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Cloning and expression of an insect Ca^{2+}-ATPase from *Heliothis virescens*

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Abstract

A complementary DNA for the Tobacco Budworm, *Heliothis virescens*, sarco(endo)plasmic reticulum-type Ca^{2+}-ATPase (HVSERCA) has been cloned and sequenced. cDNA fragments of adult rabbit fast-twitch muscle Ca^{2+}-ATPase (SERCA1a) were used as heterologous probes to isolate a partial cDNA clone coding for a protein with high homology to the Ca^{2+}-ATPase from *Drosophila melanogaster* (DRSERCA) and vertebrate ER/SR Ca^{2+} pumps. The entire cDNA clone contains an ORF encoding a protein of 1000 amino acids which shares the characteristic motifs of a P-type ATPase. HVSERCA shares 89% identity with DRSERCA, 80% identity with the *Artemia* Ca^{2+}-ATPase and 72% identity with avian and mammalian SERCAs. An insect Ca^{2+}-ATPase-specific polyclonal antiserum has been raised against a fusion protein containing sequence from the cytoplasmic domain of HVSERCA. Heterologous expression of the insect pump in COS-7 cells has been demonstrated by immunocytochemistry and the reticular pattern of staining is consistent with an ER localisation. However, the expressed enzyme from COS-7 cells does not appear to be active. © 1998 Elsevier Science B.V.

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Eukaryotic cells maintain a resting level of cytoplasmic Ca^{2+} through the activity of P-type ATPases present in the plasma membrane and endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR). The SERCA family of Ca^{2+}-ATPases maintain the ER/SR Ca^{2+} pools so that intracellular Ca^{2+} signals can be generated, for example, during excitation–contraction coupling and phosphoinositide turnover [1,2]. Sequence data is now available for over 60 P-type ATPases and has facilitated extensive sequence analyses [3]. The Ca^{2+}-ATPase consists of a single polypeptide chain of about 110 kDa. Studies of the pump, including crystallography, favour ten transmembrane helices with a large cytoplasmic region between M4 and M5 [4–7]. The cytoplasmic region comprises of three domains: phosphorylation; nucleotide binding and transduction (or β-strand). These domains contain the motifs with the highest sequence conservation across the P-type ATPase family [3].

Abbreviations: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; HV-2, HVSERCA-specific rabbit antiserum; HVSERCA, sarco(endo)plasmic reticulum-type Ca^{2+}-ATPase from *Heliothis virescens*

