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Nucleotide Sequences of Avian Cardiac and Brain SR/ER Ca^{2+}-ATPases and Functional Comparisons with Fast Twitch Ca^{2+}-ATPase

CALCIUM AFFINITIES AND INHIBITOR EFFECTS*

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Two similar forms of the cardiac/slow Ca^{2+}-ATPase (SERCA2a and SERCA2b), differing in sodium dodecyl sulfate-polyacrylamide gel electrophoresis mobility, are expressed in chicken heart and brain (Kaprielian, Z., Campbell, A. M., and Fambrough, D. M. (1989) *Mol. Brain Res.* 6, 55–60). In the current study, cDNAs encoding each form were cloned and sequenced. Chicken SERCA2a is 94% identical to its rabbit homologue, while SERCA2b has an extended carboxyl terminus with 38 of 49 amino acids identical to mammalian homologues. SERCA2b mRNA contains the SERCA2a encoding sequence within its 3′-untranslated region. Chicken genomic DNA sequence reveals that the alternate RNA splicing used to produced SERCA2a and SERCA2b subtypes involves a splice site within an exon. Tissue culture cells expressing the avian SERCA2a, SERCA2b, and SERCA1, each targetting to the endoplasmic reticulum, were used to measure Ca^{2+} affinities and inhibitor effects; no differences among the three pumps were detected.

The concentration of free Ca^{2+} ions in the cytosol is maintained at about 0.1 μM while the extracellular level is 10,000-fold higher. Cells tightly control their cytosolic calcium levels by extruding Ca^{2+} across the plasma membrane as well as by sequestering calcium in internal stores (for review see Bronner and Shamoo, 1985). These internal stores of Ca^{2+} are filled by Ca^{2+}-ATPases encoded by three separate genes (Brandl *et al.*, 1986 and Burk *et al.*, 1989).

Ca^{2+}-ATPases (designated SERCA1, SERCA2, and SERCA3)[1] are *E1-E2* type ATPases with molecular mass around 110 kDa that pump Ca^{2+} into membrane-bound compartments. Mammalian SERCA2 is expressed as two different subtypes, SERCA2a, which predominates in cardiac and slow twitch skeletal muscle, and SERCA2b, which is found in a variety of tissues (Lytton and MacLennan, 1988, Gunteski-Hamblin *et al.*, 1988; Lytton *et al.*, 1989, Eggermont *et al.*, 1989). The two subtypes are identical except that the four carboxyl-terminal amino acids of SERCA2a are replaced by 49 or 50 different residues in SERCA2b. The significance of these two forms, generated from alternate splicing of primary transcripts from the same gene, is unknown.

If the different carboxyl termini found in mammals have functionally significant roles, one might expect this to be evolutionarily conserved. Based on immunological data, our laboratory found that two slightly different forms of SERCA2 are expressed in the chicken heart and brain (Kaprielian *et al.*, 1989). One purpose of this study was to identify these different forms of chicken SERCA2 at the molecular level. Specifically, are the differences in the avian Ca^{2+}-ATPase subtypes homologous to the alternately spliced products seen in mammals? If so, are there any functional differences between the two alternate forms of SERCA2 (*e.g.* inhibitor sensitivity or Ca^{2+} affinity)?

MATERIALS AND METHODS

Enzymes and Radioisotopes—Restriction enzymes were purchased from Pharmacia LKB Biotechnology Inc., Amersham Corp., and New England Biolabs. Radioisotopes ([α-^{32}P]dATP, [α-^{32}P]dCTP, and deoxyadenosine [^{35}S]5′-(α-thio)triphosphate) were purchased from Du Pont-New England Nuclear.

cDNA Library Construction and Screening—Total RNA was extracted from the heart and brain of one adult chicken in guanidine thiocyanate as described in Taormino and Fambrough (1990). The poly(A) RNA was converted to oligo (dT)-primed double-stranded cDNA, methylated, coupled with *Eco*RI linkers, ligated into lambdaZAP phage vector and packaged (Stratagene Cloning Systems). (However, based on examination of numerous clones encoding several different proteins, it has become apparent that the cDNA used to make this library had been incompletely methylated. Many clones terminate at internal *Eco*RI sites and some have unrelated sequences ligated adjacent to the clones of interest.) For screening the libraries, the coding region of rat stomach SERCA2a (Gunteski-Hamblin *et al.*, 1988) was excised with *Pst*I and isolated as described (Davis *et al.*, 1986). The resulting probe was labeled with ^{32}P by the method of Feinberg and Volgelstein (1983). Hybridization of probe to nitrocellulose filter lifts of the plated library was performed overnight in a solution of 120 mM Tris, pH 8, 600 mM NaCl, 4 mM EDTA, and 50% formamide at 68 °C. Forty and 20 positive clones from the brain and heart cDNA libraries, respectively, were rescreened with the same rat probe. Five clones from each library were isolated by an *in vivo* excision method involving the helper phage R408 (Stratagene Cloning Systems).

cDNA Sequencing and Analysis—All but the 107 5′ most nucleotides (noncoding) of SERCA2 were sequenced on both strands by the dideoxynucleotide termination method (Sanger *et al.*, 1977) with the U. S. Biochemical Corporation Sequenase kit. Some clones were sequenced after subcloning restriction fragments. Synthetic oligonucleotide sequencing primers were made on the 391 DNA Synthesizer (Applied Biosystems) and used to sequence other clones. By the procedure in Sambrook *et al.* (1989), nested deletions were produced

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M66385.

