Structure of the Human Sarco/Endoplasmic Reticulum Ca$^{2+}$-ATPase 3 Gene

PROMOTER ANALYSIS AND ALTERNATIVE SPlicing OF THE SERCA3 PRE-mRNA*

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Human chromosome 17-specific genomic clones extending over 90 kilobases (kb) of DNA and coding for sarco/endo-sarco/endoplasmic reticulum Ca$^{2+}$-ATPase 3 (SERCA3) were isolated. The presence of the D17S1828 genetic marker in the cosmids contig enabled us to map the SERCA3 gene (ATP2A3) 11 centimorgans from the top of the short arm p of chromosome 17, in the vicinity of the cystinosis gene locus. The SERCA3 gene contains 22 exons spread over 50 kb of genomic DNA. The exon/intron boundaries are well conserved between human SERCA3 and SERCA1 genes, except for the junction between exons 8 and 9 which is found in the SERCA1 gene but not in SERCA3 and SERCA2 genes. The transcription start site (+1) is located 152 nucleotides (nt) upstream of the AUG codon. The 5′-flanking region, including exon 1, is embedded in a 1.5-kb CpG island and is characterized by the absence of a TATA box and by the presence of 14 GATA motifs, as well as single sites for Ets-1, c-Myc, and TFIIIc.

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† The abbreviations used are: SERCA, sarco/endoplasmic reticulum Ca$^{2+}$-ATPase; MOPS, 3-(N-morpholino)propanesulfonic acid; RT-PCR, reverse-transcribed-polymerase chain reaction; TFIIIC, transcription factor IIIc; aa, amino acids; bp, base pairs; cM, centimorgans; nt, nucleotides; kb, kilobase pairs; Inn, initiator.
The expression of the two isoforms is regulated by tissue-specific alternative promoters (24).

The first report describing the cloning of the SERCA3 cDNA from rat kidney (25) indicated a broad expression pattern for its 4.8-kb transcript. Recent studies demonstrated that SERCA3 is always co-expressed along with the ubiquitous SERCA2b isoform (26), and high levels of SERCA3 mRNA have been documented in the hematopoietic cell lineage, arterial endothelial and secretory epithelial cells, as well as in cerebellar Purkinje neurons (27–30). Upon expression in COS-1 cells, SERCA3 presents a much lower apparent affinity for Ca\(^{2+}\) than the other members of the SERCA family (10). We have previously identified the 97-kDa SERCA3 (999 aa) in both human and rat platelets using a set of SERCA3-specific antisera (27). Additionally, we cloned the human SERCA3 cDNA, isolated and partially characterized a genomic clone encoding all but the 5'-end of the gene, and localized the SERCA3 gene (ATP2A3) on human chromosome 17p13.3 (31).

Until very recently, there were no indications that the SERCA3 pre-mRNA was subject to alternative splicing. Two mouse nucleotide sequences coding for SERCA3a and SERCA3b have been deposited in the EMBL/GenBank\textsuperscript{TM} data bank.\footnote{Deposited in the EMBL/GenBank\textsuperscript{TM} data bank under the accession numbers U49394 and U49393, respectively, by Y. Tokuyama, X. Chen, M. W. Roe, and G. I. Bell.} So far, no indications regarding the alternative splicing mechanism were published.

We now document the complete exon/intron organization of the human SERCA3 gene. The transcription initiation site and several upstream putative cis-regulatory elements were identified. The functional promoter analysis delineates the minimal promoter region responsible for efficient transcriptional activity and suggests the involvement of the Sp1 transcription factor. We also provide evidence that the human and mouse SERCA3 gene primary transcripts are alternatively spliced, thereby generating not two but three distinct isoforms with

\[\text{FIG. 1. Restriction map of the seven chromosome 17-specific genomic clones and structure of the human SERCA3 gene (ATP2A3).}\]

The overlapping restriction maps of the clones (from left to right: ICRFc105-A09183, -G09189, -C10135, -F021, GHS3, ICRFc105-F0124, and -G1035) are shown below the scale line. The enlargement of a 50-kb genomic region (illustrated as a rectangle) encoding ATP2A3 is shown below the ICRFc105-A09183 clone. B* BamHI; C, ClaI; E, EcoRI; H, HindIII; K*, KasI and N, NotI. The asterisks denote that the indicated recognition sites are not unique ones in the cosmid contig. The 6.6-kb fragment (black box) flanked by B* and K* sites and used in the functional promoter studies is also indicated. The exon/intron layout of the gene is displayed below the restriction map of the 50-kb genomic region. The splicing pattern and the sizes (not to scale) of the 22 exons are indicated below the gene structure together with the ATG codon and polyadenylation signal (AATAAA). Exon 21 is optional, and when included, it is either 88 or 101 bp long. The inset shows the results of PCR amplifications using specific primers for the genetic marker D17S1828 and as DNA template, human genomic DNA (lane 1), ICRFc105-G1035 (lane 2), and -F10124 (lane 3) cosmid DNAs and no DNA (lane 4). PCR products were separated by 1.5% agarose gel electrophoresis. The positions and sizes (in bp) of the DNA markers (lanes M) are shown at the left of the gel. The positions of D17S1828 (small black boxes) are also indicated.
altered C termini as follows: SERCA3a, SERCA3b, and SERCA3c. Furthermore, the three mouse SERCA3 isoforms were overexpressed in COS cells and shown to be functionally active but with different apparent affinities for Ca\(^{2+}\).