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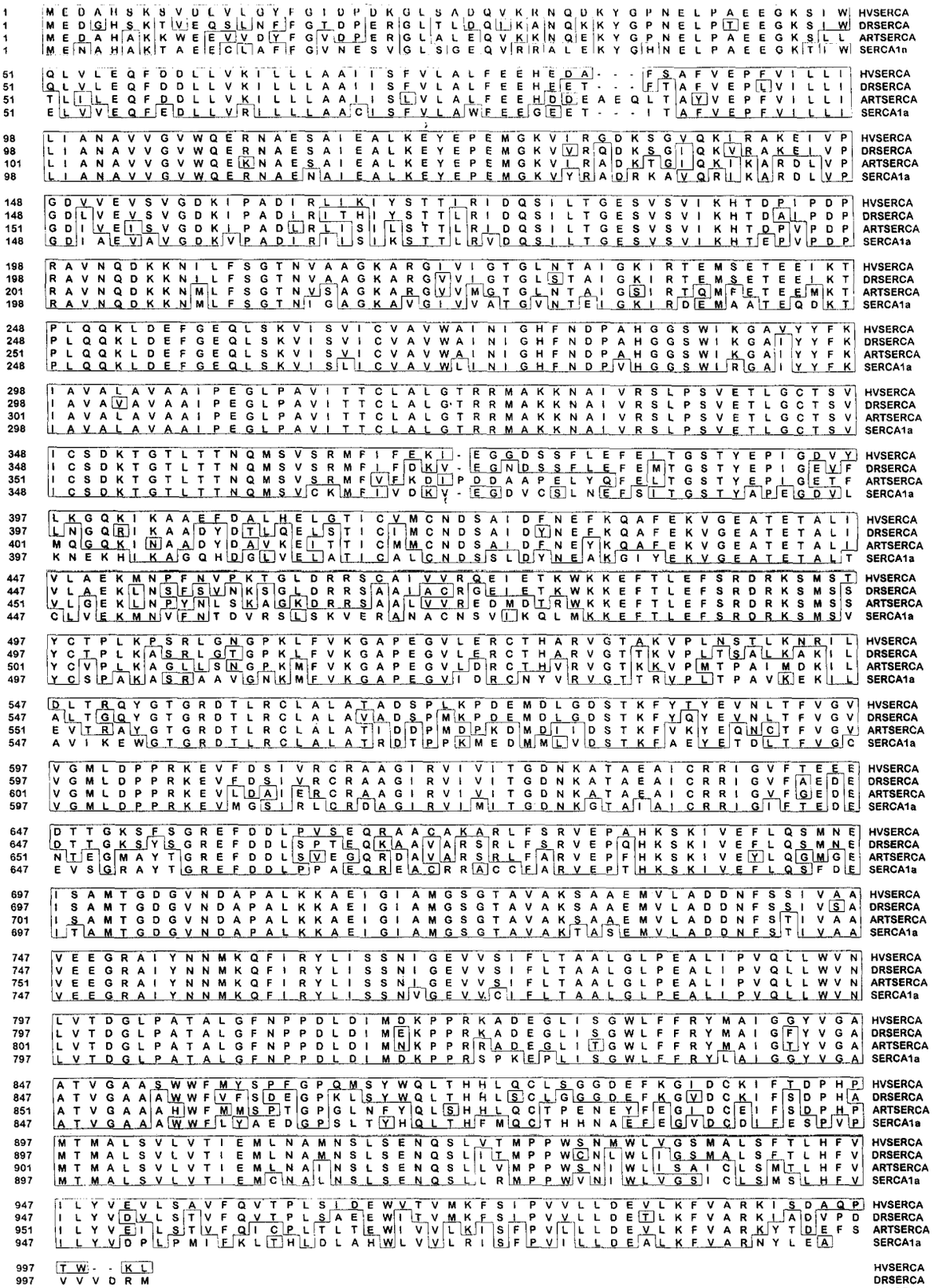
Mammals and birds have three different but related genes, *SERCA1*, *SERCA2* and *SERCA3* [4,8,9]. Differential processing of gene transcripts produces five isoenzymes that are either made in response to developmental changes, or are tissue specific. The characteristics of each of the mammalian isoforms have been compared by overexpression in COS-1 cells [10]. Invertebrates are thought to possess a single *SERCA* gene. Southern blotting under high and low stringency conditions with the same cDNA probe demonstrated the existence of only one *SERCA* gene in *Artemia* [11,12]. Similarly, the *Drosophila* genome contains a single *SERCA* gene (*DRSERCA*) as shown by Southern blot analyses [13] and chromosomal localisation [14]. The complete genes for the *Artemia* and *Drosophila* Ca^{2+}-ATPases have been isolated [11,15]. A retropseudogene containing part of the last exons of the *Artemia SERCA* gene has also been identified [12].

Northern analysis of *Drosophila* mRNA indicated the existence of a single Ca^{2+}-ATPase in the fruit fly [14]. In contrast, analysis of *Artemia* mRNA by Northern blotting detected two mRNAs of 4.5 and 5.2 kb at the embryonic stage, the longer of which became undetectable after hatching [11]. cDNA clones from both mRNAs were subsequently isolated and shown to be the products of differential splicing of a unique primary transcript. Isoform A comprises of 1003 residues, while isoform B has its last six amino acids replaced by 30 hydrophobic residues.

A random-primed cDNA library was prepared from 4th and 5th instar *Heliothis virescens*. Larval RNA was extracted by a variation of the protocol of Sambrook et al. [16] and poly A^+ RNA purified using oligo dT_{25} magnetic beads (Dynal). Random-primed cDNA synthesis was undertaken using a cDNA synthesis kit (Boehringer Mannheim) according to the manufacturer's recommendations. After phenol/chloroform extraction and ethanol precipitation of the DNA, *Eco*RI methylation was performed in 6.5 mM S-adenosyl-L-methionine, 1 times methylation buffer (100 mM Tris, pH 8.0; 1 mM Na_2EDTA), 0.4 mg/ml bovine serum albumin, 10 units *Eco*RI methylase (Pharmacia). A second phenol/chloroform extraction and ethanol precipitation was then carried out before ligation with kinased *Eco*RI linkers (Pharmacia). Excess linker was digested and the DNA applied to a Sephacryl S_{400} spin column (Pharmacia). Co-precipitation of selected cDNA and λZAP II DNA (Stratagene), digested with *Eco*RI and dephosphorylated, was performed prior to ligation according to manufacturer's recommendations. Packaging of aliquots from the ligation was carried out using the Gigapack II Packaging Extract (Stratagene).