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[1] The nomenclature used here was adopted from Burk *et al.*, (1989).

with exonuclease III to sequence one clone. The GAP program of GCG (a software package from the University of Wisconsin) was used for computer comparisons of cDNA sequence similarity across species.

RNA Blot Analyses—Total RNA was isolated from adult and embryonic heart and brain tissues by the RNAzol B method (Cinna/Biotecx Laboratories International Inc.). 30 or 45 μg of total RNA were loaded onto a 0.6% agarose gels containing formaldehyde. Probe A in Fig. 3*A* was derived from the 1.3-kb *Eco*RI fragment of clone B13[2] and labeled with ^{32}P. The remaining probes were generated by a PCR (Perkin-Elmer Cetus)[2] procedure with primers which were designed to amplify specific regions of chicken SERCA2. The primers are as follows: probe B, 5′ primer AAGAAAACAAAAGCAT (bases 3520–3535 as numbered in Fig. 2*A*), 3′ primer GAAACAATCTGACACAA (reverse complement of bases 4001–4017); probe C, 5′ primer GTAATCACTTCCTAAAC (4408–4424), 3′ primer TACATAAGCTGTTATAG (reverse complement of bases and 4820–4836); and probe D, 5′ primer CTGGCGTGTTATTTGATGCAC (bases 5183–5203), 3′ primer GAGGGATTTACAAACAATG (reverse complement of bases 5442–5460). Reaction conditions were 10 mM Tris-HCl, pH 8.3 (at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 2.5 units of Amplitaq DNA polymerase (Perkin Elmer-Cetus), with 0.07 mM non-radioactive deoxynucleotides, and 125 μCi of α-^{32}P-labeled dATP and dCTP. 30 temperature cycles of 95, 50, and 72 °C for 1 min each were performed to produce each probe. The reaction products were then passed through two Sephadex G-50 spin columns to purify the radiolabeled probes. The 5′ end-labeled probe E in Fig. 3 is an oligonucleotide 30 bases long with the sequence of ATTACTCCAGTATTGCAGGTTCCAGGTAGT. This sequence is comprised of 15 nucleotides on either side of the alternate splice site, half of which are specific for the SERCA2a terminal encoding sequence.

Gene Structure—The primers used to subclone the chicken genomic DNA that included the alternate splice site were 5′ GGAATTCATCTGGCTGGTGGAGC (an *Eco*RI linker plus bases 2788–2804) and 3′ GGAATTCATATCACTAAAGTTAG (an *Eco*RI linker plus reverse complement of bases 3101–3117). 1.5 μg of chicken genomic DNA were used as template in reaction conditions outlined above but without radiolabled nucleotides. The PCR product was digested with *Eco*RI and cloned into the vector pBluescript. Another pair of primers was designed to amplify the intron downstream of the SERCA2b unique sequence: 5′ AAGAAAACAAAAGCAT (bases 3520–3535) and CAACCTCACATTTCTGC (reverse compliment to bases 4431–4447). When the latter pair of primers was used, no product was seen when genomic DNA was used as template. However, cDNA template yielded a band of the correct size. The inability to amplify this portion of the SERCA2 chicken gene is consistent with the human gene structure which has a 3-kb intron in this region (Lytton and MacLennan, 1988).

Expression in Tissue Culture—Full-length cDNAs of SERCA2a and SERCA2b were constructed by ligating the appropriate fragments of clones B13, B14, and H14. Two "false-start" ATGs in the 5′-UT region were deleted by a PCR method. The 5′-oligo contained a *Kpn*I site, a translation initiation consensus sequence (Kozak, 1989) and the first nine coding nucleotides (TGTGTGGTACCCCGACCATGGAGAACG). The other PCR primer was based on sequence down stream of the *Spe*I site at position 463. The PCR conditions were as described above with an extention time of 30 s. The resulting product was digested with *Spe*I and *Kpn*I and cloned into pBluescript and sequenced. This was then ligated onto the 5′ end of both SERCA2a and SERCA2b. SERCA2b was digested with *Ssp*I and recloned into pBluescript in order to delete the sequence containing the SERCA2a carboxyl-terminal encoding nucleotides. The modified cDNAs of SERCA2a and SERCA2b were cloned into the *Kpn*I site of the expression vector pcDL-SRα296 (Takebe *et al.*, 1988). Chicken SERCA1 (Karin *et al.*, 1989) was cloned into the *Eco*RI site of pcDL-SRα296. COS-1 cells were transfected using DEAE dextran (Clarke *et al.*, 1989b) with the modification of substituting 10% dimethyl sulfoxide for chloroquine.

