGC CAG CAT-3′) from all tissues examined.

2.4. Genomic analysis

Primer 1 (5′-CTG CAG ATA TCT CTG GGA AGG-3′) and primer 9 (5′-CGT GGA AAA CGC GTT GTT CTG-3′) were deduced from a CCE 1 homologous cDNA fragment which was amplified from mouse brain [17]. These primers were used for amplification of fragments from the murine CCE 1 gene and primer 21 (5′-ATA TAT TCA GTT CCC TGC-3′) and primer 16 (5′-GGT TTA GGC CAT TGG CA-3′) corresponding to the peptides $A^{533}NGLN^{537}$ and $N^{487}IFSSL^{492}$ of bCCE 1 [9] for amplification of fragments from the bovine CCE 1 gene. Genomic DNA was prepared from bovine and mouse liver as described [18]. DNA fragments obtained with primer pairs 1/9 and 21/16 were 177 and 153 bp in length, respectively. Blunt end PCR products were subcloned into pUC18 and sequenced on both strands.

Using primer pair 1/9 a 129 SvJ murine genomic library in P1 phage (Genome Systems, St. Louis, MO) was screened by polymerase chain reaction. Three clones (P-25, P-242 and P-337) were isolated and subjected to Southern blot analysis using the 177 bp genomic fragment amplified with primer pair 1/9 as a probe. Hybridizing fragments of P1 clones were subcloned and sequenced.

2.5. Southern blot analysis

P1 clones digested with different restriction enzymes were electrophoresed on a 0.6% agarose gel and transferred to nylon membranes (Hybond-N). Hybridization was carried out in 0.9 M NaCl, 90 mM sodium citrate, 5×Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA for 12 h at 63°C. After hybridization, membranes were washed in 30 mM NaCl, 3 mM sodium citrate and 0.1% SDS at 68°C for 30 min.

3. Results

3.1. Identification of the 5′ truncated bCCE 1 splice variant, bCCE $1\Delta_{514}$

Screening of a oligo(dT) primed cDNA library from bovine adrenal glands with a fragment of the bCCE 1 cDNA (probe 2, Fig. 1A) yielded two different groups of clones. Clone NN44 contains 3418 bp with an open reading frame encoding 981 amino acids (bCCE 1) and is alternatively spliced upstream of the ATG triplet encoding the initiation methionine of the bCCE 1 cDNA (X 99792) originally cloned from retina. Expression of this bCCE 1 cDNA in human embryonic kidney cells induced capacitative calcium entry [9]. In addition to the full-length clone, five independent clones were obtained comprising 1772–1836 base pairs. Sequence analysis revealed that these cDNAs are identical to the 3′ portion of the bCCE 1 cDNA starting from nucleotide 1525, but lack nucleotides 1–1524 of bCCE 1 which are replaced by 83 (NN20), 100 (NN21), 103 (NN43) 119 (NN1) or 147 nucleotides (NN40) in individual clones, possibly resulting from different transcription by the reverse transcriptase during first strand synthesis. The first ATG triplet found in frame downstream of a stop codon at position −78, which is common to all five clones, leads to a protein which represents a truncated version of bCCE 1, bCCE $1\Delta_{514}$, comprising 468 amino acids with a calculated relative molecular mass of 53 503. The initiation methionine of this protein corresponds to M^{514} of bCCE 1, and according to the postulated transmembrane topology of bCCE 1, bCCE $1\Delta_{514}$ comprises two putative transmembrane segments corresponding to the predicted transmembrane segments 5 and 6 of bCCE 1 and the putative pore forming region in between (Fig. 1C).