Fragments of cDNA sequence from rabbit SERCA1a were used as probes to screen the library. Two vectors, pATH-FCA4 and pMAL-c2 PA3 (supplied by Professor D. H. MacLennan), coding for residues 615–950 and 234–607 of SERCA1a, respectively, were digested by appropriate restriction enzymes to generate DNA fragments for oligolabelling. Two heterologous probes were initially used, from 749 to 1547 (798 nt *Pst*I/*Nar*I fragment, pMAL probe) and from 1853 to 2666 (813 nt *Bam*HI/*Bgl*II fragment, pATH probe). Random-primed [γ-^{32}P]dCTP labelling was performed by a variation of Sambrook et al. [16] to a probe specific activity $> 10^8$ cpm/μg DNA.

1×10^6 clones from amplified library plates were screened with the pATH and pMAL probes. Phage DNA was transferred onto nylon membranes (Hybond-N; Amersham) followed by prehybridisation at 65°C for 4 h in 5 times Denhardt's, 5 times SSPE, 0.5% SDS and 200 μg of sonicated salmon sperm DNA. Hybridisation with the probe was performed overnight at 65°C. After autoradiography, membranes were stripped and screened with the pMAL probe. Putative positive clones identified by both probes were subsequently isolated by secondary and tertiary screens. Phagemid DNA was rescued by in vivo excision according to the manufacturer's recommendations. A partial HVSERCA cDNA clone (720–3376 nt, HVSERCA numbering) was identified and used to generate homologous probes for further screening. The 5′ end of HVSERCA was amplified directly from an aliquot of the library using a degenerate primer (5′-ATGGA[A/G]GA[T/C]GGICAC [A/T][G/C]IAA[A/G]AC-3′) based on the codons for the first 8 amino acids of DRSERCA and a 3′ HVSERCA-specific primer (5′-GTCAAGATG-GACTGATCAATACGGATG-3′). Cycling parameters were 30 cycles of denaturation at 95°C for 2 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. A further amplification of the products was performed for 20 cycles with a 3′ nested primer (5′-TGGAGTAAATCTTAATAAGACGGAT-


HVSERCA
DRSERCA
ARTSERCA
SERCA1a

GTCAG-3′) and the degenerate primer. The desired ~ 500 bp fragment was cloned with the TA Cloning Kit (Invitrogen) and used as a homologous probe to screen the library. A partial cDNA clone encoding from −157 to 2408 of HVSERCA was isolated in this way. The full-length HVSERCA cDNA was sequenced manually on both strands using the Sequenase kit (United States Biochemical Corporation). We found no evidence of any Ca^{2+}-ATPase isoforms from *H. virescens* as all cDNA clones encoded HVSERCA but this cannot be confirmed until further mRNA analysis is undertaken.

Full-length HVSERCA was constructed in the mammalian expression vector pcDNA3.1 (Invitrogen) from the two overlapping clones described above. All of the 5′ untranslated cDNA sequence apart from the Kozak translation start site [17], and much of the 3′ untranslated DNA, was deleted by PCR using Vent DNA polymerase. COS-7 cells were transiently transfected with the HVSERCA cDNA by lipofection with lipofectamine (Gibco BRL) and analyzed for expression from 15–70 h post-transfection by immunocytochemistry with an HVSERCA-specific rabbit antiserum (HV-2). The antigen was a 143 residue 6 times His fusion protein containing 86 amino acids spanning the phosphorylation site of HVSERCA from Gly-322 to Phe-407. This fusion protein had been expressed in *E. coli* using the pTrcHis vector (Invitrogen) and purified under denaturing conditions with TALON resin (Clontech).

The HVSERCA cDNA contains an ORF coding for 1000 amino acids (predicted M_r 109.5 kDa) and is the third arthropod Ca^{2+}-ATPase to be cloned. Fig. 1 displays an alignment of HVSERCA with DRSERCA [14], the *Artemia* Ca^{2+}-ATPase [18], and the chicken SERCA1a [21]. The predicted start codon lies in the sequence CCACCATGG, identical to the consensus Kozak translational start sequence [17]. HVSERCA has 89% identity and 95% similarity with DRSERCA with 78% nucleic acid sequence homology across the coding sequence. The *Artemia* Ca^{2+}-ATPase has 80% amino acid identity, compared to 72% with avian and mammalian SERCAs.