Immunofluorescence—Transfected cells were fixed (25 mM HEPES, pH 7.4, 2.5 mM Mg acetate, 25 mM KCl, 250 mM sucrose, and 1% formaldehyde at room temperature) for 10 min and permeabilized with 0.25% Saponin. The cells were then incubated with a chicken-specific monoclonal antibody to SERCA2 (CaS-3H2, Kaprielian and Fambrough, 1987) or SERCA1 (CaF3–5C3, Karin *et al.*, 1989) followed by a rhodamine-conjugated goat anti-mouse secondary antibody and photographed on an epifluorescence microscope.

Microsome Preparation—As in Clarke, *et al.* (1990), twenty 150 × 25-mm plates of transfected COS-1 cells were washed twice with 10 ml of phosphate-buffered saline, harvested in 80 ml of 5 mM EDTA in phosphate-buffered saline, and washed with 40 ml of phosphate-buffered saline. The cells were resuspended in 16 ml of 10 mM MOPS, pH 7.0, 0.5 mM $MgCl_2$, 200 KIU/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and, after 10 min to allow for hypotonic swelling of cells, homogenized with Dounce homogenizer. The suspension was diluted with 0.5 M sucrose/6 mM 2-mercaptoethanol/40 μM $CaCl_2$/300 mM KCl/20 mM MOPS, pH 7.0, and centrifuged at 10,000 × *g* for 20 min. The supernatant was adjusted to 600 mM KCl and centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended with 50 mM MOPS, pH 7.0, 10% sucrose and frozen in liquid nitrogen for later use.

Calcium Uptake Assay—5.0 ml of the reaction mixture (20 mM MOPS, pH 7.0, 60 mM KCl, 5 mM $MgCl_2$, 5 mM sodium oxalate, 0.2 mM EDTA, 2.5 mM ATP, 0.2 mM $CaCl_2$ (with 0.4 μCi/ml $^{45}Ca^{2+}$) and 10 μg/ml of microsomal protein) was equilibrated at 25 °C for 5 min. 1.0 ml aliquots were filtered (0.45-μm pore size; Millipore) and washed with 12 ml of 10 mM MOPS, pH 7.0, 2 mM $LaCl_3$. A liquid scintilation counter was used to determine amounts of $^{45}Ca^{2+}$ in each aliquot. For the cyclopiazonic acid and thapsigargin studies, the inhibitors were present during the 5-min incubation period. For the calcium concentration-dependent studies, free calcium concentration was varied as estimated from total $CaCl_2$ and EGTA in solution (Fernandez-Belda *et al.*, 1984). The data points in Figs. 6 and 7 represent averaged results obtained from two or three independent transfections and microsome preparations.

