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Characterization of the mRNAs encoding the gene 2 sarcoplasmic/endoplasmic-reticulum Ca^{2+} pump in pig smooth muscle

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The gene 2 sarcoplasmic/endoplasmic-reticulum (SR/ER) Ca^{2+} pump is expressed in slow skeletal and cardiac muscle, smooth muscle and non-muscle tissues. We have analysed the gene 2 Ca^{2+} pump mRNAs using a panel of anti-sense RNA probes which recognize either the muscle (class 1) or the non-muscle (class 2) transcript, or both. In pig smooth muscle, we confirmed the presence of the class 1 and class 2 mRNAs of 4.4 kb length and we also detected a third mRNA of 8.0 kb which reacted with both the class 1 and class 2 riboprobes. A 4.2 kb cDNA corresponding to the 3′ part of the 8.0 kb mRNA was cloned from a pig gastric smooth muscle cDNA library. Nucleotide sequence analysis of this clone revealed that the 8.0 kb mRNA (class 3 transcript) contained both the non-muscle-specific and the muscle-specific exons separated by a 2.4 kb intron which has not been removed. The class 3-mRNA-encoded SR/ER Ca^{2+} pump is identical to the class 2-encoded non-muscle isoform. Northern blot analysis demonstrated that, in cardiac muscle, the class 1 mRNA (encoding the muscle isoform) is the predominant messenger, whereas in non-muscle tissues the class 2 and 3 mRNAs (encoding the non-muscle isoform) predominate. In smooth muscle all three mRNA types are present. The tissue distribution of the mRNA types suggests a tissue-dependent processing of the primary transcript of the sarcoplasmic/endoplasmic reticulum Ca^{2+} pump gene 2.

INTRODUCTION

Various cellular responses such as contraction, secretion and metabolic processes are triggered, at least partially, by the release of Ca^{2+} from an intracellular store [1]. In both muscle and non-muscle tissues, this Ca^{2+} store must be equipped with a Ca^{2+} transport system which refills the store after it has been emptied. This store also damps the cellular response by lowering the cytosolic Ca^{2+} concentration. Ca^{2+} pumps belonging to the class of the 'E1–E2'-type cation transport ATPases have indeed been described in the sarcoplasmic reticulum (SR) of skeletal and cardiac muscle and in the endoplasmic reticulum (ER) of smooth muscle and non-muscle tissues [2].

Genetic analysis has identified three different genes for the SR/ER Ca^{2+} pump [3–14]. The expression of these genes is regulated at both the transcriptional and the post-transcriptional level. Gene 1 is expressed exclusively in fast skeletal muscle and its pre-mRNA is alternatively spliced in a developmentally regulated way resulting in either a neonatal or an adult isoform [3–6]. Gene 2 is expressed in slow skeletal muscle, cardiac muscle, smooth muscle and non-muscle tissues [3,6–13]. Tissue-dependent alternative splicing of the gene 2 transcript results either in a muscle isoform, present in slow skeletal and cardiac muscle, or in a 'non-muscle' isoform which was originally detected in non-muscle tissue [3,6–8]. In smooth muscle, cDNAs encoding the muscle isoform (class 1 cDNA) as well as cDNAs for the non-muscle isoform (class 2 cDNA) have been detected [9–11]. Finally, the gene 3 SR/ER Ca^{2+} pump is expressed in muscle and in non-muscle tissues [14].

We have now analysed the different smooth-muscle mRNAs for the gene 2 SR/ER Ca^{2+} pump in further detail. This has led to the cloning of a third SR/ER Ca^{2+} pump messenger. Furthermore, we demonstrate that cardiac muscle, smooth muscle and non-muscle tissues contain different sets of the SR/ER Ca^{2+} pump gene 2 mRNAs, illustrating that the processing of the gene 2 transcript is tissue-dependent.

MATERIALS AND METHODS

cDNA library synthesis and screening

The synthesis of a λgt 11 cDNA library derived from pig stomach (antral part) smooth muscle has been described previously [9]. Probes specific either for class 1 cDNA (the 3′-terminal *Sph*I–*Eco*RI fragment nt 3385–3781, of class 1 cDNA [9]) or for class 2 cDNA (the 3′-terminal *Xba*I–*Eco*RI fragment, nt 3250–3959, of class 2 cDNA [9]) were nick-translated [15] with [α-^{32}P]dCTP (400 Ci/mmol) up to a specific radioactivity of 10^8 c.p.m./μg of DNA. Filters were prehybridized, hydribized and washed as described before [9].

Sequencing procedures

Clone ER-15 was plaque-purified and subcloned in pGEM-Z7f(+) (Promega, Madison, WI, U.S.A.). Sets of overlapping clones were generated by progressive deletion with ExonucleaseIII/S1 Nuclease [16] using the

Abbreviations used: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; nt, nucleotide(s); aa, amino acid(s).

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X16951.

Erase-a-Base kit (Promega). Sequence analysis was performed on double-stranded plasmid DNA [17] using the Sequenase method [18] (United States Biochemical Corporation, Cleveland, OH, U.S.A.). The ER-15 clone was sequenced in both directions except for the first 300 nt, which were only determined in the sense direction.