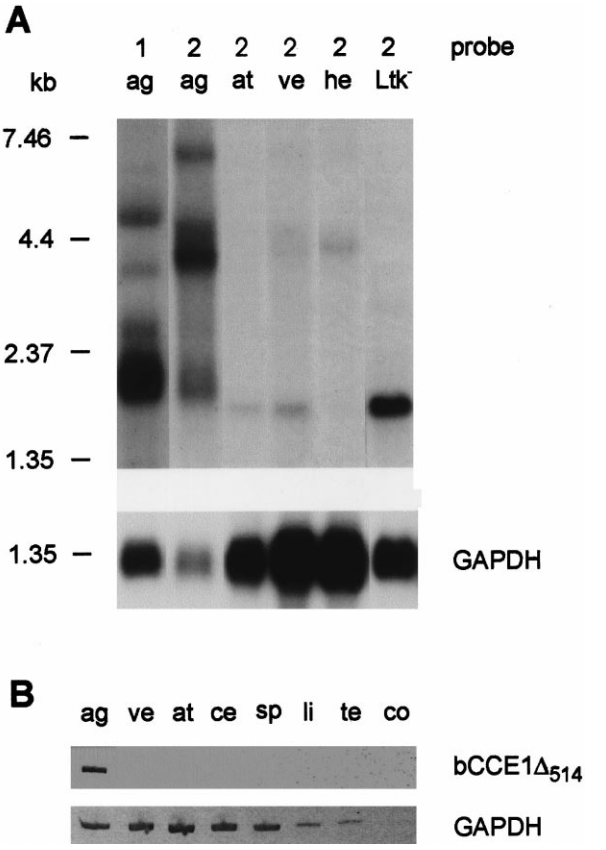

Fig. 2. Analysis of tissue distribution of bCCE 1 and bCCE $1\Delta_{514}$ mRNA using Northern blot analysis (A) and RT-PCR (B). A: Poly$(A)^+$ RNAs from bovine adrenal gland (ag, 16 μg), atrium (at, 20 μg), ventricle (ve, 20 μg), rabbit heart (he, 15 μg) and mouse fibroblasts (Ltk^-, 15 μg) were hybridized with one probe specific for bCCE $1\Delta_{514}$ (probe 1) and one derived from bCCE 1 which covers part of bCCE $1\Delta_{514}$ (probe 2, Fig. 1A). Size markers are indicated (upper panel). The lower panel shows control hybridization with a GAPDH probe. Autoradiograms were digitized, processed with the aid of Corel Photo-paint software and printed with a HP Photo Smart printer. B: Revers transcribed poly$(A)^+$ RNA from bovine ventricle (ve), atrium (at), cerebellum (ce), spleen (sp), liver (li) and testis (te) was used as template for PCR with a bCCE $1\Delta_{514}$ specific (upper panel) and GAPDH specific (lower panel) primer pair. Amplification products of 212 and 238 bp, respectively, were obtained. Control reactions devoid of template (co) are shown.

3.2. bCCE $1\Delta_{514}$ is specifically expressed in the bovine adrenal glands

The tissue distribution of bCCE 1 and bCCE $1\Delta_{514}$ was examined by both Northern blot analysis (Fig. 2A) and RT-PCR (Fig. 2B). We used two different cDNA probes, one specific for bCCE $1\Delta_{514}$ (probe 1, Fig. 1A) and one derived from bCCE 1 which also covers part of bCCE $1\Delta_{514}$, and