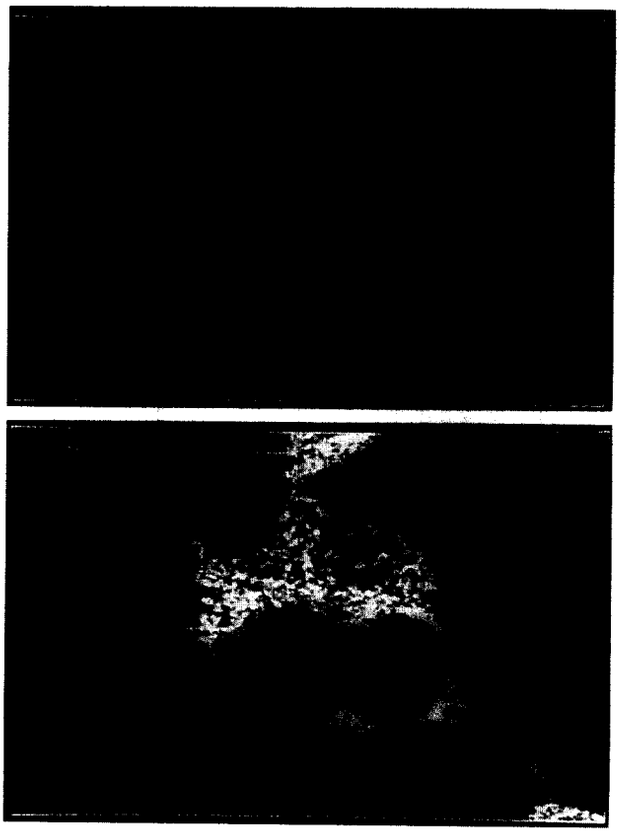


Fig. 2. Confocal micrographs showing the reticular immunofluorescence pattern of heterologously expressed HVSERCA and rabbit SERCA1a in COS-7 cells. (A, top). High magnification image of HVSERCA expression. Cells were treated with HV-2 antiserum (1:10 dilution) and sheep anti-rabbit IgG FITC conjugate (Sera Labs; 1:100 dilution). (B, bottom). Expression of rabbit SERCA1a. Cells were treated with monoclonal antibody Y/1F4 [20] and sheep ant–mouse IgG FITC conjugate. The bar shows 10 μ.

HVSERCA contains all the key functional motifs of an organellar Ca^{2+}-ATPase. If the primary sequence is compared to the sequence alignments of Fagan and Saier [3], it is consistent with the observed conserved residues found in Ca^{2+} pumps and all P-type ATPases. All 51 residues so far defined by site-directed mutagenesis as being functionally important [19] are also fully conserved.

Heterologous expression of HVSERCA in COS-7

Fig. 1. Alignment of the deduced amino acids sequences of HVSERCA, *Drosophila* Ca^{2+}-ATPase (DRSERCA) [14], the *Artemia* Ca^{2+}-ATPase (ARTSERCA) [18], and chicken SERCA1a (CHICK1A) [21]. The alignment was determined using the clustal method with the PAM250 residue weight table. Identical residues are boxed.

cells can be demonstrated by immunocytochemistry. Transfected cells express a protein specifically recognised by HV-2 antiserum as shown in Fig. 2. This has been verified by Western blotting (results not shown). Immunoblotting of microsomal proteins from *S. frugiperda* 9 cells (a line derived from ovarian cells of the related Fall Armyworm) with HV-2 also detects a band of around 110 kDa, likely to be the endogenous insect Ca^{2+}-ATPase. The antiserum does not show detectable cross-reactivity with endogenous COS cell Ca^{2+}-ATPases, nor purified or COS-expressed rabbit SERCA1a. The reticular immunostaining of HVSERCA is typical of an ER-localised protein and is morphologically identical to the pattern of SERCA1a expression in these cells. Despite the high homology between HVSERCA and mammalian SERCAs we have been unable to detect functional expression of the HVSERCA and it would seem that HVSERCA may be structurally defective in this system.

In summary, the sequence conservation between HVSERCA and other SERCA enzymes, combined with the reticular pattern of the pump in COS cells, strongly indicates that the cDNA isolated encodes an ER/SR Ca^{2+}-transporting ATPase from *Heliothis virescens*. Although heterologous expression does not appear to be functional, the use a suitable expression system (e.g. baculovirus) will enable the biochemical characterisation of an organellar insect Ca^{2+} pump.

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