A truncated isoform of Ca^{2+}/calmodulin-dependent protein kinase II expressed in human islets of Langerhans may result from trans-splicing

Maria A. Breen, Stephen J.H. Ashcroft *

Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

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Abstract Calcium/calmodulin-dependent protein kinase II (CaM kinase II) has been proposed to play a key role in glucose stimulated insulin secretion. Using the rapid amplification of cDNA ends technique we amplified the 3′ end of the CaM kinase II γ gene from human islet RNA. A novel cDNA was detected composed of 5′ sequence from the human CaM kinase II γ gene joined to the 3′ end of the human signal recognition particle 72 (SRP72) gene. We predict that this mRNA species will code for a truncated form of CaM kinase II, designated γ_{SRP}, comprising the entire catalytic and regulatory domains of the protein and with a predicted molecular weight of 37 kDa. We mapped the human SRP72 gene to chromosome 18 and, as the CaM kinase II γ gene was previously mapped to human chromosome 10q22, we suggest this novel cDNA may have resulted from trans-splicing.

Key words: CaM kinase II; Signal recognition particle; Trans-splicing; Pancreatic β-cell; Human islets of Langerhans; Protein phosphorylation

1. Introduction

It is well established that a rise in intracellular free Ca^{2+} plays a key role in glucose-stimulated insulin secretion [1–3] but the cellular events linking increased Ca^{2+} concentration to the release of insulin are unknown. One possible mediator is calcium/calmodulin-dependent protein kinase II (CaM kinase II) [4]. CaM kinase II is a ubiquitous serine/threonine protein kinase which has been implicated in diverse effects of hormones and neurotransmitters that utilise Ca^{2+} as a second messenger, ranging from muscle contraction, secretion, synaptic transmission to gene expression (for review see [5]). The enzyme is an oligomeric protein of M_r 500–600 kDa composed of distinct but related subunits arranged in a hub-and-spoke pattern and comprises a multigene family in which each of the isoforms (α, β, γ, and δ) is encoded by a separate gene. Cells can contain more than a single isoform of the kinase, and it is likely that both homomultimers and heteromultimers of the kinase exist. Alternatively spliced variants of each subunit, differing in insertions or deletions within the variable and association domains of the enzyme, are expressed in different tissues [6,7], suggesting tissue-specific functions for each isoform or combination of isoforms.

Evidence for the involvement of a CaM kinase in insulin secretion has been obtained by studies in which the inhibition of insulin release by the diabetogenic agents alloxan [8] and dehydrouramil [9] was correlated with the inhibition of β-cell CaM kinase activity. Insulin release has also been shown to be inhibited by the CaM kinase II inhibitor KN-62 [10,11]. These findings are however confused by additional effects of alloxan and KN-62 on glucokinase [12] and Ca^{2+}-channel activity [11], respectively. More convincing evidence was provided by the patch clamp technique; a specific peptide inhibitor of CaM kinase II (residues 290–309) markedly reduced insulin secretion from a single cell determined by capacitance measurements without affecting the Ca^{2+} current [13]. Furthermore, glucose has been shown to activate CaM kinase II in isolated rat islets and the extent of activation correlates closely with insulin secretion [14,15].

Western analysis and cDNA cloning have demonstrated the expression of a number of different isoforms of CaM kinase II in rodent islets and β-cell lines [6,15–18]. We have previously reported the cloning of a partial cDNA encoding the γ isoform of CaM kinase II from human islets [19]. In this study we isolated the 3′ end of the γ CaM kinase II gene from human islet RNA using the rapid amplification of cDNA ends (RACE) technique and demonstrate the expression of a novel truncated isoform of CaM kinase II in human islets of Langerhans

2. Materials and methods

2.1. Materials

The human islets were supplied by Dr. Derek Grey, Islet Transplantation Laboratory, Churchill Hospital, Oxford and the human islet cDNA library by Dr. Hiroshi Sakura, University Laboratory of Physiology, Oxford. The somatic cell hybrid mapping panel was supplied by the Human Genome Mapping Project (HGMP) resource centre, Hinxton Hall, Cambridge. Nucleotide sequencing was carried out using the dideoxy chain termination method with the Sequenase version 2.0 system (Amersham Life Sciences) on both strands. Taq DNA polymerase was purchased from Perkin Elmer and all other reagents were from Promega or Sigma except where specified otherwise.