RNA isolation

Pig gastric smooth muscle (antral part) was prepared as described previously. Pig ileal longitudinal smooth muscle was obtained by selectively stripping the outer longitudinal smooth-muscle layer from the rest of the ileum. Pig liver, kidney and heart total RNA and pig ileal and gastric smooth-muscle total RNA were prepared according to the Chirgwin procedure [19]. Poly(A)$^+$-enriched RNA was prepared with oligo(dT)–cellulose [20] using a spun-column procedure (Pharmacia LKB Biotechnology, Uppsala, Sweden). For Northern blots, only one round of oligo(dT) purification was performed. This results in a RNA fraction which consists of approx. 50% of poly(A)$^+$ RNA.

Northern blotting

Anti-sense RNA probes were used for Northern blot analysis [21]. For construction of transcription vectors, the following restriction fragments were subcloned in pGEM-7Z(f)+ : pER, the 5′ *Eco*RI–*Pst*I fragment of clone ER-6 (nt 62–1093) of class 1 and 2 cDNAs [9]; pER-1, *Dra*I–*Sph*I (nt 3182–3385) of class 1 cDNA [9]; pER-2, *Xba*I–*Sca*I (nt 3250–3611) of class 2 cDNA [9]; pER-3, *Bam*HI–*Dra*III (nt 1120–1539) of clone ER-15. The linearized plasmids were used as templates for transcription *in vitro* following a standard protocol [21] with the exception that 240 μCi of [α-^{32}P]CTP (800 Ci/mmol; 40 mCi/ml) per transcription reaction was used and that the total concentration of CTP was adjusted to 20 μM with unlabelled CTP. SP6 RNA polymerase was used to synthesize pER, pER-2 and pER-3. T7 RNA polymerase was used for pER-1 synthesis. After DNAase digestion, probes were purified with a Sephadex G-50 spun column [22]. RNA probes were labelled with up to 10^9 c.p.m./μg of RNA.

Glyoxylated RNAs were separated on a 1% agarose gel in a 10 mM-sodium phosphate buffer (pH 7.0) which was continuously recirculated [23]. RNAs were transferred on to Hybond-N$^+$ membranes (Amersham International, Amersham, Bucks., U.K.) in 50 mM-NaOH for 3 h. Membranes were prehybridized for 3 h at 65 °C in a solution containing 5 × SSPE, 50% formamide, 5 × Denhardt's solution, 0.1% SDS, 50 μg of denatured salmon sperm DNA/ml and 0.2 mg of yeast tRNA/ml. Afterwards, RNA probes were added to the prehybridization mixture to a final concentration of approx. 10^7 c.p.m./ml and hybridization was performed overnight at 65 °C. Membranes were washed for 2 × 15 min with 1 × SSPE/0.1% SDS at room temperature, for 2 × 15 min with 0.1 × SSPE/0.1% SDS at 65 °C and finally for 2 × 15 min with 0.1 × SSPE at room temperature. A 0.24–9.5 kb RNA ladder (Gibco BRL, Gaithersburg, MD, USA) was used for sizing.

Unless stated otherwise, solutions (SSPE and Denhardt's solution) were of standard composition and experiments were carried out following standard procedures [22].

RESULTS

Screening of a pig smooth-muscle cDNA library had previously resulted in the detection of two distinct transcripts (named class 1 and 2) which were derived from the SR/ER Ca^{2+} pump gene 2 [9]. To study the tissue distribution and the relative amount of the corresponding mRNAs, we analysed smooth-muscle poly(A)$^+$-enriched RNA on Northern blots with three different anti-sense RNA probes: pER was a non-discriminating riboprobe, pER-1 a class-1-specific riboprobe and pER-2 a class-2-specific riboprobe (Fig. 1). Both pER-1 and pER-2 detected a 4.4 kb messenger in gastric and ileal smooth muscle, which confirmed the presence of the class 1 and 2 mRNAs in these tissues. In addition, all three probes also reacted with an 8.0 kb RNA. Taking into account the exon/intron structure of the human SR/ER Ca^{2+} pump gene 2 [7], we hypothesized that the 8.0 kb RNA corresponded to an incompletely processed gene transcript which still contained both the class 1- and the class 2-specific exons. To clone cDNAs derived from this putative mRNA, we subsequently screened our gastric smooth-muscle cDNA library with a combination of probes. A total of 5×10^5 plaques were first tested with a class 1-specific probe (a 3′ *Sph*I–*Eco*RI fragment of class 1 cDNA) which resulted in the detection of 21 positive clones. After rescreening them with a class 2-specific probe (a 3′ *Xba*I–*Eco*RI fragment of class 2 cDNA), only one clone which reacted with both probes was retained. This clone, ER-15, was purified and its restriction map and nucleotide sequence were determined (Figs. 2 and 3).