### MATERIALS AND METHODS

**Isolation and Characterization of Genomic Clones**—To isolate the entire gene, a human chromosome 17-specific library from Reference Library Data Base, ICRF (32), was screened with the 1482-bp EcoRI fragment from FIG. 2. Exon/intron boundaries of the human SERCA3 gene. The nucleotide sequence of the exon/intron boundaries is shown, and the determined or estimated (\(-\)) intron sizes are indicated in kilobases (kb). Exon sequences are shown in **uppercase letters** and intron sequences in **lowercase letters**. The numbers above the exon sequences denote the nucleotides where splicing occurs (numbering relative to the ATG codon). The deduced amino acid sequences at each junction are displayed below the exon sequences. Exon/intron boundaries are indicated by **diagonal lines**, and the conserved GT and AG nucleotides are shown in **boldface**. The **asterisk** in the last intron indicates the presence of an optional exon (exon 21). The sizes of all exons are shown in Fig. 1.

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**FIG. 2.** Exon/intron boundaries of the human SERCA3 gene. The nucleotide sequence of the exon/intron boundaries is shown, and the determined or estimated (\(-\)) intron sizes are indicated in kilobases (kb). Exon sequences are shown in **uppercase letters** and intron sequences in **lowercase letters**. The numbers above the exon sequences denote the nucleotides where splicing occurs (numbering relative to the ATG codon). The deduced amino acid sequences at each junction are displayed below the exon sequences. Exon/intron boundaries are indicated by **diagonal lines**, and the conserved GT and AG nucleotides are shown in **boldface**. The **asterisk** in the last intron indicates the presence of an optional exon (exon 21). The sizes of all exons are shown in Fig. 1.
size-fractionated by 1.5% agarose gel electrophoresis. The positions and sizes (in bp) of the DNA markers (unpublished data.

Analysis of the putative transcription factor binding sites was performed using the Wisconsin Package Version 9.0 program from Genetics Computer Group (GCC, Madison, WI).

Primer Extension Analysis—Poly(A) RNA was isolated from human tonsils (31).

Using the Wisconsin Package Version 9.0 program from Genetics Computer Group (GCC, Madison, WI).

The resulting plasmid, p6.6BK, was used as a template for further analysis. The location of the extension primer is indicated in Fig. 5c. The position of the extended product (46 nt long) is indicated at the right of the autoradiogram together with an enlargement showing the sequence (5′ to 3′) surrounding the transcription start site (+1). The coding strand is boxed.

Berthold, Bad Wildbad, Germany) and corrected for protein concentration, as determined by the bicinchoninic acid method (Pierce), using bovine serum albumin as standard. Luciferase activities are expressed relative to the β-galactosidase activities and normalized to the value obtained with the promoterless pGL3 basic vector which is set at 1.

Tissue Distribution of Human SERCA3 mRNA—The human RNA Master Blot™ (CLONTECH, Palo Alto, CA), to which high quality poly(A) RNAs from 50 different adult and fetal tissues have been immobilized along with several controls (Fig. 6), was hybridized following the manufacturer's protocol. The synthesis of a 5′-end probe by PCR

3 Available at the following on-line address: censor@charon.girinst.org.

4 F. Bulens, I. Van Nerum, P. Merchiers, A. Belayew, and D. Collen, unpublished data.
was described earlier (31). The probe corresponds to the nucleotides 3033–3405 (accession number Z69881) found in the 5'-untranslated region of human SERCA3 cDNA. The blot was analyzed by means of a PhosphorImager model STORM 840 ( Molecular Dynamics, Sunnyvale, CA). A common SERCA3b/SERCA3c probe (90-bp long) was PCR-amplified using a 5' primer (5'-GAGGTCAGTGCTGTCAGTCCTGC-3') and the 3' primer P1 (5'-GGGCTATCTTCTCTCGTGTCG-3') and the GHSI clone as template DNA; these primers (Fig. 8a) span the exon/intron junctions involved in the alternative splicing. PCR amplification was carried out for 20 cycles, each cycle consisting of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C.