2.2. Rapid amplification of cDNA ends

Amplification of the 3′ end of the human CaM kinase II gene from human islet RNA was carried out using Clontech's 3′ AmpliFINDER RACE system. Single stranded cDNA was prepared from 25 ng of poly A^- RNA following the manufacturer's instructions and one twentieth of this reaction was used as the target for two rounds of nested PCR. The nucleotide sequences of the primers used were derived from the CaM kinase II γ cDNA partial sequences previously isolated from a human islet cDNA library [19] (GenBank accession No. U66064). Primer 1 (P1) (5′-AAGCGCATCACGGCTGACCAGGCTC-3′) corresponded to nucleotides 817–836 and primer 2 (P2) (5′-TCCACGGTGGCATCCATGAT-3′) to nucleotides 878–897 of the library clone. Thirty-two cycles of amplification were carried out using 1μl (5 units) of Taq DNA polymerase for each of four independent RACE reactions. Each cycle consisted of 60 s denaturing

*Corresponding author. Fax (44) (1865) 221 834.

Abbreviations: CaM kinase II, calcium/calmodulin-dependent protein kinase II; RACE, rapid amplification of cDNA ends; SRP72, the gene coding for the 72-kDa protein of the signal recognition particle

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at 95°C, 120 s annealing at 60°C and 180 s extension at 72°C. One μl of the primary PCR reaction was used as the target for the secondary PCR reaction.

2.3. Polymerase chain reaction (PCR)

PCR primers were designed that would amplify a region of the CaM kinase II γ_{SRP} isoform (see below), spanning the junction of the human SRP72 sequence and the human CaM kinase II sequence (sense primer = 5′-GAGAAAACTGAAGGGTGCCA-3′, antisense primer = 5′-TAACTCCTCTATGCTTCTC-3′). Thirty-two cycles of amplification were carried out with one unit of Taq DNA polymerase per reaction. The samples were boiled for 5 min to disrupt the phage particles before addition of the Taq DNA polymerase. Each cycle consisted of 1 min denaturing at 95°C, 2 min annealing at 52°C and 3 min extension at 72°C and a final 10 min extension at 72°C. Either 1μl or 0.5μl of undiluted cDNA library was used as the target for the PCR reactions and all reactions were carried out in duplicate.

2.4. Genetic mapping

A PCR-based assay was developed using primers M1 (5′-AAGATATTCACACCCTGGCACAGC-3′) and M2 (5′-CACCAGCAGAATTTTCAAGAGCC-3′) that detected the presence of the human SRP72 gene in an inter-species somatic cell hybrid panel. Reactions were carried out in a 40μl reaction volume with 33 cycles of amplification, each consisted of 1 min denaturing at 95°C, 2 min annealing at 62°C and 3 min extension at 72°C and a final 10 min extension at 72°C. The PCR products amplified were analysed by electrophoresis in 4% agarose gels and visualised using ethidium bromide staining. All reactions were carried out in triplicate.

2.5. Western analysis

50 μg samples of human islet protein were subjected to SDS-polyacrylamide gel electophoresis through a 7.5% acrylamide gel for 90 min at 150 V. The protein was transferred from the gel to a polyvinylidene difluoride membrane using a semi-dry electroblotter. Incubation of the membrane with a CaM kinase II specific antibody was carried out overnight at 4°C. The presence of CaM kinase II protein on the membrane was then detected using the ECL chemiluminescence system (Amersham Life Sciences) according to the manufacturer's instructions. The primary antibody used was a rabbit polyclonal IgG (Upstate Biotechnology, Lake Placid, USA) raised against two epitopes; (1) sequence = CTRTDEYQLFEEL, (residues 7–20 of rat CaM kinase II δ isoform, and (2) sequence = EETRVWHRRDGKWQNVHFHC (residues 514–533 of rat CaM kinase II β isoform). The amino acid sequences of these regions of CaM kinase II are 70–90% homologous to corresponding regions in the isoforms of CaM kinase II (γ and β3) expressed in human islets [20].