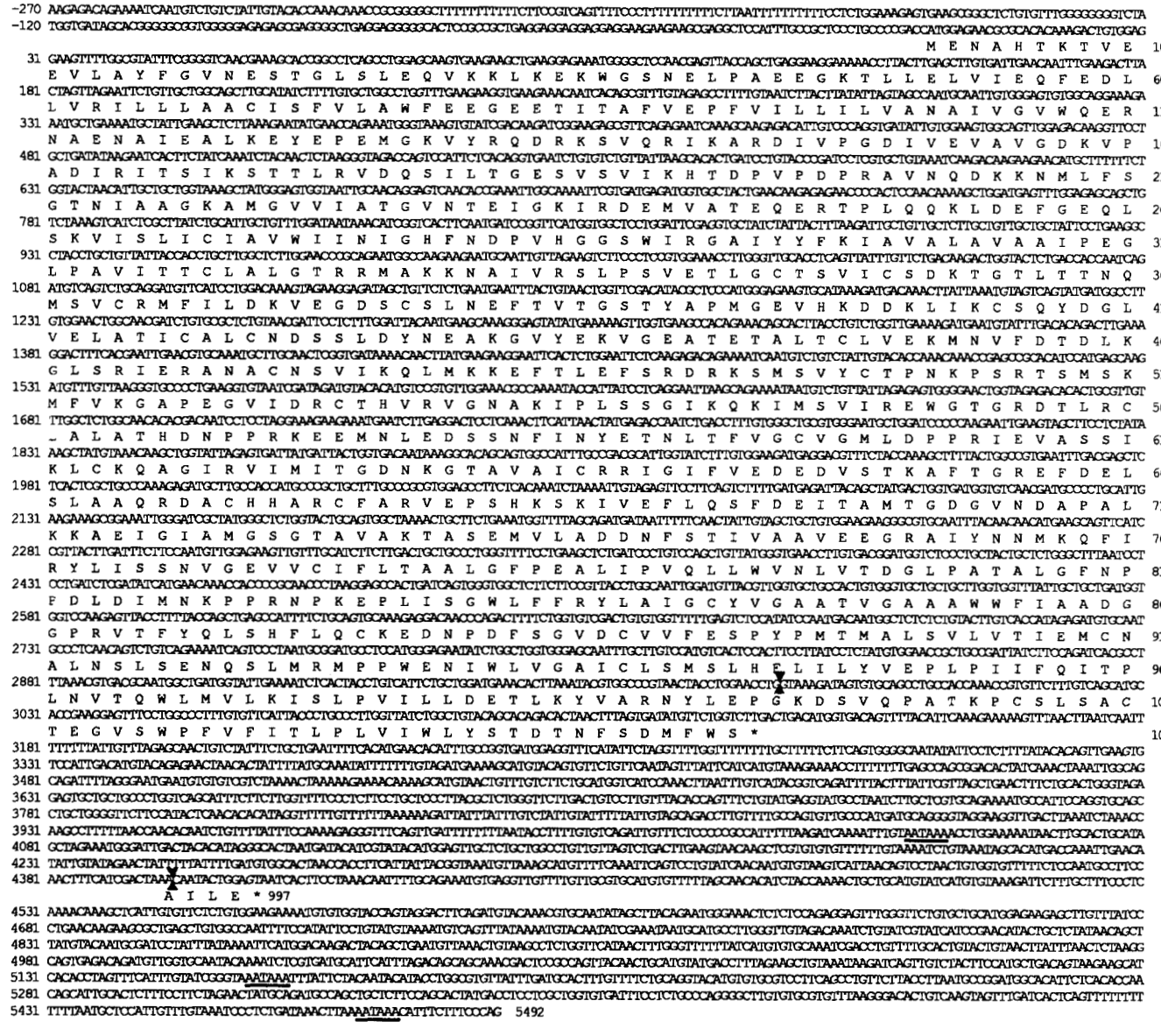
RESULTS

cDNA Sequence and Analysis—A cDNA probe encoding rat SERCA2a was used to screen chicken heart and brain cDNA libraries at high stringency (68 °C, 50% formamide). A number of overlapping cDNA clones from each library were excised *in vivo* (see "Materials and Methods") and sequenced to yield complete nucleotide sequences encoding chicken SERCA2a and SERCA2b. Clone B11[2] did not contain the complete coding sequence but it was of particular interest since it was the only clone isolated from the brain cDNA library which encoded the SERCA2a carboxyl terminus. No poly(A) tails were found in any of the clones, perhaps due to the presence of an *Eco*RI site between the polyadenylation signal and the poly(A) tail in combination with partial methylation of the cDNA during construction of both libraries (see Materials and Methods).

The nucleotide and deduced amino acid sequences of chicken SERCA2a are shown in Fig. 1*A*. The amino acid sequence is 94% identical to mammalian homologues (MacLennan *et al.*, 1985, Lytton and MacLennan, 1988, and Gunteski-Hamblin *et al.*, 1988, Eggermont *et al.*, 1989). Of the variant amino acids, conservative changes account for nearly half of the substitutions.

In addition to the cDNA clones that encode SERCA2a, the avian cDNA homologue of mammalian SERCA2b was also sequenced. The nucleotide and deduced amino acid sequences of SERCA2b are presented in Fig. 1*A*. The 44 additional carboxyl-terminal amino acids account for the difference in apparent molecular mass between SERCA2a and SERCA2b seen by SDS-PAGE (110 *versus* 115 kDa). When the carboxyl terminus of avian SERCA2b is compared to homologous mammalian sequences, 38 of 49 amino acids are identical with six out of the 11 substitutions being conservative ones. Among

[2] The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; 3′-UT and 5′UT, 3′- and 5′-untranslated; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TG, thapsigargin; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; Clones beginning with the letter "B" are from the brain cDNA library while those with an "H" are derived from the heart library.



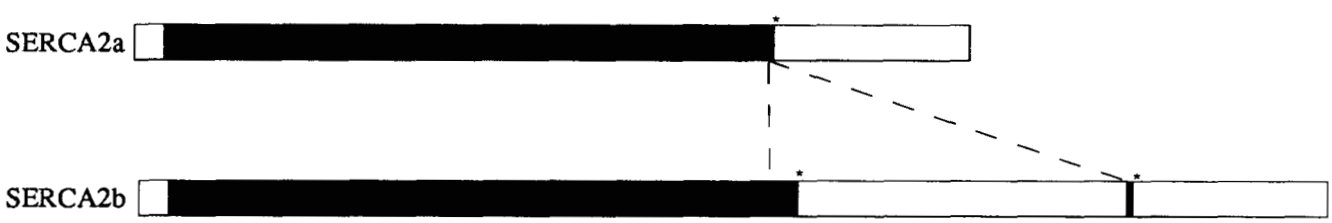


FIG. 1. **Nucleotide and deduced amino acid sequences of chicken SERCA2 cDNAs.** *Panel A*, nucleotides are *numbered* on the *left* and amino acids on the *right*. Amino acids are identified by *single letter abreviations* below their codons, *stars* denote stop codons, and consensus polyadenylation signals are *underlined*. The alternate splice site occurs at base 2980. The SERCA2b unique sequence is located between the two (▼ ▲) symbols and is absent from SERCA2a mRNA as a result of alternate splicing. The SERCA2a carboxyl terminus is written below its encoding sequence with the first base of the first codon located 5′ of the alternate splice site. *Panel B*, the diagram depicts mRNAs transcribed from SERCA2. The *black boxes* represent coding sequences of SERCA2 with *open boxes* indicating the UT portions. The *dashed lines* indicate the relationship of the SERCA2b unique sequence to the SERCA2a mRNA.