**Reverse Transcriptase-PCR Analyses—Total RNA (0.5 μg) from mouse pancreatic islets (gift from D. L. Eizirik and D. Pipeleers, Department of Metabolism and Endocrinology, Vrije Universiteit, Brussel, Belgium) and 0.5 μg of poly(A)+ RNA from human kidney (CLONTECH) were reverse-transcribed in an oligo(dt)-primed reaction as described (27). The mouse SERCA3 primers used are as follows: a 5' primer M1 + 1 (5'-GGGGGCGTGCTCTCGGCAGTTGC-3') corresponding to nucleotides 2848–2872 in mouse SERCA3a and SERCA3b nucleotide sequences (accession numbers U49394 and U49383, respectively) and a 3' primer M1 – 1 (5'-GGCAAAATGCCTGCTGCTCAGTG-3') corresponding to the inverse complement of nucleotide stretches 3086–3110 and 3159–3183 in mouse SERCA3a and SERCA3b cDNA nucleotide sequences, respectively. A specific 5' primer for the mouse SERCA3c isoform, P3 (5'-GGGGCGTGCTCTTGACGAAGAGCCAC-3') spans the splice boundary between the last exon and an optional exon. PCR amplifications were carried out for 35 cycles, each cycle consisting of 30 s at 94 °C, 30 s at 68 °C, and 30 s at 72 °C for both M1 + 1/M1 – 1 and M1 + 1/P3 pairs. The human SERCA3 primers used are as follows: a common 5' primer 22 (5'-CTGCACTTCTCATTCTGCTG-3') corresponding to nucleotides 2535–2584 and a 3' primer 22 (5'-ATGCCAGCTACCTCAGTG-3') corresponding to the inverse complement of the nucleotide stretch 3040–3060; numbering according to the nucleotide sequence deposited under accession number Z69881. Two additional 3' primers specific for the human SERCA3b and SERCA3c isoforms were designed as follows: the above-mentioned primer P1 and the primer P2 (5'-GGGCTATCTTCTGCTCAGTG-3'), respectively. The PCR conditions were the same for the 3 pairs of primers (22+/1+, 22+/P1, and 22+/P2). 35 cycles, each cycle consisting of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C. All PCR amplifications were performed using a mixture of Pwo (proofreading activity) and Taq polymerases from Boehringer Mannheim, Brussels, Belgium, M1 + 1, M1 – 1, P2, and P3 primers are also represented in Fig. 8a. PCR fragments were gel-purified and subcloned, and for each fragment several individual clones were sequenced.

**Various Analyses Using PCR Techniques—Amplification of the genomic marker D17S1828 (accession number, 602622) from human genomic DNA, ICRF105-G1035 and -F10124 cosmid clones was performed using 5' primer L (5'-TGCACTGACATCGAGTTGC-3') and the 3' primer L (5'-CTAAGCAATTTGCTCAGT-3') for 35 cycles, their positions are indicated below the appropriate codons. The exon/intron junction is indicated by a diagonal line. The nucleotide at position +1 denotes the cap site. The boxed sequence represents a putative initiator (Inr) element, where the A represents the transcription start site often used in other genes. The thin arrow indicates the nucleotide region complementary to the extension primer. Putative transcription factor binding sites are indicated above their underlined sequence; CACCC box and its inverse complement are underlined only. The Alu Type S sequence is shown in bold. The restriction enzymes used to generate the promoter constructs (c) are indicated above their recognition sites, c, functional promoter activity. A schematic representation of the 5'-flanking region of the gene, including exon 1 (5'-untranslated region as a black box and the coding one as an open box), is shown at the left of the panel. The transcription (nt +1) and translation (nt +153) initiation sites as well as the restriction enzymes used to generate the seven SERCA3-luciferase constructs are indicated, and their names and 5'-most positions are indicated at the left margin of each construct. The 3'-end at position +55 is the same in all constructs as a result of filling in the KasI site at position +51. After nucleotide +55, the stretch of 20 bp (small black boxes) derived from the pBluescript vector (used for an intermediate construct) is also indicated. pGL3 basic indicates the promoterless luciferase vector. The transcriptional activity for all SERCA3 promoter constructs in Jurkat E6.1 cells (as relative luciferase activities) is expressed as the -fold induction over the background activity of pGL3 basic, and the results from three different experiments are shown at the right of the panel as black, gray, and open bars for each construct.

**Fig. 5. Structural and functional analysis of the 5'-flanking region of the human SERCA3 gene.** 

**a.** CG (%) content analysis of an 11-kb nucleotide sequence surrounding the first exon. Exon 1 is shown schematically below the plot. The detected repetitive sequences are illustrated at the bottom of the panel: S, Alu type S; J, Alu types J and I, mammalian wide interspersed repeats. b shows the nucleotide sequence of the 5'-end of the gene. The nucleotide sequence of the first exon is in uppercase letters and the first intron and 5'-flanking sequences in lowercase letters. The amino acids encoded by exon 1 and
obtained from human kidney mRNA (CLONTECH) after reverse transcriptions from both human genomic DNA and first-strand cDNA. The cycling conditions were as follows: 35 cycles, each cycle consisting of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C.

The entire coding regions of the mouse SERCA3a, SERCA3b, and SERCA3c cDNAs were amplified by PCR from mouse pancreatic islets with either the primer MMLD/P3 primer pairs. PCR reactions were carried out with either the primer MMLD/P3 primer pairs. PCR products were separated by 1% agarose gel electrophoresis, gel-purified, blunt-ended, phosphorylated, and transferred into the EcoRI-cut, dephosphorylated, and blunt-ended mammalian expression vector pMT2 (from R. J. Kaufman, Genetics Institute, Boston, MA). The cloning of the pig SERCA2b cDNA in pSV57 expression vector was described earlier (11). COS-1 cell culture and DEAE-dextran-mediated DNA transfections were performed as described (11).

Membrane Preparations, Immunoblotting Analysis, and Ca2+-Transport Assays—Microsomes were isolated from COS-1 cells expressing mouse SERCA3a, SERCA3b, SERCA3c, and pig SERCA2b according to Verboom et al. (11). Preparation of the N89 anti-SERCA3 antibody, denaturing gel electrophoresis on 0.75-mm-thick 7.5% polyacrylamide slab gels, semi-dry blotting onto Immobilon-P membranes (Millipore, Bedford, MA), and immunostaining of the blots were done as reported earlier (27). Oxalate-stimulated Ca2+-uptake was measured by a rapid filtration method in the absence or presence of 5 mM ATP at 27 °C as described (12).

