Three *vha* Genes Encode Proteolipids of *Caenorhabditis elegans* Vacuolar-type ATPase

**GENE STRUCTURES AND PREFERENTIAL EXPRESSION IN AN H-SHAPED EXCRETOY CELL AND RECTAL CELLS***

(Received for publication, May 13, 1997, and in revised form, July 7, 1997)

Toshihiko Oka, Ryuji Yamamoto, and Masamitsu Futai‡

From the Department of Molecular Cell Biology, Division of Biological Science, Institute of Scientific and Industrial Research, Osaka University, Osaka 567, Japan

The proteolipids of the vacuolar-type H\(^{+}\)-ATPase (V-ATPase) are major components of the integral membrane sector. The *vha*-1 and *vha*-2 (vacuolar-type H\(^{+}\)-ATPase) genes in *Caenorhabditis elegans* encode putative 16-kDa proteolipids and are tandemly localized on chromosome III. The *vha*-2 gene has three exons, whereas *vha*-1 has no introns. The deduced amino acid sequences of the two genes exhibit about 60% identity with the homologues from yeast, mouse, and cow. The mRNAs of both *vha* genes are trans-spliced to spliced leaders, suggesting that these genes constitute a polycistronic transcriptional unit. The *vha*-4 gene consists of four exons and is very similar to the yeast VMA16 gene that codes for the 23-kDa proteolipid. This is the first example of three distinct V-ATPase proteolipids being identified in higher eukaryotes. Northern blot and transgenic analyses show that the three *vha* genes may be highly expressed in the H-shaped excretory cell, rectum, and a pair of cells posterior to the anus. These results suggest that the V-ATPase activity may be important for exporting toxic compounds or metabolic wastes in this organism.

Vacuolar-type H\(^{+}\)-ATPase (V-ATPase)\(^1\) is a multi-subunit enzyme responsible for acidification of eukaryotic intracellular organelles (1–3). V-ATPase-dependent organelle acidification is essential for intracellular processes such as protein sorting, zymogen activation, and receptor-mediated endocytosis (4–6). The proton electrochemical potential generated by the V-ATPase is coupled to transmitter uptake in neurosecretory granules, synaptic vesicles, or synaptic-like microvesicles and energizes secondary active transport in plasma membrane (7, 8).

The V-ATPase is composed of peripheral (V\(_1\)) and integral (V\(_0\)) membrane sectors similar to the F\(_{1}\)F\(_{0}\)-ATPase (9). The V\(_0\) sector creates a proton pathway across the membrane and is formed from the 16-kDa proteolipid and other subunits (3, 10). The proteolipid is a major component of V\(_0\), and chemical modification with N\(_{2}\),N\(_{2}\)-dicyclohexylcarbodiimide and directed mutagenesis experiments have identified a glutamate residue essential for proton transport (2, 3, 11, 12). cDNA clones encoding the proteolipids have been obtained from a number of species (13–17). In *Saccharomyces cerevisiae*, two genes (VMA3 and VMA11) encode 16-kDa proteolipids, and both gene products are essential for a functional V-ATPase (18–20). Furthermore, a 23-kDa proteolipid encoded by VMA16 was also found to be required for function (12). Up to now, multiple proteolipid isoforms have not been found in higher eukaryotes.

From the contiguous sequence of *Caenorhabditis elegans* chromosome III, a putative proteolipid gene has been identified (21), but the open reading frame of 302 amino acid residues is much longer than expected based on homologous proteins from other organisms. We have reassessed the *C. elegans* genomic sequence and surmised that the chromosome III locus actually contains two 16-kDa proteolipid genes. In this study, we demonstrate the existence of the two genes named *vha*-1 and *vha*-2, which are tandemly localized as a polycistronic unit. The *vha*-1 and *vha*-2 gene products are homologous to those of other organisms. In addition, a third proteolipid gene, *vha*-4, was identified on chromosome II, and its 23-kDa proteolipid product shares a high degree of homology with the yeast VMA16 protein. The promoters of three *vha* genes together with that of the V\(_1\) sector B subunit are predominantly active in an H-shaped excretory cell of the adult worm.

**EXPERIMENTAL PROCEDURES**

*General Maintenance of Worm Strains—*Wild-type Bristol N\(_{2}\) was cultured and maintained as described (22). Animals were transformed using the selectable marker plasmid, pRf4 (23).