ER-15 contains 4211 nucleotides which are followed by a poly(A) tail. A comparison of the ER-15 nucleotide

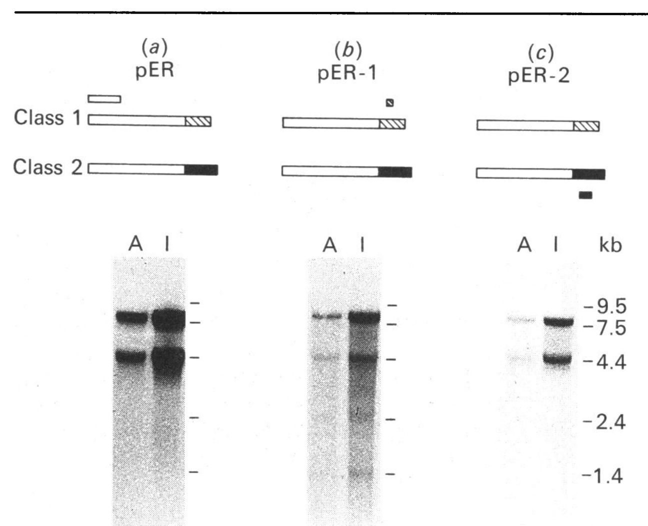

Fig. 1. Northern blot analysis of the gene 2 SR/ER Ca^{2+} pump mRNAs in pig smooth muscle

The different anti-sense RNA probes are represented in comparison with the class 1 and 2 cDNAs in the upper part of the Figure. pER (*a*) is a non-discriminating probe, whereas pER-1 (*b*) and pER-2 (*c*) are specific for class 1 and 2 mRNAs respectively. Parallel blots containing poly-(A)-enriched RNA of pig stomach (antral part) smooth muscle (lane A; 5 μg) and of ileal smooth muscle (lane I; 10 μg) were hybridized with the respective probes. Note the presence of a 8.0 kb mRNA reacting with all three probes.

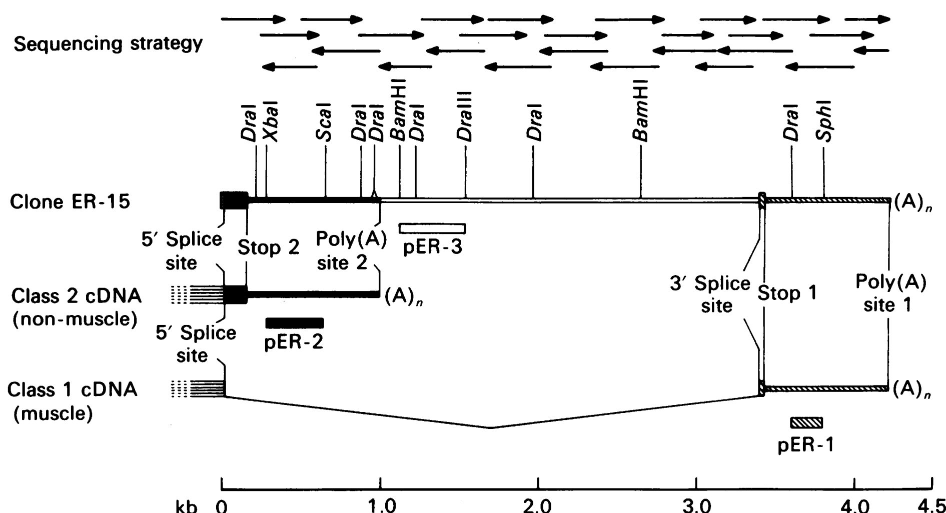

Fig. 2. Domain structure of the pig clone ER-15 and schematic alignment with the pig class 1 and 2 SR/ER Ca^{2+} pump cDNAs

Clone ER-15 is shown schematically: domain 1 (nt 1–17), ▤; domain 2 (nt 18–997), ■; domain 3 (nt 998–3405), □; domain 4 (nt 3406–4211), ▧. Wide boxes represent open reading frames, and small boxes represent untranslated regions. Class 1 and 2 cDNAs are aligned in the lower part and identical segments are indicated with the same symbols. Functionally important sites are indicated as follows. Stops 1 and 2 correspond to the stop codons of the class 1 and 2 open reading frames respectively. Poly-(A) sites 1 and 2 represent the cleavage/polyadenylation sites of, respectively, the class 1 and 2 cDNAs. The 5′ and 3′ splice sites denote the boundaries of the optional intron. The positions of the specific RNA probes pER-1, pER-2 and pER-3 are also indicated. The restriction map of clone ER-15 and the sequencing strategy are shown in the upper part.