RESULTS
Isolation and Characterization of Human SERCA3 Genomic Clones—We have previously described the isolation and partial characterization of the first genomic clone (GH3, approximately 40 kb in length) specifying the 3′ region of the human SERCA3 gene and localized the gene by fluorescence in situ hybridization to human chromosome 17 (31). Subsequent screening of a human chromosome 17-specific cosmid library from the Reference Library Data Base, ICRF (32) with a probe corresponding to the 5′-coding region of human SERCA3 cDNA resulted in the isolation of six new overlapping cosmid clones, whose restriction maps are illustrated in Fig. 1. The cosmid contig covers a genomic region of about 90 kb. The exon/intron organization of the human SERCA3 gene and the sizes of all exons are shown in Fig. 1. The gene is divided in 22 exons distributed across 50 kb of genomic DNA. Exon 21 is optional and, when retained, consists of 88 or 101 bp due to the use of an internal donor splice site (see below). The positions and sizes of introns were determined by PCR analyses using SERCA3-specific primers derived from exonic sequences homologous to those flanking the exon/intron junctions in human (3) and rabbit SERCA1 (2), for which the complete gene structures are known. The exon sequences obtained by genomic sequencing perfectly match those from the cDNA (accession number Z69881), except for a single polymorphism in which a C replaces a T at position 1361 of the cDNA sequence. This point mutation does not change the amino acid sequence at position 453 (Asn). The nucleotide sequences of the exon/intron junctions as well as the position and size of each intron in relation to the amino acid stretch are shown in Fig. 2. However, the last intron (intron 20, 3,218 kb long) contains an optional exon (exon 21). The inset in Fig. 1 documents the presence of the D17S128 marker in the cosmid clones ICRFc105-G1035 and D17S1828, containing the dinucleotide repeat (CA)22, has been mapped by Genethon 11 cM from the top of the short arm p of chromosome 17. The amplified product (215 bp) was used as a probe in Southern blot hybridization analysis and assigned to a position approximately 20 kb downstream of the SERCA3 gene (Fig. 1).

Comparison of Exon/Intron Boundaries of SERCA Genes—The exon/intron structure of the human SERCA3 gene is compared in Fig. 3 with that of the indicated SERCA genes. The positions of 13 out of 20 introns present in the human SERCA3 gene occur in equivalent positions in the Artemia gene, whereas in the Drosophila gene only six introns occur in the same position as in human SERCA3. Two introns in Drosophila and four in Artemia (arrowheads in Fig. 3a) are not present in human SERCA3. Prior to this elucidation of the human SERCA3 gene structure, all known exons of mammalian SERCA genes were found in conserved positions, with one minor exception at the junction between exons 12 and 13 in the human and rabbit SERCA1 genes, where splicing in the human
transcript occurs one nucleotide downstream of the rabbit splicing site (2). We found that this junction is conserved in both human SERCA1 and SERCA3 (Fig. 3a). The complete analysis of the exon/intron layout of the human SERCA1 and SERCA3 genes indicated that the positions of all junctions are conserved except for one boundary, which is found in the SERCA1 gene between exons 8 (298 bp) and 9 (167 bp), but not in the SERCA3 gene. This boundary is also absent from the Artemia and Drosophila genes. PCR amplifications from both human genomic DNA and human kidney first-strand cDNA with human SERCA2-specific primers (Fig. 3b) documented the absence of an intervening sequence in human SERCA2 at this position, too.

Structural and Functional Analyses of the 5'-End of the Gene—The cosmid contig described in Fig. 1 contains approximately 22 kb of genomic DNA upstream of the translation initiation site, of which the proximal 4447 nt were sequenced. In order to determine the transcription initiation site for the SERCA3 mRNA, primer extension analysis was performed with an antisense 22-nt long extension primer, stretching from −107 to −128 nt upstream of the ATG site, and using poly(A)+ RNA from human tonsils. The result in Fig. 4 shows a single extension product of 46 nucleotides, thus locating the transcription initiation site revealed that its proximal part from −455 to −1 (GC-rich region with 72% G + C) concentrates consensus sequences for several potentially important cis-regulatory elements (Fig. 5b). The Sp1-binding site 5'-GGGCAG-3', present in constitutively expressed genes (37), and its inverse complementary sequence 5'-CCGCCA-3' are present 13 times. One Sp1-like element 5'-GGAGG-3' was also found at position −49 to −39. The sequence 5'-CACCC-3' and its inverse complement 5'-GGGTG-3' occurred 11 times. This motif was reported to be potentially associated with a glucocorticoid receptor binding site (38) and to be functionally important in the human and mouse β-globin promoter, where it can be bound by both an erythroid Kruppel-like protein and the ubiquitously factor Sp1 (39). In contrast, the human SERCA2 promoter (19) is characterized by the presence of only one 5'-CACCC-3' box. The sequence 5'-CANNTG-3' (3 times) corresponds to the consensus binding site of muscle-specific transcription factors of the MyoD family (40). The binding site 5'-CCGGCC-3' (455 to 452 bp) from position −887 to −880, 447–454, and 454–461. This motif, known to behave as a cis-acting element in the β-globin gene promoter (42), was also present in the 5'-flanking region of SERCA1 (2) and SERCA2 (14) genes. The GATA motif 5'-GATAAG-3', currently associated with hematopoietic and endothelial expressed genes (43), was found in the SERCA3 gene immediately upstream of the 5'-GGCTGGG-3' element at position −886 to −881. The sequence 5'-GAGGGTTC-3' known to bind c-Myc protein (44) was found at position −831 to −825. The sequence 5'-CAGGGCTT-3', representing the inverse complement of the TFIIIC consensus binding site, was found at position −119 to −112 (45). The Ets-1 cis-element 5'-GAGGAG-3' (46) was found at position −1181 to −1175. A trinucleotide repeat (GAA)n is found at position −1450 to −1404. A poly(dA-dT) stretch (47), 16 bp long, was found within the Alu sequence at position −1634 to −1619. Another poly(dA-dT) stretch, 19 bp long, is flanking the 3'-end of the Alu sequence at position −1449 to −1437. Such stretches were also reported in the 5'-flanking region of rabbit SERCA2 (14) and Artemia SERCA (22) genes.