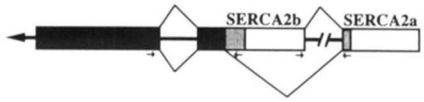


FIG. 2. **Alternate splicing of SERCA2.** The diagram shows the primary transcript with exons as *boxes* and introns as *thick lines*. The *black boxes* indicate coding sequences 5′ of the alternate splice site. The *stippled boxes* are the coding regions for the two different carboxyl termini. *Open boxes* are 3′-UT regions. The splicing pattern on top results in SERCA2b encoding mRNA and the bottom pattern results in SERCA2a encoding mRNA. The *small arrows* mark the location and direction of the PCR primers used to identify the gene structure.

the various chicken cDNA clones, there are three base substitutions occurring in the coding region. These changes do not alter the primary structure of the protein and are probably caused by two different alleles being expressed by a heterozygous animal used for construction of the libraries. The changes are from C^{1014} to A, T^{1971} to C and C^{2767} to A.

Previous publications of SERCA2 sequences (Lytton and MacLennan, 1988, Gunteski-Hamblin *et al.*, 1988, Lytton *et al.*, 1989, and Eggermont *et al.*, 1989) had not shown the correct relationship between SERCA2a and SERCA2b mRNA. The fact that the 3′-UT region of SERCA2b message also contained the sequence which encodes the SERCA2a carboxyl terminus was not realized. But as shown in Fig. 1, *A* and *B*, the SERCA2a terminal encoding cDNA is downstream of the SERCA2b cDNA. In order to translate SERCA2a, the primary transcript must be spliced so that all of the SERCA2b unique sequence is excised. By removing the SERCA2b unique sequence, the encoding sequence of the four terminal amino acids of SERCA2a becomes contiguous with the bulk of the coding region, thus allowing SERCA2a translation. Therefore, primary transcripts of the SERCA2 gene contain the encoding sequences for both SERCA2a and SERCA2b. After processing the RNA, the 3′-UT region of SERCA2b mRNA still contains the SERCA2a-terminal encoding sequence while SERCA2a mRNA has had its SERCA2b unique portion excised.

mRNA Processing—To delineate exact intron/exon boundaries surrounding the chicken alternate splice site within the gene, PCR was employed to amplify a portion of the gene. The deduced gene structure and RNA splicing pattern are shown in Fig. 2. Primers were designed to amplify both the alternate splice site and the intron upstream of the SERCA2b unique sequence. The resulting PCR product was cloned and sequenced. Between nucleotides G^{2859} and A^{2860} is a 119-base pair intron[3] which begins with GT and ends in AG. An intron occurs at the homologous position in the human SERCA2 gene (Lytton and MacLennan, 1988). There is no intron at the alternate donor splice site used to generate SERCA2a mRNA.

A series of probes to different regions of the 3′-UT regions of SERCA2 were hybridized to RNA blots to determine which of three potential polyadenylation signals are used in mRNA processing. Probe A was the *Eco*RI fragment depicted in the diagram in Fig. 3. The resulting bands (Fig. 3, *blot A*) show the relative amounts of heart and brain mRNA loaded in blots A through E. Probe B, which hybridizes to a SERCA2b specific portion of the sequence, does not hybridize to the heart RNA but does detect high levels of SERCA2b mRNA isolated from brain tissue. Embryonic brain also contains mRNAs that include the sequence unique to SERCA2b; only SERCA2a mRNA was detected in embryonic heart (data not

[3] The intron sequence is in lower case letters and exon boundaries are in upper case letters. The sequence is: GCCGgtaagtgcttctgtctta-catccatccctgagctcagaggtggagcagaaaaccttgtgtggagcataaggcaagggacgga-ggtgtagcaaagctgtaatgcttggctgttctttcagATTA.

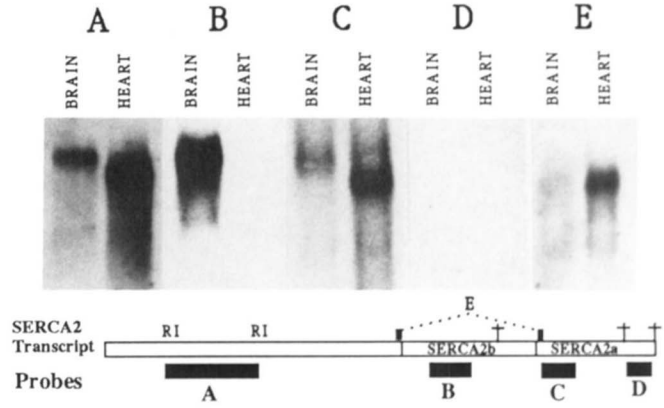


FIG. 3. **SERCA2 RNA processing in heart and brain.** This autoradiograph is an RNA blot probed with ^{32}P-labled probes. One 0.6% agarose gel was loaded with replicates of 45 μg of adult brain and 30 μg of adult heart RNA as indicated. The approximate sizes of the probes are 1.2 kb (*A*), 500 (*B*), 400 (*C*), 250 (*D*), and 30 (*E*) nucleotides. The molecular size markers are 4.6 and 1.8 kb. The location of each probe is indicated in the figure below with the three potential polyadenylation signals indicated by *crosses*.