sequence with the class 1 and 2 sequences shows that it can be divided in four domains (for a schematic alignment, see Fig. 2). Domain 1 (nt 1–17) is common to both class 1 and class 2 cDNAs. Domain 2 (nt 18–997) is identical to the unique 3′ end of the class 2 cDNA. Whereas the class 2 cDNA is polyadenylated after nt 997 [...AATG-$(A)_n$] [9], the ER-15 clone continues beyond this point in spite of the canonical polyadenylation signals [24], both upstream (...AATAAA...: nt 981–986) and downstream (a GT/T-rich cluster; ...TGTCTTGTTTTT...: nt 1014–1025) of the class 2 cleavage site. Domain 3 (nt 998–3405) is unique for the ER-15 clone. When the nucleotide sequence of domain 3 was compared with the human genomic sequence of the corresponding SR/ER Ca^{2+} pump [7] (alignment not shown), a 75–80 % identity was found with an intron which in the human gene separates the non-muscle-specific exon b′ (domain 2 in the pig ER-15) from the muscle-specific exon c (domain 4 in the pig ER-15). The alignment was limited to the 5′ end (75 % identity) and the 3′ end (80 % identity) of domain 3, because only the sequence of the 5′ and 3′ boundaries of the human intron has been reported [7]. The comparison with the human genomic sequence also indicates that domains 2 and 3 (nt 18–3405) of clone ER-15 correspond to an optional intron which, in the case of clone ER-15, has not been removed, although its 5′ (.../GTAAAG...: nt 18–23) and 3′ (...CTCTTTCCTTTTCAG/...: nt 3391–3405) boundaries correspond to the consensus sequences for the 5′ donor (.../GTAAGT...) and 3′ acceptor (...Y_{11}NCAG/...) splice sites [25]. Domain 4 (nt 3406–4211) is identical to the unique 3′ end of the class 1 cDNA. Both class 1 cDNA and ER-15 end with a poly(A) tail which is preceded by a canonical upstream polyadenylation signal (...AATAAA...: nt 4188–4193) [24]. It should be noted that the poly(A) tail of the ER-3 clone (class 1 cDNA) starts after nucleotide 4206 {...ATGAT-$(A)_n$: see [9]} whereas ER-15 has an additional 5 nt [...ATGAT*AAGTT*-$(A)_n$: see Fig. 3). Whether this represents a biological variation in poly(A) site usage rather than a cloning artifact is not known.

When poly(A)+-enriched smooth-muscle RNA was analysed on Northern blots with an anti-sense RNA probe complementary to the ER-15 unique domain 3, one major signal of length 8.0 kb was detected. This band comigrated with the 8.0 kb signal seen with the pER, pER-1 and pER-2 probes (Fig. 4). Therefore the ER-15 clone corresponds to the 3′ end of a gene transcript which is polyadenylated at the poly(A) site 1 [the poly(A) site 2 must have remained cryptic], but which is incompletely spliced as it still contains an unremoved intron at its 3′ end. We will refer to this 8.0 kb messenger as the class 3 mRNA. Clone ER-15 starts with an open reading frame encoding 54 amino acids (see Fig. 3) which are identical to the 54 *C*-terminal aa of the class 2 Ca^{2+} pump (NYLEP/GKEC...41 aa...MFWS) [9]. Thus the class 2 and 3 mRNAs encode an identical ER Ca^{2+} pump which corresponds to the non-muscle isoform (1042 aa; M_r 115000).

We then examined the tissue distribution of the class 1, 2 and 3 mRNAs. Northern blots containing total RNA of cardiac muscle and poly(A)+-enriched RNA of non-muscle tissues (liver and kidney) and of smooth muscle (antrum and ileum) were tested with a panel of anti-sense RNA probes, pER being a non-discriminating probe, and pER-1, -2 and -3 being specific for the class 1, 2 and 3 mRNAs respectively (Fig. 4). The SR/ER Ca^{2+} pump gene 2 is expressed in all five tissues (Fig. 4*a*). Cardiac muscle clearly contains class 1 transcripts (a 4.4 kb messenger reacting with pER-1), but class 2 and 3 transcripts are hardly detected. Therefore cardiac muscle predominantly expresses the muscle isoform of the gene

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                 5' donor splice site
                       |
   1 CAACTACCTGGAACCTG/GTAAAGAGTGTGTGCAGCCTGCCACCAAATCCTGCTCGTTCTCGGCATGTACCGATGGGATTTCCTGGCCCTTTGTGCTGCTCATAATGCCGCTGGTGATCTG
 989   N  Y  L  E  P  /G  K  E  C  V  Q  P  A  T  K  S  C  S  F  S  A  C  T  D  G  I  S  W  P  F  V  L  L  I  M  P  L  V  I  W

 121 GGTCTATAGCACAGACACTAACTTTAGTGATATGTTCTGGTCTTGACTGACAGTTTTACATAAAGATGTTTAACTTAATCAATTAATTTTTTTATTGTTTAAAGCAACTGTCTATTTCTG
1029   V  Y  S  T  D  T  N  F  S  D  M  F  W  S  *  1042

 241 CTGAATTTTCACATGAACATATTGGCTGGTAAAGGAGGTTTCATACTCTAGATTTTGTTTTGCTTTTTCTGACTCCAGTGGGGCAAGATTTTCCTTTTTTATACACATAATTAAAGTGTC

 361 CATTGACATGTACAGAGAACTAACACTATTTTATGCAAATATTTTTTTGTAGATGAAAAAGCATGTACAGTGTTCTGTTTAATACTCATCCTTGTATAAAAAAAAAAAGAGTTGAGCCAG