3. Results

3.1. Cloning of a novel isoform of human islet CaM kinase II

By screening a human islet cDNA library we previously isolated a partial cDNA clone encoding the γ isoform of CaM kinase II [19]. To obtain full length sequence we now have used RACE to amplify the 3′ end of the human CaM kinase II γ gene from human islet RNA. Full length cDNA sequences were then deduced from the overlapping sequences of the RACE products and the partial cDNA clone. Two sequences were identified as the γ_B [7] and γ_E [21] isoforms of CaM kinase II. The 5′ sequence of a third RACE product comprised 164 bp which were 99.5% homologous to the sequence of all known γ isoforms of human CaM kinase II. A single base pair variation was observed at position 956 resulting in an amino acid change of Ser (AGC) to Asn (AAC). The remaining part of the sequence (804 bp) was 93.9% homologous to a region of the canine gene encoding signal recognition particle 72 (SRP72; GenBank accession No. X67813) and contained sequence 99.4% homologous to a partial cDNA (483 bp) encoding the human SRP72 (GenBank accession No. N55583). The deduced full length cDNA sequence, shown in Fig. 1, is predicted to code for a truncated form of CAM kinase II of 330 amino acids with a molecular mass of 37 kDa. This novel isoform of CaM kinase II which we designate γ_{SRP} contains the catalytic and regulatory regions of γ CaM kinase II plus an additional 6 novel amino acids before a stop codon is reached. Homology searches were performed for this new region of CaM kinase II (RNNYKL); neither BLAST nor Motifs searches revealed any significant homology to any known protein motifs.

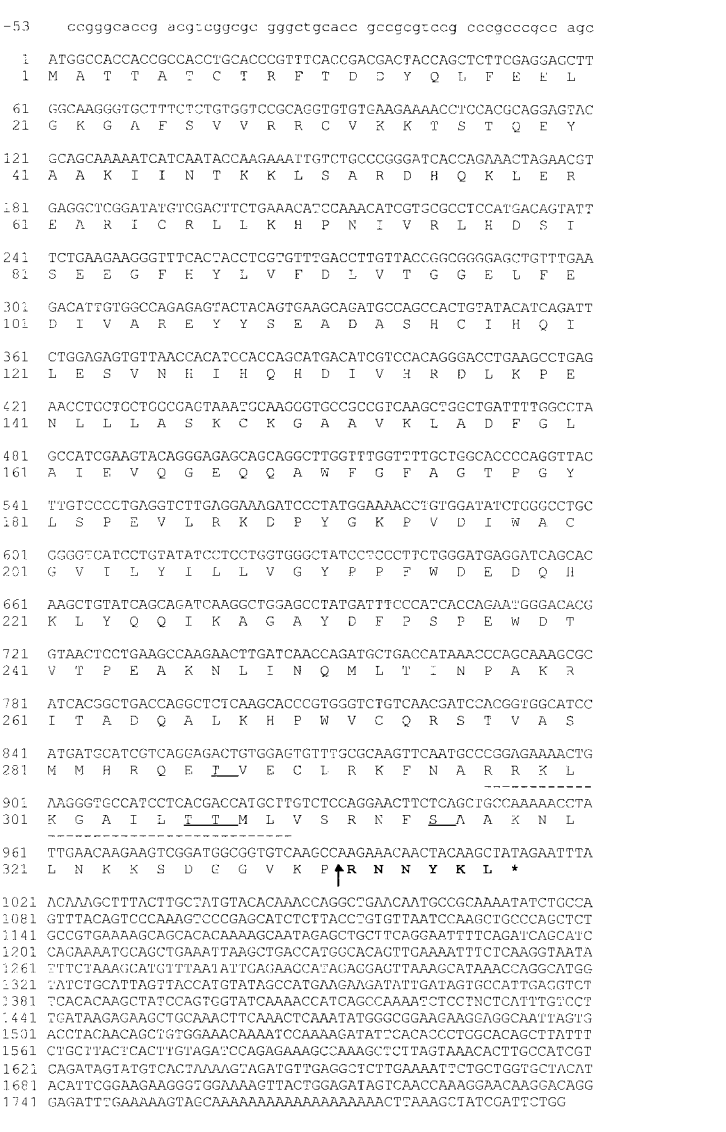

Fig. 1. Nucleotide sequence of the human γ_{SRP} isoform of CaM kinase II. The position where the sequence of the human γ CaM kinase II gene ends and the sequence of the human SRP72 gene begins is indicated by an arrow. Amino acids with solid underline are predicted autophosphorylation sites based on similarity to α CaM kinase II and the calmodulin binding site is underlined in dashes [7].