shown). The first consensus sequence for polyadenylation occurs at nucleotide 4048, within the 3′-UT region unique to SERCA2b. Little if any SERCA2b mRNA uses the consensus sequence at 4048 since the band detected with probe B has an apparent size 1 kb greater than that predicted for messages terminating at 4048. In RNA blots from both heart and brain, bands are evident when probe C was used but not probe D. These data show that for both SERCA2a and SERCA2b mRNAs, the AATAAA at 5158 is used as the predominant polyadenylation signal rather than the consensus sequence at position 5473. It is interesting to note that the rarely used polyadenylation signal sequence at 5473 is not present in homologous mammalian cDNAs. Heart transcripts occasionally use the signal sequence at 5473 (or at some position further downstream) since one cardiac cDNA clone (H14) was found to contain the sequence downstream of the predominantly used polyadenylation signal at base 5158. (A faint signal in the heart RNA lane was detected when probe D was used but was too faint to appear in the photograph.) There is no evidence that brain messages ever use the polyadenylation signal at nucleotide 5473. Probe E, an oligonucleotide which spans the alternate splice site and is specific for SERCA2a mature mRNA, hybridizes to RNA of similar size in both brain and heart lanes. This verifies that brain does express SERCA2a but at a much lower level than SERCA2b. The brain SERCA2a mRNA was not detected in *panels A* or *C* probably because of the low expression level and smeared signal. In summary, both brain and heart transcribe messages which predominantly use the polyadenylation signal at nucleotide 5158, though heart infrequently uses the polyadenylation signal at nucleotide 5473. SERCA2a mRNA was detectable in both brain and cardiac lanes while SERCA2b message was seen in brain RNA only.

Expression and Analysis of cDNA Clones in Tissue Culture—In order to examine functional differences among Ca^{2+} pumps, cDNAs encoding each SERCA2 subtype as well a chicken SERCA1 were expressed in tissue culture (see "Material and Methods"). Full-length constructs encoding either SERCA2a, SERCA2b, or SERCA1 were transiently expressed in COS-1 cells. The transfected cells were fixed, permeabilized, and labeled with a chicken specific anti-SERCA2 or anti-SERCA1 monoclonal antibody and rhodamine-conjugated secondary antibody. High levels of expression were

obtained for all three avian Ca^{2+}-ATPases. An immunofluorescent staining pattern indicative of the endoplasmic reticulum was observed. This is best seen at the thin edges of cells as shown in Fig. 4, *A-C*. These results show that SERCA1 and the SERCA2 subtypes are capable of targeting to the appropriate organelle when transfected into non-muscle tissue cultured cells. Microsomes made from similarly transfected cells were analyzed by SDS-PAGE and immunoblots. Using protein blots from 6% polyacrylamide gel electrophoresis and probing with avian-specific monoclonal antibodies, it was possible to demonstrate clearly the expressed avian Ca^{2+}-ATPases (Fig. 4*D*).

To ensure that the ER localization was not merely due to accumulation of misfolded protein, microsomes of cells transfected with SERCA1, SERCA2a, or SERCA2b were assayed for their ability to sequester $^{45}Ca^{2+}$. Fig. 5 shows that all three pumps are functional. The apparent lower activity of SERCA2a is due to lower yields of SERCA2a protein/milligram of total microsomal protein (see Fig. 4*D*). Equal amounts of total microsomal protein were analyzed by immunoblots to quantify relative amounts of SERCA2a and SERCA2b. There is 1.7-fold less SERCA2a than SERCA2b in the respective microsomes (data not shown). Therefore, with a factor of 1.7 to correct for the lower expression of SERCA2a, all three pumps sequester about 1100 nmol of Ca^{2+}/mg protein/h. This is an order of magnitude greater than the rate of Ca^{2+} sequestration by microsomes from nontransfected COS-1 cells or cells transfected with the SERCA2a cDNA cloned into the expression vector in the reverse orientation.