*Sequencing cDNA Clones—*yK185dd8, yK100 p12, and y167f7 (lambda ZAP II cDNA clones of *vha*-1, *vha*-2, and *vha*-4, respectively) were kindly provided by Y. Kohara and were converted to recombinant plasmids using the Rapid Excision kit (Stratagene). The resulting plasmid pCVA-1 carried the cDNA from *vha*-1, pCV10 carried the cDNA from *vha*-2, and pCVC-1 carried the cDNA from *vha*-4. Nucleotide sequences were determined using the Dye Terminator DNA sequencing kit (Applied Biosystems). The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank™ nucleotide sequence data bases with the following accession numbers: *vha*-1, AB000917; *vha*-2, AB000918; and *vha*-4, AB000919.

*Amplification of the 5' Ends of the vha Transcripts—*Total RNA from *C. elegans* were prepared from a liquid culture of mixed growth stages using TriZOL™ LS reagent (Life Technologies, Inc.). After first-strand cDNA was synthesized with SuperScript II reverse transcriptase (Life Technologies, Inc.), PCR was performed with the following cycle conditions: 94 °C for 30 s at 68 °C for 30 cycles with SL primers (SL1 or SL2 are equivalent to the *C. elegans* spliced leader sequences) plus gene-specific primers ( *vha*-1, 5'-ctcaatgatatagctgg-3'; *vha*-2, 5'-gggaactgtaagctggtgg-3'; or *vha*-4, 5'-gatcgggtgtgagata-3').

*This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (to T. O.) and the Human Frontier Science Program Organization (to M. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*To whom correspondence should be addressed: Tel.: 81-6-879-8480; Fax: 81-6-875-5724; E-mail: m-futai@sanken.osaka-u.ac.jp.

\(^1\)The abbreviations used are: V-ATPase, vacuolar-type H\(^{+}\)-ATPase; SL, spliced leader; PCR, polymerase chain reaction; RT, reverse transcription; PCR, polymerase chain reaction; GFP, green fluorescent protein; kb, kilobase(s); bp, base pair(s).

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TABLE I

Predicted proteolipid genes in *C. elegans* chromosome

| Proposed gene structure | Gene | Predicted protein | Reference |
|-------------------------|------|-------------------|-----------|
| (A)                     | R10E11.2 | 302 a.a. | Wilson et al. (1994) |
| (B)                     | vha-1 | 169              | This study |
|                         | vha-2 | 161              |           |

Construction of the GFP Reporter Plasmids—To construct the translational GFP fusion genes, *vha-1*:GFP, *vha-2*:GFP, and *vha-4*:GFP, genomic fragments that included the upstream region and sequences encoding the first two amino acids of each *vha* gene were subcloned in-frame into the GFP (S65C mutation) reporter vectors2 (24, 25). The 6.2-kb BamHI fragment, which includes all of the *vha-1* and *vha-2* genes, was taken from cosmid R10E11 and ligated into pBluescript II to make the clone pCV-F. To create the *vha-1*:GFP fusion plasmid pCV01, the 1.3-kb BamHI to Sall fragment of pCV-F was inserted into pPD95.67. To create the *vha-2*:GFP fusion plasmid pCV02, the 2.2-kb BamHI to Sph I fragment was ligated into pPD95.70. In the case of the *vha-4*:GFP fusion, the 5.2-kb BamHI to EagI fragment of cosmid T01H3, which includes a part of the *vha-4* gene, was inserted into pPD95.67 to create pCV03. To make the GFP fusion with the putative V-ATPase B subunit gene F20B6, the 3.2-kb HindIII fragment, which includes the upstream and coding sequences for the first four amino acid residues, was taken from genomic clone F20B6 and ligated into pPD95.70.

Northern Blot Analysis—Total RNA was electrophoresed on a 1.5% agarose, 6% formaldehyde gel and transferred to a Hybond-N membrane (Amersham Life Science, Inc.). Probes were digested from cDNA clones (*vha-1*, bp +6 +281; *vha-2*, bp +5 +283; or *vha*-4, bp +21 to +359 (numbering from the first letter of the initiation codon)) and 32P-labeled using the Random Primed DNA labeling kit (Boehringer Mannheim). Hybridizations were carried out using the QuikHyb solution (Stratagene).