 481 CAGACATTGTCAGCAAATTAATTGGCAGCAGATTTTAGGAAATGAATGTGTGTGGTTTTTTCTAAGACTAAATAGCATGTATTGTGTCTTCTGCATGATCATCTGGATTTAATCTGAGAT

 601 CACAGTCTAATTTTTATTCATAAGCCAATTTTTCTGCACTGAGCAGAGTACTGCTACCTCAGTCAGTATTTTTTGGTTTGCCACCCCCCCGTCACCCTTCTCAGCCCTTGTCACCTCCAC

 721 CCCATCCTCCACCCCCGCCCTGCTCGGCTGCTTCTCCTGTAGGGTTTTGATGGTTCTGTTTACATCAGTCTTAACAAGAGGTATGCCTGTTCTCGCTTGTGCAGAAAACATTGTTCCAGA

 841 TTCAATCAACTGGGTTCGTGTCCCCTCATGTAGTTTTTAAGGTTATTTATTTAAATGTCTAATGTATTTTATTGTAACAGACATTGTTTTGCCAACATTGCCTATTTCAGTGGCACTTCA

                                         poly(A)-site 2
                                         |
 961 CAATCTAGTTTAAAAAGGAAAATAAACATTTTAAATG|GACAGAGAAAAATAACTGTCTTGTTTTTAACTCTTATGTGGCTTAGTTTGAACTAATATGTCCAACTTTCTCTTAAGTTCTTA

1081 GCTCAGAAATTTTAGTTTTATTGTGCTTGAGGGGAAGAAGGATCCTGTTCTGCTGCATAGGGAGCTGTAGGGATTTTATTTTTAATGTATAGGCACCAGTTGTCATCTGTGATACATTTT

1201 ATGCAAGTTTCTGCTGGCCTGTTTTTAAAGCCTATGTAGAGGACATAAGGGCAAGTGTGTGTGCGTGTGTGTGTGTGTGTGTGTTTTGTAAAATCTGTAAATAGCACATGACCAAATG

1321 AACATATTGTATAGAACTATTTTTATTTGAATGTGGCACTAACCACCACCATCATTACTATGATAAATGTTTGTGCATGTTCGAGATCAGTCTTACCAACAGTGTATAAGTCATTAACAG

                                              5' donor site in clone RB 2-5
                                              |
1441 TCCTAACTGTGGTGTTTTCCTCCAATGCCTTCCAACATCCATCAACTAACGTGAGTATCCCCTGGAATTTGGATGCTTTAGCCTAAAGGTGACTGCCACTAAGTGACCTGGCTATACGAA

1561 CACTAACGAATAAGCCCCTTGTGTCGGGCACCCTGAGAGCAGCAAGGGCACAACCTTGCTGGGTGAGCCACTATGGACACCACACACTGCATAGGACAGTGGCACAGCCAGGTGTCAGGC

1681 AAGGGCCTGGGGGAAGAGAAACTCCTGCTTTGGTCAGACGACAAGTGCCTTCACAAGTGCTGAGGTATGGAGTCACAAAAACTTGTCATGGTTCCCTTTAACTTTGTTAGCTTAAAGGAC

1801 TCATTCCAGAATCTTGTTAGCTGGTTCATTAAAAAGCAAAAGGCAAGTGAAAGCAATTAACTGAACCAGGAAACTCAGGCCTCCATTCATTCCTCTTAAATAGGCTGCCTCTTTTCACCT

1921 TCTAGGTCATATCCTTTGTGGCTGAGACTTGTTTTTTCTCTCCAGGGTACATTTAAAGCACAGTTACTGGGCCATTGTTCTGCTCAGCCAGCAAACAACCAGCCTAGTAGTTAGGGAGCA

2041 AGAGGGCCCAGGCAGCTGCTTGTATTCAGGCTGGCAGCTCCTCCGTGAATGAAGCCCCGAGTGTTCACATGAAGGTTCTCGGGTTACCGGGCCAGCCAGAAATGCTGGGAGTCTGGGGCA

2161 AGGTTCCTGTTTAAGAGGATAGAAGAGGCACATGGAAACGGATTCTTAATGCAGAGTCCGGGCTTGGCTCACTGAGGCCTCTCAGAGGATGGGGGAGGCTGTTTTGCTTGGCAGAAAAAT

2281 GTAGGGGTAGGTTTTGCCAGTGTTAGTTAGCATTATGAGGTATAGACAGAAACAGGTTGGGTAGATCAAATGCAAGGAGTGTCTGGGCTGCAAACCAAAACAGGACGTCTGAGCTTGTTG

2401 GGTGGAGTGCAGTTCAATCCCAGACTAACCAAATGTTCGACTTTTTCCTGACTCAGTTCTTTGATTTATTGGCTTTTACAAAGTTGAGTGGGACGGGCTGCTCGAGGAGCCATGCACAGC

2521 CCCAGGTGGCCCCGGGAGGTTGTTGCCTTGTGGTTTTTCAGCCTGTCCAGCCCACAGAGGCAGTTTGGATGCCCACTTCCCTCAACCCAGACAGCTGGGAGTGGGGAAGACGCAGTGCCG