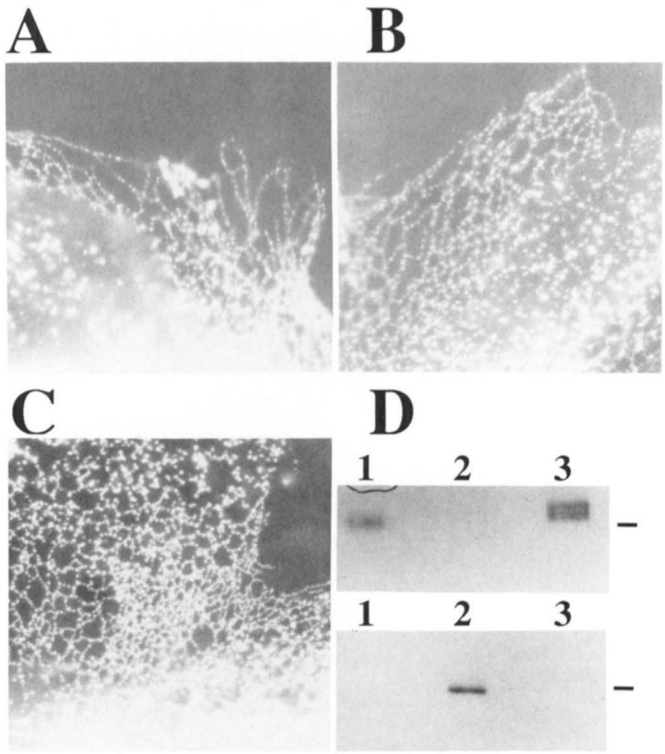


FIG. 4. **Expression of chicken SERCA2a and SERCA2b cDNAs.** Immunofluorescence micrographs of the thin, extreme margins of COS-1 cells transfected with SERCA2a (*A*), SERCA2b (*B*), SERCA1 (*C*) and labled with a monoclonal antibody specific for chicken SERCA2 (*panels A* and *B*) or avian SERCA1 (*panel C*) followed by a rhodamine-labled goat anti-mouse IgG. *Panel D* shows autoradiographic results of microsomes of transfected COS-1 cells analyzed by SDS-PAGE, blotted onto nitrocellulose, probed with either SERCA2- (*top*) or SERCA1- (*bottom*) specific antibodies and incubated with ^{125}I-labled secondary antibodies. The molecular mass marker is 106 kDa and the *lanes* are as follows: *lane 1*, SERCA2a; *lane 2*, SERCA1; and *lane 3*, SERCA2b.

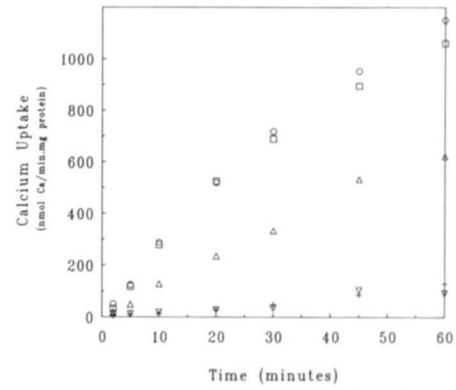


FIG. 5. **Time course of calcium uptake by microsomes.** Calcium uptake assays were carried out as described under "Materials and Methods." The curves are labeled as follows: △, SERCA2a; □, SERCA2b; ○, SERCA1; +, reverse orientation of SERCA2a in the expression vector; ▽, transfected cells.

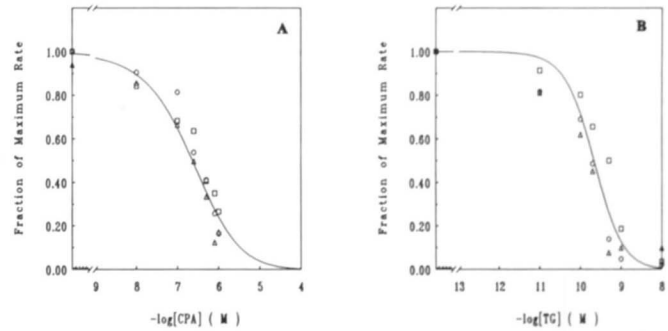


FIG. 6. **Initial rates of calcium uptake.** The same reaction conditions were used as in Fig. 5 in the presence of *A*, cyclopiazonic acid (*CPA*), or *B*, thapsigargin (*TG*). The results are standardized as fraction of maximal rate and plotted against concentration of inhibitory reagent added to the reaction mixture. The curves are labeled as follows: △, SERCA2a; □, SERCA2b; ○, SERCA1. Experimental points were fitted with the Hill equation, assuming 2.19×10^{-7} M for the apparent IC_{50} of CPA (*A*) or 1.49×10^{-10} M for the apparent IC_{50} of TG (*B*).

There are two toxins reported to be SR/ER Ca^{2+} pump inhibitors. Both thapsigargin (TG, Thastrup *et al.*, 1990) and cyclopiazonic acid (CPA, Seidler *et al.*, 1989) are believed to act upon SERCA-type ATPases but not the plasma membrane calcium pumps. When calcium uptake was measured for SERCA2a, SERCA2b, and SERCA1 over a range of inhibitor concentrations, no significant differences were detected in the sensitivity of the three isoforms to the inhibitors (Fig. 6).