In order to delineate the core promoter region responsible for human SERCA3 gene expression, six defined 5' deletions, ranging from position −1313 to −31 relative to the transcription initiation site, were generated from the pGL3 basic-derived plasmid p6.6BK. The various constructs and the promoterless pGL3 basic vector were used to transiently transfected cells.
of the human Jurkat E6.1 cell line. The results of three independent experiments are shown in Fig. 5c. Significant transcriptional activity was obtained with each construct, except for the Smal-del. Despite the differences in the relative luciferase activities obtained for each construct among the three experiments, a 3.1-fold induction is obtained with the full promoter construct, p6.6BK. This demonstrates that the 6.6-kb BamHI-KasI genomic fragment contains the required nucleotide sequence information for SERCA3 gene transcription. A maximum level was reached when the region from −6600 to −135 was removed (corresponding to PstI-del construct). Due to experimental fluctuations, the existence of as yet unspecified regulatory elements in the region between −6600 and −135 cannot be ruled out. A total loss of transcriptional activity was observed for the shortest construct, Smal-del, whose 5′-end extended to −31. Based on this functional analysis, the shortest genomic segment, still carrying all the promoter elements (core promoter region) needed for the efficient transcription of the SERCA3 gene, can now be assigned to the GC-rich (87% G + C) region from −135 to −31.

Tissue Distribution of Human SERCA3 mRNA—To determine the relative levels of human SERCA3 mRNA in different tissues, we used a human mRNA Master Blot (dot blot), on which the applied mRNA amounts are normalized for eight housekeeping genes, thus minimizing the tissue-specific variations often related to the expression of any single housekeeping gene. Our dot blot hybridization analysis, using a 3′-end probe, first demonstrated that SERCA3 mRNA is expressed in the human adult and fetal non-muscle tissues shown in Fig. 6, and second, revealed that the expression levels dramatically vary from tissue to tissue as follows: with high levels in thymus, trachea, salivary gland, spleen, bone marrow, lymph node, peripheral leukocytes, pancreas, and colon and intermediate to low levels in the rest of the tissues.

Alternative Splicing of the SERCA3 Primary Transcript Generates Three Variants in Human and Mouse—Recently, two nucleotide sequences encoding mouse SERCA3a and SERCA3b isoforms were deposited in the EMBL/GenBank® data base under accession numbers U49394 and U49393, respectively, and RT-PCR analysis indicated that SERCA3b is co-expressed with SERCA3a in mouse pancreatic islets of Langerhans. Insertion of a 73-bp optional exon in SERCA3b occurs immediately after nt 2980 (relative to the ATG codon) which, interestingly, also represents the point of divergence between the different splice variants in the related SERCA1 and SERCA2 genes. Retention of this additional nucleotide stretch results in a shift in the open reading frame, so that the last 6 amino acids of SERCA3a are replaced by a 45-aa tail in the SERCA3b isoform. We confirmed the existence of the two SERCA3 transcripts in mouse islets by means of RT-PCR using M + 1 and M − 1 as primers (Fig. 7a, lane 1). Equally intense bands of 163 and 236 bp were detected. Subcloning and subsequent sequencing confirmed that the 163-bp fragment, indeed, corresponded to SERCA3a. Remarkably, the 236-bp band proved to represent a heterogeneous population, consisting of a fragment of 236 bp (SERCA3b-specific) contaminated with a 249-bp long fragment. The latter represented a novel variant, SERCA3c, in which the 3′-end of the SERCA3b optional exon is extended with an additional 13-bp stretch (see also Fig. 8e). The SERCA3c-specific amplification from mouse islets became possible by using a mouse SERCA3c-specific primer, P3 (Fig. 7a, lane 2). Analysis of the genomic sequence of the 3′-end of the human SERCA3 gene indicated that the generation of the three SERCA3 splice variants is theoretically possible. The hybridization of the human mRNA Master Blot with a common human SERCA3b/ SERCA3c probe indicated that SERCA3b and/or SERCA3c are mainly expressed in human kidney, thymus, salivary gland, trachea, and colon but at much lower levels than the predominant SERCA3a mRNA (Fig. 7b). RT-PCR from human kidney performed with the human-specific primers, 22+ and 1− (Fig. 7c, lane 1), encompassing the optional exon(s), could in principle amplify all three SERCA3-specific variants (expected lengths: 228, 316, and 329 bp for SERCA3a, SERCA3b, and SERCA3c, respectively). However, only a 228-bp SERCA3a-specific product was detected; amplification of SERCA3b- and SERCA3c-specific products (Fig. 7c, lanes 2 and 3, respectively) became possible by using primer 22+ in combination with splice variant-specific primers P1 and P2, respectively. Fig. 8a compares a 3218-bp long human genomic fragment spanning the intervening region between exons 20 and 22 (31), with a partial mouse genomic sequence derived from a 3-kb PCR product amplified from mouse genomic DNA with the M + 1 and M − 1 primers (data not shown). An optional exon (exon 21) is found 334 bp (in human) and 387 bp (in mouse) downstream of the conserved point of divergence (nt 2980). The human exon 21 is 15 nt longer than the mouse one, because an additional 3′ acceptor splice site is found in the human sequence 15 nt upstream of the one used in mouse. Exon 21 contains an internal 5′ donor splice site (designated D1 in Fig. 8, a and b). D1 and D2 are conserved in human and mouse. If D1 is used, the size of exon 21 is 88 or 73 bp in human or mouse, respectively, giving rise to the SERCA3b isoforms. If D2 is used, exon 21 reaches its maximum size in both human (101 bp) and mouse (86 bp), thereby giving rise to SERCA3c. An illustration of how