The following criteria were used to establish that the RACE products we obtained were genuine. (1) They were generated from four independent reactions. (2) They were primed from the expected position in the RNA (site at which primer P2 binds, see Section 2). (3) Their sequences overlapped that of the library clone previously obtained (see above). (4) They contained a poly-A tail plus the sequence of the anchor primer.

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[RACE]  104 TCTCCAGGAACTTCTCAGCTGCCAAAAACCTATTGAACAAGAAGTCGGATGGCGGT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
[PCR]     1 TCTCCAGGAACTTCTCAGCTGCCAAAAACCTATTGAACAAGAAGTCGGATGGCGGT

                  ↓
GTCAAGCCAAGAAACAACTACAAGCTATAGAATTTAACAAAGCTTTACTTGCTATGTACACAAACCAG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GTCAAGCCAAGAAACAACTACAAGCTATAGAATTTAACAAAGCTTTACTTGCTATGTACACAAACCAG

GCTGAACAATGCCGC  242
|||||||||||||||
GCTGAACAATGCCGC  139
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Fig. 2. Comparison of the nucleotide sequence of the γ_{SRP} RACE product with that of the PCR product amplified from a human islet cDNA library. The part of the sequence spanning the junction between the γ CaM kinase II sequence and the SRP72 sequence is shown and the junction indicated by an arrow.

The sequence of the novel RACE product was submitted to GenBank and assigned the accession No. U81554.

3.2. PCR

The polymerase chain reaction was used to confirm the expression of a γ_{SRP}-like cDNA species in the human islet cDNA library. A band of the expected size (434 bp) was amplified. This band was isolated and subcloned and plasmids containing inserts of the expected size were identified by restriction enzyme digestion and sequenced. The nucleotide sequence of these clones was 100% identical to the nucleotide sequence of the clone isolated by the RACE method (Fig. 2).

3.3. Genetic mapping

In an attempt to elucidate if the γ_{SRP} cDNA was produced by alternative splicing of a single large transcript from one gene or by some other method, we mapped the human SRP72 gene. A monochromosomal hybrid mapping panel was used to map the γ_{SRP} gene. The basis of the mapping strategy was that SRP72 specific primers MI and M2 would amplify bands of different sizes from human and rodent DNA, allowing us to determine if the gene coding for SRP72 was present in the human DNA contained within each hybrid. As each hybrid contained the DNA from only one human chromosome, we could determine which human chromosome contained the gene coding for SRP72. Primers MI and M2 amplified a single band of size 144 bp from total human genomic DNA (containing all chromosomes), a much larger band from total hamster genomic DNA and no product from total mouse genomic DNA (lanes 1, 2 and 3 in Fig. 3). In Fig. 3, lanes f, g, l–p, t–v and w contain PCR reactions carried out with hybrids containing DNA from individual human chromosomes 6, 7, 12–16, 20–22 and X, respectively, on a mouse genetic background. Using primers MI and M2 no product was amplified from these samples (Fig. 3). Lanes a–e, h–k, q, s, t and x (Fig. 3) contained PCR product amplified from hybrids containing DNA from individual human chromosome 1–5, 8–11, 17, 19–20 and Y, respectively, on a hamster genetic background. Using primers MI and M2 a single band corresponding in size to the hamster genetic background was amplified from these hybrids. Lane j contained DNA from human chromosome 10 only, on a hamster genetic background. As only one band corresponding in size to the hamster genetic background was amplified from this hybrid and no band corresponding in size to the human gene (144 bp), we conclude that the gene encoding SRP72 is not present on human chromosome 10. Lane DL18TS (Fig. 3) contained the PCR product from a reaction carried out with hybrid DL18TS. This hybrid contained the DNA from human chromosome 18 on a hamster genetic background [22]. Two bands were amplified from hybrid DL18TS – a band of the size expected for the human SRP72 gene and a band of the size expected for the hamster gene. It was concluded that the human SRP72 gene is located on human chromosome 18.