2641 CCCTGAGCAGGATCCCTTTGTTCTGCTCCAGGCTTTGCCACAGAAAAACATGTCTGCACTGGCATCTCCTTAGCAGTTATGCTGCTCTGTCTTATCGGGAGTGGCGGGTGCCTGCAGGAA

2761 GGCACTGTCAGCCTCTCGACTTTTGTCTCCATCAGTAACTGGATGGAGTGGTGCCATGCTCAGACCTACCGGGGCAGACCTCAGGGATAGCCGAGACCACGAGCATTAGCTCAGTGTCTG

2881 CCGAGCCCACTGTGTCCGCACCACATTCAGTCAGAACCACCAGCCTGCGGTGAACAGTGAGGGTAATCTGCAGCCTTCTTTCCTGAAAGAGAATGGTGACAAACAGCTCCTCTTCTAGGA

3001 TTGAGCAGGGCTGGGCCCCAGGGAAGGAACGGGAGGGCGCTGGAGGCTTGGCGCTGATTACTACGCTGCTCACTGCTTTGCCTTCCTTATCCTCTGCACTGCTCACCCAAAACTCTTCCT

3121 GCTCAGCTTCTGTTACACCTGAAAATGATTAAATTCCTGTGAGCCAGGGGTGACCTTTGCTCTGTGAGCTAGTCAGTATGGGGGTCTGTGTGGCCGAGCCAGTGCTTCAAGATGCTACCC

3241 TGCGTGGGCAGCAGCTCAGGGACAGTTAATCAGAAATGCTGGTCTTACTCTTAAAAAATCAAACTGAATATTCCTTTTCATCTGTCACTATTAATCTTAAATAGTTATTCTAACATTTCC

                                           3' acceptor splice site
                                           |
3361 TTCTTGTATTTCATAAAGGTGATTTCCTCTCTCTTTCCTTTTCAG/CAATACTGGAGTAACCGCTTCCTAAACCATTTTGCAGAAATATAAGGGTGTTCGGTTGCGTGCATGTGCGTTTTT
                                             /A  I  L  E  *  997

3481 AGCAACACATCTACCAGCCCTCTGCATGATTGATGTTGGGGGTAAAAGAAAATTAGAAAAAAGTCCCCAATTCATTGTGTGTTACGTGGAGGAAACGTGTATTACCAGTGGGGTTTTTTA

3601 GCTTTTAAATCAAAATAATAATTACAAATGTACAATTTAGCTTAATGGATCAGAAATCCTCTCCAGAGAAGTTTGGTTTCTTTGCTGCAAGAAGAATGAGGTTCTGAAACCTTACCCAAG

3721 AACTTAGAAGCCATCAGCCAAGTATCCATGTTTCTCTGTGCAAAATGTCATAGCTTAAATAAATGTACAATATTCAATTGTAATGCATGCCTTCAAGTTGTAAGTAGCCAGATTTCTCTA

3841 TGATGTCATTGAAACATGCTACTTTTTAATTGGCCCTGTACAGTTTGCTTATTTATAAATTTATTGAAAACACTACAGGTGTTGGTTAAAATGTAGGCTTCTAGCTCATAACTTCAGTTA

3961 TTTTTTGAGTGTGCAAACAGCTATTTTGCACTGTATTAAATGTAACTTATTTAATGAAATCAAAAGCAGTAGACAGATGTTGGTGCAATACGAATATTGTGATGCATTTATATTAATAAA

4081 ATGCTAAACGTCAATTTATCACAACGCATGTTTGACTTTAGACTGTAAATAGAGATCAGTTTATTTCTGTGCTGGTAAAAAGTATTGCACAGACATGGTTTCAGGTAAATAAATCTATTC

            poly(A)-site 1
            |
4201 TATGATAAGTT-(A)n
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Fig. 3. Nucleotide sequence of the pig ER-15 cDNA clone

The nucleotide sequence of clone ER-15 is shown continuously. Nucleotides are numbered at the left side. The amino acid sequence is given where the open reading frame corresponds to the class 1 or 2 open reading frame. Amino acids are numbered on the left side, residue 1 being the start methionine of the class 1 and 2 open reading frame. Sites for cleavage and