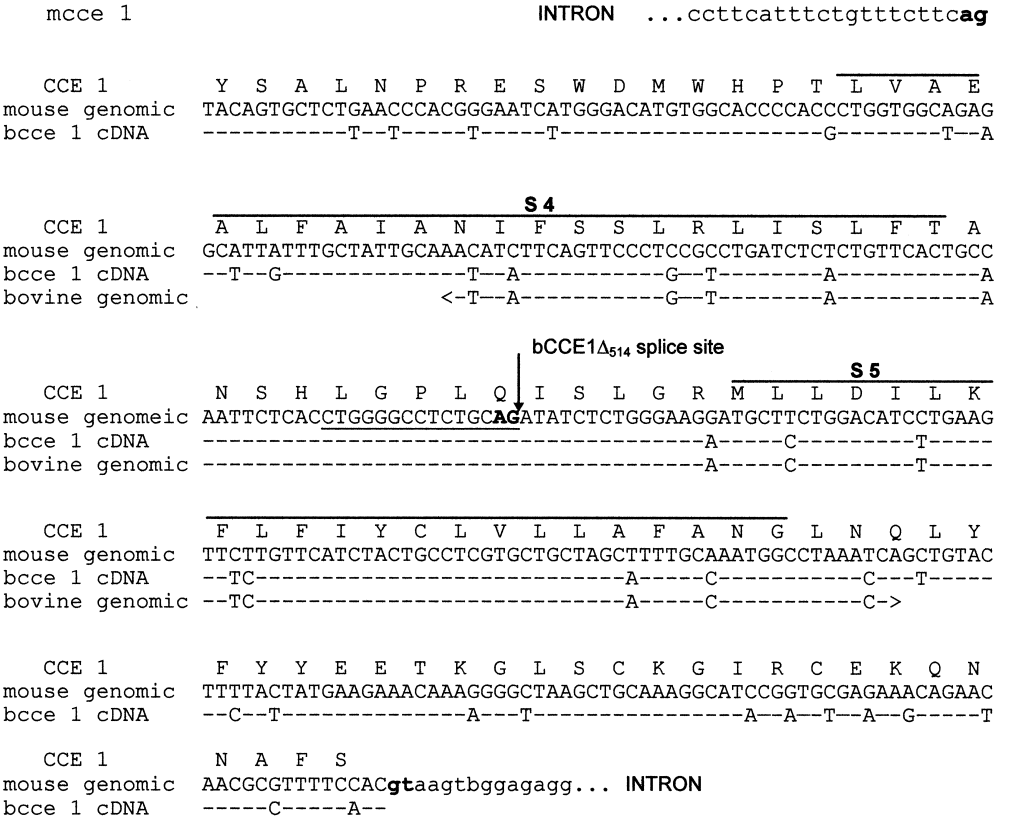

Fig. 3. A: Genomic nucleotide and derived amino acid sequences of the translated exon encoding transmembrane domains 4 and 5 of the mouse CCE 1 gene (mouse genomic) aligned with the corresponding bovine cDNA (bCCE 1 cDNA) and the sequence amplified from bovine genomic DNA (bovine genomic). The 5′ and 3′ splice regions of introns are in lower case. Identical nucleotides are represented by -. Nucleotides matching the 3′ splicing site of an intron are underlined. The translated amino acid sequence is given above the nucleotide sequence of the coding exon in single letter code. bCCE $1\Delta_{514}$ splice site and transmembrane domains are indicated. The limits of the obtained sequences are shown as $< >$.

which was initially used to screen the adrenal gland cDNA library (probe 2, Fig. 1A). mRNA species of 1.9, 3.8, 4.8 and 7.0 kbp length were detected in the adrenal gland with probe 2. Transcripts of similar size were also detected in bovine cardiac ventricle (3.8, 4.8 and 7.0 kbp) and rabbit heart (3.8 kbp) but not in mouse fibroblasts. In addition, probe 2 hybridized to transcripts of ~1.7 kbp in bovine atrium and ventricle and mouse fibroblasts (Ltk^- cells). These ~1.7 kbp transcripts are shorter in length than those seen in the adrenal gland. In contrast to probe 2, probe 1, which should specifically recognize bCCE $1\Delta_{514}$ transcripts, predominantly hybridizes to the 1.9 kbp mRNA species. The size of the 1.9 kbp transcripts is consistent with the size of the cloned bCCE $1\Delta_{514}$ cDNA (1772–1836 nucleotides) indicating that this mRNA species corresponds to the truncated cDNA clones. The identity of the 3.6 and 5.0 kbp mRNA species recognized by probe 1 are not known but may represent alternative splicing products containing sequences related to probe 1 but not to CCE 1.

We further investigated the expression pattern of bCCE $1\Delta_{514}$ by RT-PCR (Fig. 2B). With a sense primer specific for bCCE $1\Delta_{514}$ and an antisense primer derived from a region where bCCE $1\Delta_{514}$ and bCCE 1 sequences are identical, a specific 212 bp product can be amplified only from RNA of the adrenal glands, but not from RNA isolated from six other bovine tissues including heart indicating that the 1.9 kbp species is different from the 1.7 kbp transcripts identified by Northern blot analysis in bovine heart tissues.

3.3. Genomic analysis of the murine and bovine CCE 1 gene indicates an alternative splicing mechanism for the formation of bCCE $1\Delta_{514}$

In order to elucidate a possible mechanism for the formation of bCCE $1\Delta_{514}$ transcripts, we investigated the bCCE 1/bCCE $1\Delta_{514}$ splicing region of the CCE 1 gene. With different sets of primers including primer pair 1/9 (see Section 2), a 177 bp cDNA fragment could be amplified from mouse genomic DNA. The amplified cDNA encodes the predicted transmembrane segment 5 and shares 88.2 and 99% identical nucleotides and amino acids, respectively, with the bovine CCE 1 cDNA, indicating that it represents part of the mouse orthologue of bovine CCE 1. Using the sequence of the mouse cDNA fragment, several clones were obtained from a murine 129 SvJ genomic library. Subcloning of genomic DNA fragments identified by Southern blot analysis with the amplified mouse cDNA fragment revealed that transmembrane domains 4 and 5 are encoded by a common exon (Fig. 3). However, the nucleotide sequence 5′ of nucleotide 1525 of clone NN44 in the bCCE 1 cDNA (Fig. 3, underlined) matches the consensus sequence of the splice acceptor site of an intron-exon junction which is $(C/T)_nN(T/C)\underline{AG}G$ 3′ with $n \geq 11$ [19]. The consensus run of 11 or more pyrimidines may con-