3.4. Western analysis

Although we had established the expression of a γ_{SRP} cDNA in human islets we did not know if this cDNA was stable and if it would be transcribed to produce a protein product. On Western analysis with a CaM kinase II specific antibody and human islet protein, four bands were detected (Fig. 4). The molecular weight of the smallest band was de-

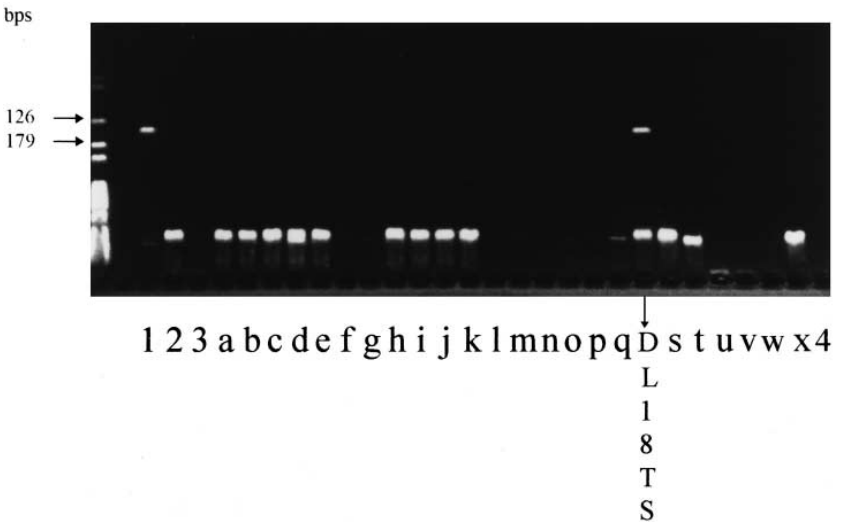

Fig. 3. Genetic mapping of the human SRP72 gene using the monochromosomal hybrid mapping panel. The first three lanes 1, 2 and 3 contain PCR products amplified from the control reactions, 1, human genomic DNA; 2, hamster genomic DNA; and 3, mouse genomic DNA. Lanes a–q and s–v, w and x contain PCR products amplified from the monochromosomal hybrids containing DNA from individual human chromosomes 1–17, 19–22, X and Y, respectively. Lane DL18TS contains the product amplified from hybrid DL18TS. Lane 4 is the negative control containing PCR reaction carried out using no target DNA (water). The sizes (bp) of the nucleic acid markers are indicated on the left.

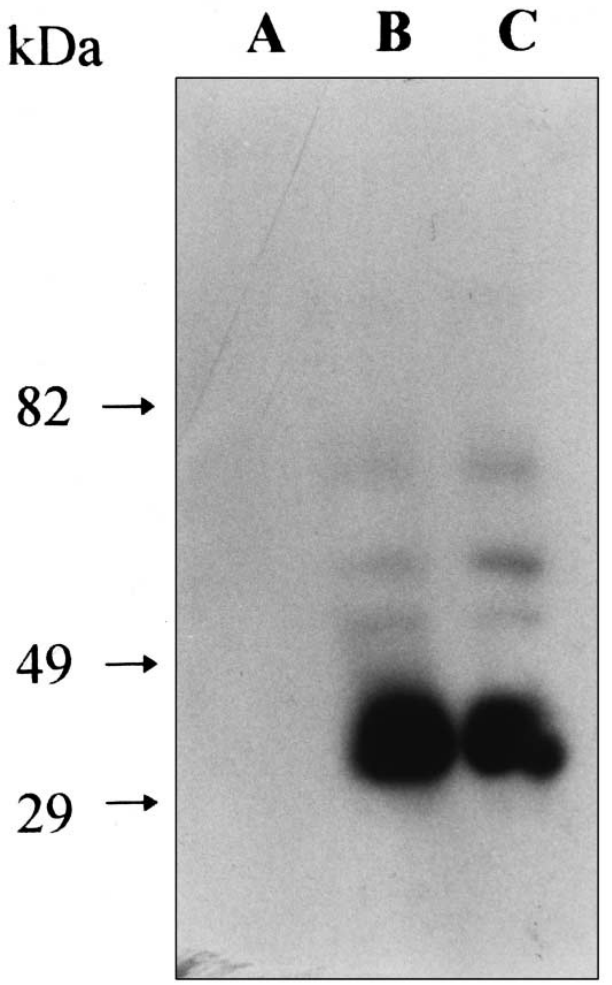

Fig. 4. Western blot of human islet protein using a CaM kinase II specific antibody. Lane A contains a negative control (water) and lanes B and C contain human islet protein. The sizes of the appropriate molecular weight markers are indicated on the left.