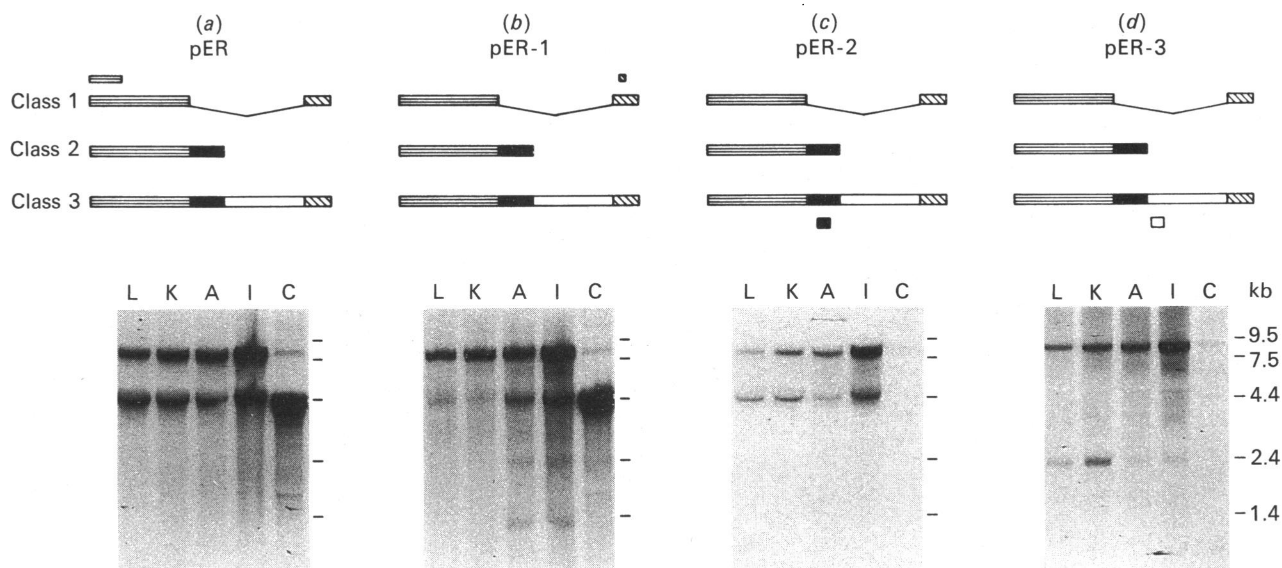

Fig. 4. Characterization of the pig gene 2 SR/ER Ca^{2+} pump mRNAs in cardiac muscle, smooth muscle and non-muscle tissues

The anti-sense RNA probes are represented in the upper part of the Figure. pER (*a*) is a non-discriminating probe, whereas pER-1 (*b*), pER-2 (*c*) and pER-3 (*d*) are specific for, respectively, class 1, 2 and 3 mRNAs. Poly(A)-enriched RNA (10 μg) of non-muscle tissues (lane L; liver; lane K, kidney) or smooth muscle (lane A, antrum; lane I, ileum) and total RNA (5 μg) of cardiac muscle (lane C) were applied. Parallel blots were hybridized with the respective probes. Note the near absence of a class 2 and 3 mRNA in cardiac muscle and the near absence of a class 1 mRNA in liver and kidney.

2 Ca^{2+} pump. In contrast, the class 2 and 3 transcripts are clearly present in liver and kidney, whereas only a faint signal corresponding to the class 1 mRNA can be seen. Thus these non-muscle tissues mainly express the non-muscle isoform of the gene 2 Ca^{2+} pump. Smooth muscle represents an intermediary situation, as the three mRNA classes are present, which means that smooth muscle expresses both the muscle (class 1 mRNA) and the non-muscle (class 2 and 3 mRNA) isoform of the gene 2 Ca^{2+} pump. A 2.4 kb band reacting with pER-3 was also observed in the non-muscle tissues and to a lesser extent in smooth muscle. The nature of this band remains enigmatic.

DISCUSSION

We have identified a third transcript of the SR/ER Ca^{2+} pump gene 2. This transcript is 8.0 kb long and contains the class 1- and class 2-specific exons separated by a 2.4 kb intron. If translated it encodes the non-muscle isoform (1042 aa; M_r 115000) of the gene 2 SR/ER Ca^{2+} pump. One could argue that the 8.0 kb transcript is not a messenger as such, but only a non-translated nuclear precursor of the class 1 mRNA. However, cardiac muscle contains only a small amount of the 8.0 kb transcript although the fully spliced class 1 mRNA is strongly represented. In contrast, non-muscle tissues such as liver and kidney contain only small amounts of the class 1 mRNA, yet they contain an appreciable amount of the 8.0 kb transcript. Therefore there is no strict precursor–end-product relationship between the class 1 mRNA and the 8.0 kb transcript, and we consider the 8.0 kb transcript to be a third SR/ER Ca^{2+} pump messenger (class 3 mRNA).

Our results indicate that the 3′ part of the primary transcript of the SR/ER Ca^{2+} pump gene 2 can be processed in three different ways (Fig. 5). Splicing of the transcript removes the poly(A) site 2 and, consequently, only the poly(A) site 1 is available for cleavage/polyadenylation. This results in a class 1 mRNA which is 4.4 kb long and which encodes the muscle isoform of the gene 2 SR/ER Ca^{2+}-pump (997 aa; M_r 110000). However, if splicing is omitted, the transcript can be cleaved/polyadenylated at either poly(A) site 1 or 2. Polyadenylation at site 2 results in a class 2 mRNA of 4.4 kb. Polyadenylation of a non-spliced transcript at poly(A) site 1 yields a class 3 mRNA of 8.0 kb. Both class 2 and class 3 mRNAs encode the non-muscle isoform of the gene 2 Ca^{2+} pump (1042 aa; M_r 115000).