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5 G. I. Bell, unpublished observations.
Alternative splicing of the SERCA3 pre-mRNA in human and mouse. a, the genomic nucleotide sequence of the 3' end of the human SERCA3 gene is compared with its mouse counterpart. The exon sequences are shown in uppercase letters and the introns in lowercase letters. For mouse, only the exons and the ends of the introns are aligned; the dashes in the mouse sequences denote the nucleotides that are identical with the human ones. To maintain the partial alignment, a single gap (depicted by a thin vertical arrow) was introduced in the mouse sequence downstream from a putative branch point sequence, 5'-ctctgac-3' (shown in a rectangle with round corners). The 5' donor sites D1 and D2 are indicated above their boxed sequences. If D1 is used, then the sequence of the exon 21 indicated in bold is joined to exon 22. When D2 is used, then the sequence in italics is included in exon 21 and then joined to exon 22. The human (hA) and mouse (mA) 3' acceptor sites used are also indicated. The boxes Sa and Sb denote the overlapping human and mouse stop codons used in SERCA3a and SERCA3b, respectively. The human and mouse stop codons for SERCA3c (in italics) are shifted and shown in small boxes. The nucleotide numbering is shown relative to the human ATG codon in bold and italics for SERCA3b and SERCA3c, respectively. The determined or estimated (\textit{;}) sizes of the human and mouse introns and of the last human exon are also indicated. P1 (thin arrow), P2 (dashed arrow), P3, N1, M1, and M11 (thick arrows) primers are...
SERCA3 splice variants are generated is shown in the upper part of Fig. 8b.

Functional Analyses of the Three SERCA3 Isoforms Transiently Expressed in COS-1 Cells—To characterize functionally the three SERCA3 isoforms, we have employed the COS-1 cell expression system and measured the oxalate-stimulated Ca\(^{2+}\) uptake into the microsomal fraction. For this purpose, we PCR-amplified the coding regions of SERCA3a, SERCA3b, and SERCA3c cDNAs from mouse pancreatic islets first-strand cDNA (data not shown) and subcloned them into the expression vector pMT2. Since the higher GC content of the 5′-untranslated region of the human SERCA3 gene relative to the mouse one causes premature termination of the reverse transcription reaction (data not shown), the amplification of the complete human SERCA3 cDNAs coding for their corresponding isoforms was not possible so far. Therefore, COS-1 cells were transfected with each of the mouse SERCA3 constructs and, for comparison, also with the pig SERCA2b construct. Fig. 9a shows a typical immunoblot analysis of the mouse SERCA3 isoforms expressed in microsomes isolated from COS-1 cells transfected with the corresponding SERCA3 cDNAs. The immunoblot was stained with the polyclonal antibody N89, which was raised against an epitope close to the N terminus of rat SERCA3 (27). The epitope amino acid sequence is also conserved in both human and mouse SERCA3 isoforms. The time course of oxalate-stimulated Ca\(^{2+}\) uptake into microsomal vesicles isolated from COS-1 cells transfected with SERCA2b and each of the SERCA3 cDNAs (Fig. 9b) demonstrates the ability of each of the SERCA3 isoforms to function as a Ca\(^{2+}\) pump. The apparent affinities for Ca\(^{2+}\) of the SERCA3 and SERCA2b isoforms were also deduced (Fig. 9c). We confirm that SERCA3a presents, in this COS-1 cell system, a lower Ca\(^{2+}\) affinity with respect to SERCA2b (K\(_{50}\) = 2.2 versus K\(_{50}\) = 0.19 μM), but the obtained values differ slightly from those reported earlier: K\(_{50}\) = 1.1 μM for rat SERCA3 (10) and K\(_{50}\) = 0.27 or 0.24 for SERCA2b (10, 12). Interestingly, SERCA3b and SERCA3c show much lower apparent affinities for Ca\(^{2+}\) than SERCA3a. The K\(_{50}\) values for SERCA3b and SERCA3c cannot be determined, since the saturation plateau for either SERCA3b or SERCA3c was not reached under our experimental conditions and the use of still higher free Ca\(^{2+}\) concentrations was incompatible with the calcium oxalate precipitation technique.