Finally, the apparent Ca^{2+} affinity for each isoform was determined. Equal amounts of microsomes were incubated with varying free Ca^{2+} concentrations. There was no appreciable difference in the Ca^{2+} activation patterns of the Ca^{2+}-ATPases as shown in Fig. 7.

DISCUSSION

Previous work has shown that two subtypes of SERCA2 with different M_r are expressed in the chicken (Kaprielian *et al.*, 1989). This paper demonstrates that the difference is due to alternate splicing at an intraexonic donor site in the primary transcript. SERCA2a can only be expressed when a splice site donor, which occurs within the exon coding for the carboxyl terminus of SERCA2b, is used for RNA processing. Only SERCA2a was detected in heart, while both forms of SERCA2 were expressed in brain with SERCA2b being the

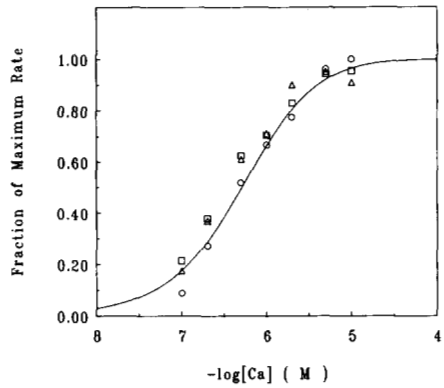


FIG. 7. **Calcium concentration dependence of initial rates of calcium uptake.** Reaction conditions are the same as used in Fig. 6 except the concentration of free calcium was varied as described under "Materials and Methods." The results are standardized as fraction of maximal rate and plotted against free calcium concentration. The curves are labeled as follows: △, SERCA2a; □, SERCA2b; ○, SERCA1. Experimental points were fitted with the Hill equation, assuming 4.17×10^{-7} M for the Ca^{2+} concentration yielding half-maximal activation.

predominant form. This means that the internal RNA splice site donor is used much less often in brain and that the carboxyl-terminal coding sequence of SERCA2a usually appears within the 3′-UT region of SERCA2b mRNA. A similar, though more complex, splicing pattern of RNA has recently been reported for mammals (Plessers *et al.*, 1991). Unlike mammalian SERCA2 mRNA expresssion, there are only two forms of avian SERCA2 mRNA. The only detected SERCA2b mRNA always contained within its 3′-UT region the SERCA2a terminal encoding nucleotides. Therefore, alternate splicing via an internal donor site appears to be the mechanism to produce alternate carboxyl termini in avian as well as mammalian SERCA2.

When SERCA2a and SERCA2b were expressed in COS-1 cells, the Ca^{2+}-ATPases were targeted to the endoplasmic reticulum as evident by the immunofluorescent staining pattern (Fig. 4, *A-C*). This localization is not due to accumulation of misfolded protein since similarly transfected cells synthesized functional enzymes. In the photomicrographs, there is some punctate staining in addition to the reticular network. This could be due to incomplete fixation and vesicularization of the ER and/or capping of the Ca^{2+}-ATPases within the ER. The antibody's epitope is glutaraldehyde and methanol sensitive so other fixation protocols were unsuccessful. The possibility of lateral mobility within the ER of SERCA2 proteins is under investigation.

We have compared the expression and activities of the three chicken isoforms in a number of ways. When analyzed by immunoblots (Fig. 4*D*), the bands in the SERCA2 lanes appear as broad bands. These data, in addition to some prelimonary data, suggest that the calcium pump might be a glycoprotein. Functionally, the three Ca^{2+}-ATPases are very similar in their sensitivity to Ca^{2+} as an activator and to CPA and TG as inhibitors. Since SERCA2a and SERCA2b differ only at their carboxyl termini, it is not surprising that the ATPases are indistinguishable in their apparent Ca^{2+} affinities and inhibitor sensitivities. Although there is a 15% amino acid sequence difference between SERCA1 and SERCA2, the similar effects of TG and CPA suggest that neither inhibitor interacts with isoform-specific residues. By comparing primary sequences and pharmacological sensitivities of SERCA-type pumps from a variety of species, it may be possible to predict which regions interact with TG and CPA.

In order to understand Ca^{2+}-ATPases more fully, it is helpful to compare primary sequences across a wide range of species. Chicken SERCA2a is 94% identical to its mammalian homologue while the carboxyl terminus of SERCA2b is also highly conserved. A series of mutagenesis studies has furthered our understanding of the structure/function relationship (*e.g.* Clarke *et al.*, 1989a, 1989b; Maruyama *et al.*, 1989; Vilsen *et al.*; 1989, Andersen *et al.*, 1989; Clarke, *et al.*, 1990). Of the residues shown by other laboratories to be required for the function of the Ca^{2+}-ATPase, all are completely conserved in chicken SERCA2. There is no evidence which shows that the carboxyl terminus of a calcium pump is vital for function and yet diverse species have conserved, through millions of years, alternate SERCA2a and SERCA2b termini. It remains to be determined why there is a selective advantage for birds and mammals to retain multiple isoforms of the Ca^{2+}-ATPase.

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