The alternative polyadenylation and/or splicing is clearly tissue-dependent. Cardiac muscle predominantly forms class 1 mRNAs, which means that in heart the transcripts are nearly always spliced. In contrast, non-muscle tissues such as liver and kidney contain class 2 and 3 mRNAs and only limited amounts of class 1. Thus splicing is a rare event in non-muscle tissues, but they can use both polyadenylation sites. Smooth muscle expresses all three types of mRNA, implying that some but not all

polyadenylation are indicated by a vertical line. Upstream (AATAAA) and downstream consensus (GT/T-rich cluster) polyadenylation signals [24] are singly underlined. The 5′ and 3′ boundaries of the optional intron are indicated by a slash (/). Consensus sequences for 5′ donor (/GTAAGT) and 3′ acceptor (Y_{11}NCAG/) splice sites are doubly underlined [25]. The position of the 5′ splice site used in clone RB 2–5 of Gunteski-Hamblin *et al.* [8] is also indicated.

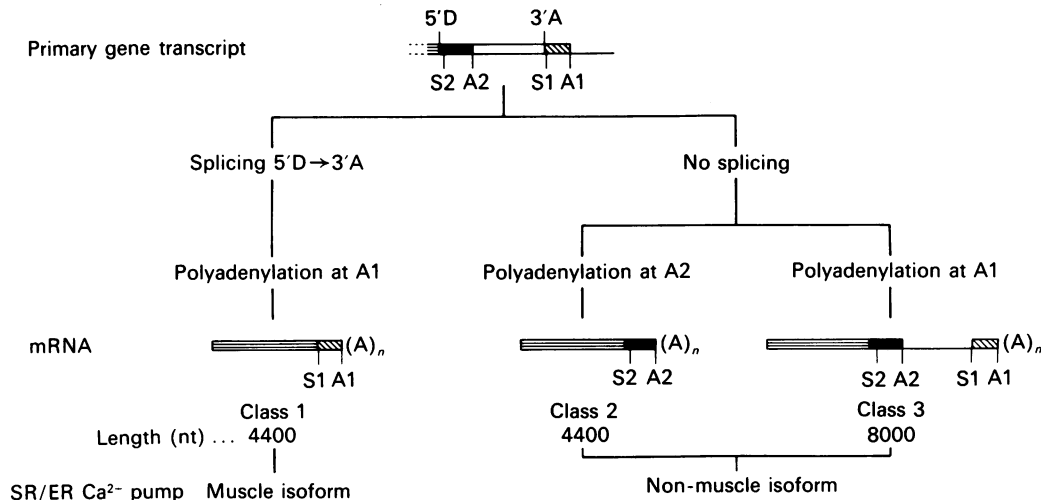

Fig. 5. Processing of the gene 2 SR/ER Ca^{2+} pump transcript

The 3′ part of the primary gene transcript is shown in the upper part of the Figure, and the different mRNA types are shown below. Poly(A) sites 1 and 2 (A1 and A2), 5′ donor (5′D) and 3′ acceptor (3′A) split sites as well as stop codons of the class 1 and 2 open reading frames (S1 and S2) are indicated. If the pre-mRNA is spliced, it can only be polyadenylated at the poly(A) site 1. This results in an 4.4 kb class 1 transcript. Alternatively, splicing can be omitted, in which case either the poly(A) site 1 or 2 can be used, which results in a class 3 or 2 mRNA. Although splicing precedes polyadenylation in the depicted scheme, this does not mean that regulation of transcript processing is achieved at the level of splicing. The Figure is only meant to illustrate the relationship between the different mRNAs.

of the transcripts are spliced and that both poly(A) sites can be used. However, on the basis of these experiments, it cannot be discriminated whether the three mRNAs are co-expressed in one smooth-muscle cell or whether smooth muscle contains several distinct cell types each expressing a particular mRNA. The tissue distribution of the mRNA types suggests a tissue-dependent processing of the primary transcript which may be regulated by tissue-specific factors.

An analysis of rat cDNA libraries revealed four distinct transcripts of the SR/ER Ca^{2+} pump gene 2, three of them corresponding to the porcine class 1, 2 and 3 mRNAs [8]. The fourth transcript, clone RB 2-5 (5.6 kb), results from polyadenylation at poly(A) site 1 and splicing so that only part of domain 3 is removed (the corresponding 5′ donor site is indicated in Fig. 3: nt 1490...*C*/GTGAGT...; the 3′ acceptor site corresponds to the first nucleotide of domain 4 :...CAG/*C*...). We could not detect a corresponding mRNA in the porcine tissues we examined. Indeed, a Northern blot analysis of several rat tissues has very recently shown that the expression of the 5.6 kb transcript is limited to rat brain [14]. Finally, it should also be mentioned that our results on the pig smooth-muscle SR/ER Ca^{2+} pump mRNAs differ from these obtained on rat and rabbit smooth muscle. On Northern blots we clearly found in smooth muscle three types of mRNAs encoding either the muscle isoform (class 1 mRNA) or the non-muscle isoform (class 2 and class 3 mRNA). However, S1 nuclease mapping of rabbit [10] and rat [11] smooth-muscle RNAs revealed only messengers encoding the non-muscle isoform (i.e. class 2 and 3 mRNAs). This discrepancy may be related to the different species used.

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