**DISCUSSION**

We have isolated a total of seven genomic clones spanning a DNA region of 90 kb, of which 50 kb encode the human SERCA3 gene. So far, four other SERCA genes have been completely characterized as follows: the rabbit (23 kb; Ref. 2), human (11 kb; Ref. 3, 26, 39), rat (16 kb; Ref. 16), and pig (15 kb; Ref. 15) SERCA3 genes, and the unique SERCA3 gene. So far, four other SERCA genes have been completely characterized as follows: the rabbit (23 kb; Ref. 2) and human (26 kb; Ref. 3) SERCA1 genes, and the unique SERCA genes in the crustacean Artemia franciscana (65 kb; Ref. 22) and in the fruit fly Drosophila melanogaster (7.1 kb; Ref. 23). For SERCA2 only a partial exon/intron characterization of the human (7), rabbit (14), rat (16), and pig (15) genes has been reported. The estimated size of the mammalian SERCA2 gene is between 45 and 50 kb, which is comparable to that of human SERCA3. The human SERCA3 gene consists of 22 exons with an average exon size of 219 bp. In comparison, the rabbit and human SERCA1 genes count 23 exons. In both genes, the penultimate exon (exon 21 in SERCA3 and exon 22 in SERCA1) is alternatively spliced. Analysis of the exon/intron boundaries showed (Fig. 3e) that all the intron positions are conserved between the SERCA3 and SERCA1 genes, with the exception of one boundary that is present only in SERCA1 between exons 8 and 9. In the SERCA3 gene, the corresponding exonic sequences are joined in one exon, i.e. exon 8. We now provide evidence (Fig. 3b) that this junction is also absent from the human SERCA2 gene. The comparative junction analysis suggests that SERCA2 and SERCA3 would have diverged through gene duplication mechanisms from a common ancestor gene prior to the SERCA1 separation. Recent phylogenetic tree analyses based on the amino acid sequence comparison of the invertebrate and vertebrate SERCA pumps are in line with this conclusion (48). The localization of SERCA3 gene on human chromosome 17 (31) was further confirmed by the isolation of six overlapping genomic clones from a chromosome 17-specific library. In this study (inset in Fig. 1), we showed that the genetic marker D17S1828 was found approximately 20 kb downstream of the SERCA3 gene, which means that the gene encoding the SERCA3 pump can now be mapped 11 cM from the top of the short arm p of chromosome 17. D17S1828 and D17S1798 microsatellite markers have been recently demonstrated to flank a genetic region of 1 cM, which represents the interval where the cystinosis gene locus has been mapped (49). It remains an open question whether ATP2A3 is included in the genomic region associated with cystinosis, an autosomal recessive disorder caused by a defect in the transport of cystine from lysosomes to the cytosol.

To investigate the regulation of the human SERCA3 gene expression, primer extension, nucleotide sequence, functional promoter, and mRNA dot blot hybridization analyses were performed. The primer extension analysis indicated that the transcription site (nt +1) is located 152 nt upstream of the AUG codon. No TATA element was found 25–30 bp upstream of the cap site. We have, however, identified a sequence 5′-CCAACGCA-3′ extending from +7 to +13 nt (represented as a box in Fig. 5b) that matches the consensus initiator (Inr) sequence YNYNT/AYY (35), where the A represents the transcription start site frequently used in other genes. For SERCA3, the A is found at position +9. It has been previously demonstrated that an Inr element can enhance the promoter strength even if it is shifted a few bases upstream or downstream with respect to the transcription initiation site (50). The SERCA3 promoter falls in the TATA- Inr category of promoters (51), whereas the SERCA1 and SERCA2 genes in human and rabbit (2, 3, 13, 14) have TATA Inr-type promoters. It should be noted that almost every Inr element described so far functions in connection with upstream Sp1-binding sites (52). It has also been shown that a distance of 40–50 bp between the Sp1 element(s) and the cap site is optimal for accurate transcription initiation (53). Analysis of the CpG dinucleotide distribution within the first 11 kb of the SERCA3 gene (Fig. 5a) showed that the 5′-end of the gene is embedded in an 1.5-kb well defined CpG island (36). Within the CpG island, a total of 14 putative DNA-binding sites for Sp1 were identified. Eight of them were found immediately upstream of the cap site, in the region between −267 and −39. Moreover, three adjacent Sp1 elements were clustered in the region −57 to −39, i.e. within depicted above or below their nucleotide sequence. The polyadenylation signal is underlined; n.d., sequence not determined. b, in the upper part the alternative splicing pattern is schematically illustrated. The exons (Exons 29, 21, and 22) are represented as boxes and introns as straight lines joining the exons. 3a, 3b, and 3c indicate the splicing pattern for SERCA3a, SERCA3b, and SERCA3c, respectively. The donor splice sites, D1 and D2, the stop codons for SERCA3a (Sa), SERCA3b (Sb), SERCA3c (Sc), and the polyadenylation signal are indicated. Below the diagram, the C-terminal parts of the human and mouse SERCA3 isoforms are compared. The sequence in bold, up to aa 993, is encoded by the last constitutively spliced exon (exon 20). The common sequences of the SERCA3b and SERCA3c isoforms, encoded by exon 21 up to D1 site, are underlined in both human isoforms and in their mouse counterparts. The size (in aa) of each isoform is indicated at the right of its corresponding sequence.
the optimal distance range with respect to the Inr element. Transient transfections in Jurkat cells via electroporation were performed using seven chimeric promoter constructs. The results obtained with the PstI- and SmaI-del constructs were the most informative ones. The PstI-del construct (from +55 to −135) gave the maximum transcriptional activity, whereas a total loss of activity was obtained for the SmaI-del construct (from +55 to −31). One pertinent conclusion deduced from the functional promoter analysis is that the GC-rich region (87% G + C) from −135 to −31 is of critical importance in initiating SERCA3 gene transcription. This region contains six putative Sp1 motifs, an inverse complement for the CACCC box, and single potential binding sites for AP-2 and TFIIC. The results of our functional analysis are in line with a transcription model in which Sp1 protein mediates the transcription initiation through complex interactions involving the Inr element and the cellular transcription machinery. Besides Sp1, several additional transcription factors are likely to be involved in the modulation of the core promoter activity. In contrast to the SERCA2 genes, no thyroid-responsive elements were identified in the 5′-flanking region of the SERCA3 gene. This suggests that SERCA3 expression is not under the control of thyroid hormone as is the case for SERCA2. We conclude that the existence of a TATA−Inr−promoter, which seems to be prevalent among the hematopoietic lineage-specific genes in mammals (54), together with the several putative cis-regulatory elements identified in the 5′-flanking region of the SERCA3 gene might account for the observed tissue-restricted expression pattern of SERCA3 (Fig. 6). On the contrary, a TATA−Inr−promoter (like the one characterizing the SERCA2 gene) might be responsible for a lineage-independent expression (54).