termined to be between 29 and 49 kDa by reference to the molecular weight standards used. This band was identified as a γ_{SRP}-like isoform of CaM kinase II, based on the following criteria: (1) it was immunoreactive with the CaM kinase II specific antibody; (2) its apparent molecular weight was smaller than that of any other isoform of CaM kinase II thus far identified; and (3) it was within the range of the predicted molecular weight of γ_{SRP} (37 kDa). The gel was slightly overloaded in order to show the presence of other full length isoforms of CaM kinase II in human islets. Three further bands observed in the size range 49–82 kDa are thought to represent the β3 (65 kDa), γ_E (61 kDa) and γ_B (57 kDa) isoforms of CaM kinase II also shown to be present in human islets [20].

4. Discussion

All isoforms of CaM kinase II can be divided into three functional domains – an N-terminal catalytic region, and a C-terminal association domain flank an intervening variable region [5]. The association domain may not have a catalytic or regulatory role but is thought to play a part in holoenzyme formation [23]. The novel truncated isoform (γ_{SRP}) of CaM kinase II demonstrated here in human islets of Langerhans is predicted to contain the catalytic and regulatory domains, the calmodulin-binding site and the auto-inhibitory domain of the kinase (Fig. 1). Therefore we expect CaM kinase II γ_{SRP} to have similar catalytic properties and be regulated in the same manner as the holoenzyme. However as this isoform lacks an association domain it is likely to exist as a monomer. Consistent with these ideas is the fact that a 30-kDa active fragment of CaM kinase II produced by chymotryptic digestion exists as a monomer and phosphorylates synapsin I at the same site as does the native CaM kinase II [23]. Expression studies will be needed to confirm these predictions and to assess the function of the 6 amino acids (RNNYKL) in CaM kinase II γ_{SRP} derived from the SRP72 gene. The expression of a truncated CaM kinase II may have important functional consequences for targeting the enzyme.

Using a rodent-human somatic cell hybrid panel the human SRP72 gene was mapped to human chromosome 18. The CaM kinase II γ gene has previously been mapped to human chromosome 10 [24]. Thus we conclude that the γ_{SRP} cDNA could not have resulted from the alternative splicing of a single large transcript from one chromosome. It may however have resulted from a chromosomal translocation event or more probably from trans-splicing. The γ_B and γ_C isoforms of CaM kinase II differ from each other by an insertion/deletion of 69 base pairs at position 1012 (γ_B) [7]. As these two isoforms of the kinase are thought to result from alternative splicing of a single RNA transcript, the nucleotides close to position 1012 of γ_B may represent a splice junction. The SRP72 gene is joined to the CaM kinase II gene at a position corresponding to nucleotide 1014 in γ_B and therefore may be at a splice junction. Although in both cases the consensus exon/intron boundary sequence can be identified ($^{C}_{A}$AG/GT$^{A}_{G}$AGT), the consensus sequence for an intron/exon boundary (($^{T}_{C}$)$_n$N$^{C}_{T}$AG/G) can not [25]. The intron/exon structure of neither the SRP72 gene nor the CaM kinase II γ gene has been elucidated. The identification of the splice donor and acceptor sites of the CaM kinase II and SRP72 genes will need to be investigated, in order to confirm that the γ_{SRP} fusion RNA expressed in human islets is formed by trans-splicing.

Multiple isoforms of CaM kinase II are expressed in different tissue types. These various isoforms differ in insertions and deletions within the variable and association domains of the protein and are thought to be produced by alternative splicing of single RNA transcripts [6,7]. The present study suggests that trans-splicing may represent an alternative process by which isoforms of CaM kinase II may be produced in normal adult tissue. To date the expression of cDNA produced by trans-splicing appears to be limited to proteins of undetermined function in diseased human adult tissue [26], transgenic mice [27] or rat fetal tissues [28]. We believe that γ_{SRP} may represent the first example of trans-splicing producing a potentially functional protein in normal adult human tissue.

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