tain as many as six purines and, accordingly, 15 out of 16 nucleotides of the genomic mouse DNA and of the bovine cDNA match this consensus sequence of a 3′ splice region emphasizing that the formation of bCCE $1\Delta_{514}$ clones is due to an alternative splicing event at this position within the S4/S5 exon.

To exclude species differences regarding the structure of the S4/S5 coding exon, a 153 bp fragment of the CCE 1 gene was amplified from bovine liver with primers deduced from the S4 and S5 region of the bovine CCE 1 cDNA. The sequence of the amplified DNA was identical to the corresponding cDNA and contained no additional intron sequences (Fig. 3) demonstrating a identical exon structure of this part of the CCE 1 gene in both mouse and cow.

4. Discussion

This study reports the cloning and expression pattern of bCCE $1\Delta_{514}$, a 5′ truncated splice variant of the recently identified mammalian *trp* homologue bCCE 1 which has been shown to function as a capacitative calcium entry channel [9]. It is shown that bCCE $1\Delta_{514}$ represents an alternatively spliced product of the CCE 1 gene which gives rise to the expression of a $\sim$1.9 kbp transcript.

In contrast to the six transmembrane segments predicted to be present in full-length bCCE 1, bCCE $1\Delta_{514}$ contains only three hydrophobic segments corresponding to transmembrane segments 5 and 6 and the putative pore-forming region in between. This membrane topology is reminiscent to that of the inward rectifier-type K^+ channel (Kir) gene family which also encodes proteins of less than 500 amino acids with two putative transmembrane spanning domains M1 and M2 and a hydrophobic segment in between contributing to ion conducting pore formation [20]. Accordingly, the ion conducting properties of the bCCE 1 protein might still be preserved in bCCE $1\Delta_{514}$, although with different regulation mechanisms arising from the lack of the greater part of the amino-terminus of bCCE 1.

In Northern blot experiments using a bCCE 1 specific probe representing the six transmembrane segments, mRNA transcripts of 1.9 kbp and 1.7 kbp were identified in adrenal gland or heart and fibroblasts, respectively, in addition to mRNA species of 3.8, 4.8, 7.0 kbp in adrenal gland. The 3.8 kbp mRNA species corresponds to the full-length bCCE 1 cDNA ($\sim$3.4 kbp, clone NN44 and [9]) whereas the 4.8 and 7.0 kbp transcripts may result from alternative RNA processing within the 5′ non-coding sequence of bCCE 1 [9]. The 1.9 kbp species is also detected, in even higher abundance in relation to larger transcripts, by a probe specific for bCCE $1\Delta_{514}$, and may represent bCCE $1\Delta_{514}$ transcripts which contain 1772–1836 base pairs. The slightly smaller transcripts of 1.7 kbp detected in heart tissues and fibroblasts may not correspond to bCCE $1\Delta_{514}$ mRNA, because bCCE $1\Delta_{514}$ cDNAs could only be amplified from adrenal gland mRNA but not from other bovine tissues. Thus, expression of bCCE $1\Delta_{514}$ appears to be restricted to the adrenal gland and the 1.7 kbp transcripts present in heart and fibroblasts (LTK^- cells) may arise from alternative mRNA processing of the CCE 1 gene or from genes related to bCCE 1.

Genomic sequences isolated in order to analyze the murine CCE 1 gene revealed that transmembrane domains 4 and 5 are encoded by a common exon. It is shown that this exon contains a consensus sequence of a 3′ splice region of an intron which might be used for alternative splicing in adrenal gland leading to the formation of truncated bCCE 1 transcripts. The isolation of five independent bCCE $1\Delta_{514}$ cDNA clones from an oligo(dT)-primed adrenal gland cDNA library make this proposed splicing event of CCE 1 RNA a conceivable mechanism for the formation of bCCE $1\Delta_{514}$ transcripts.

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