Interestingly, the SERCA gene from A. franciscana comprises two promoters (24) as follows: a TATA−Inr−promoter, controlling the expression of a housekeeping isoform, and a TATA−Inr−promoter involved in the expression of the muscle-specific isoform. We might speculate that the evolution from a unique SERCA gene in invertebrates to the multigene SERCA family in vertebrates was also accompanied by rearrangements of the TATA and Inr promoter elements.

The alternative processing of the SERCA3 pre-mRNA can give rise to three SERCA3 isoforms, which, like the other SERCA family members, differ solely in their C-terminal amino acid sequences, found downstream of amino acid 993. Like in SERCA1, an optional SERCA3 exon (exon 21) can be skipped, thereby generating the SERCA3a splice variant, but it can be retained partially (in SERCA3b) or entirely (in SERCA3c) due to the alternative use of an internal 5′ donor splice site (D1) as is the case for SERCA2. Moreover, the alternative splicing mode for SERCA3 resembles that of the plasma membrane Ca2+-ATPase 1 gene. In the latter case, four isoforms with different C-terminal parts can be generated by alternative exclusion, inclusion, or partial inclusion of a single exon in the 3′-end of the gene.

Both human and mouse SERCA3 genes are transcribed and processed in the same way, according to the splicing scheme...
illustrated in Fig. 8b. We have identified in both human and mouse introns preceding the corresponding exon 21 a sequence, 5′-CTCTGAC-3′, that matches the consensus branch point sequence 5′-YNURAC-3′ (complementary to the U2 snRNA sequence) in which the A is involved in the first transesterification reaction of the pre-mRNA splicing and lariat RNA formation. However, human SERCA3b and SERCA3c splice variants structurally differ from their mouse counterparts; this difference is caused by the occurrence of an additional 3′ acceptor splice site in the human genomic nucleotide sequence, located 15 nt upstream of the site used in mouse. This explains why the optional exon (exon 21; 101 bp) in the human gene is 15 bp longer than in mouse (86 bp). Moreover, the 15-nt sequence, representing the 5′-end of exon 21, encodes a new stretch of five amino acids, ACLYP998. This stretch, present in both human SERCA3b and SERCA3c isoforms, is inserted immediately downstream of amino acid 993, which is encoded by the last constitutively spliced exon (exon 20). Both in human and mouse, when present, SERCA3b and SERCA3c are always co-expressed in the same tissue along with the SERCA3a isoform. However, in mouse pancreatic islets of Langerhans, SERCA3a and SERCA3b are expressed at nearly equal levels, whereas in human kidney SERCA3b is expressed at lower levels than SERCA3a. SERCA3c was also found to be expressed at much lower levels than SERCA3a in all human and mouse tissues examined so far. The tissue expression pattern of SERCA3b and/or SERCA3c mRNAs seems to be much more restricted than that of SERCA3a. As a result of alternative splicing, the SERCA3a-specific C terminus comprising the last six amino acids (from 994 to 999 aa) is replaced either by a tail restricted than that of SERCA3a. SERCA3c was also found to be expressed at much lower levels than SERCA3a in all human and mouse tissues examined so far. The tissue expression pattern of SERCA3b and/or SERCA3c mRNAs seems to be much more restricted than that of SERCA3a. As a result of alternative splicing, the SERCA3a-specific C terminus comprising the last six amino acids (from 994 to 999 aa) is replaced either by a tail

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