RESULTS

Presence of Two Genes for the 16-kDa Proteolipids of the *C. elegans* V-ATPase—Analysis of the contiguous sequence of chromosome III led to the identification of a gene in genomic clone R10E11 that was believed to encode a V-ATPase proteolipid-like protein (21). Interestingly, the open reading frame encoded a protein of 302 amino acids that is much longer than known V-ATPase proteolipids. For this reason, we reanalyzed the genomic sequence and found reasonable evidence that the chromosomal segment actually contains two genes very similar to each other (Table I). Both genes encode V-ATPase 16-kDa proteolipids with significant similarity to those known from other organisms. To verify this possibility, Northern blot analysis was carried out using probes that would differentiate between the putative genes. The two probes hybridized with distinct transcripts of different lengths (Fig. 1). These results indicate that the genomic clone R10E11 carries two genes, both of which are transcribed in vivo. The two genes were named *vha-1* (0.8-kb transcript) and *vha-2* (0.9- and 1.0-kb transcripts).

A Single Polycistronic Transcription Unit of *vha-1* and *vha-2*—*C. elegans* genes are often transcribed in clusters, and polycistronic RNAs are processed to individual mRNAs by transsplicing (26). One of two spliced leaders, SL1 or SL2, is attached to the 5'-end of almost all processed transcripts (27).

2 A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication.

3 Y. Kohara, unpublished results.
the \textit{vha-1} and \textit{vha-2} gene products are hydrophobic and possess four putative transmembrane domains. The proteins share 60\% identity with each other and exhibited 55–67\% similarity with 16-kDa proteolipids of yeast, mouse, and cow (Fig. 3B).

Especially noteworthy is the high degree of sequence conservation in the fourth transmembrane segment, which includes the putative \textit{N,N'-dicyclohexylcarbodiimide}-reactive glutamic acid (Glu-153 in \textit{Vha1} and Glu-145 in \textit{Vha-2}). This segment shares about 80\% identity, and all remaining residues are conservatively substituted.

\textit{vha-4} Codes for the 23-kDa Proteolipid—The \textit{C. elegans} genome project\textsuperscript{4} predicted that the T01H3 cosmid clone contains another potential proteolipid gene, T01H3.1, which is similar to the yeast VMA16 protein (12). Based on the Expressed Sequence Tag data base of \textit{C. elegans}, T01H3.1 was believed to be covered by five lambda cDNA clones. We sequenced both terminal regions of all cDNA inserts and selected the longest clone, \textit{yk167f7}, for further study. The full sequence was determined, and the 1002-bp insert was found to contain an open reading frame for a 214-amino acid polypeptide with five putative transmembrane segments but without a spliced leader. This gene, named \textit{vha-4}, contains three introns and maps to chromosome II.

For analysis of the 5' upstream region of the \textit{vha-4} mRNA, RT-PCR was performed using primers specific for SL1 and SL2. Only SL1 spliced leader sequence was found associated with \textit{vha-4} mRNA, suggesting that the \textit{vha-4} gene is localized on the first gene of a polycistronic unit (Fig. 4A). SL1 was located four nucleotides upstream of the initiation methionine codon.

Homology of 23-kDa Proteolipid of \textit{C. elegans} and Other Sources—As shown in Fig. 4B, comparison of deduced amino acid sequences found that the Vha-4 protein exhibited 52\% identity with the yeast VMA16 protein (12) and 64\% identity with a human homologue.\textsuperscript{5} A glutamic acid at position 100 in the middle of the third transmembrane segment is conserved in all four species. The asterisk denotes the putative \textit{N,N'-dicyclohexylcarbodiimide}-reactive glutamate residue. Putative transmembrane domains (I-IV) were defined by hydropathy analysis.

\textsuperscript{4} J. Burton, unpublished results.

\textsuperscript{5} H. Nishigori, S. Yamada, A. A., Fernald, M. M., Lebeau, T. Takeuchi, and J. Takeda, manuscript in preparation.
the three proteolipids. This residue was shown in the yeast V-ATPase to be critical for function (12).

Characterization of Three vha Transcripts—To determine the sizes of the vha-1, vha-2, and vha-4 mRNA, total RNA prepared from populations of mixed stages were hybridized with the 5' region of each cDNA. Single mRNA bands of about 0.8 kb corresponding to vha-1 and 1.0 kb corresponding to vha-4 were found and were consistent with the size of their cDNA clones (Fig. 1, lanes 1 and 3). On the other hand, two transcripts of 0.9 kb and 1.0 kb were detected by a vha-2-specific probe (Fig. 1, lane 2). The size of the longer RNA agreed well with that of the vha-2 cDNA isolated above. To assess whether the vha-2 gene contains the two polyadenylation sites, RT-PCR was carried out using a primer including an oligo(dT) sequence. Two amplified products with about a 100-bp difference were obtained. Sequence analysis showed that the polyadenylation of the shorter RT-PCR product was located 135 bp upstream of that of the longer one, which was identical to the isolated vha-2 cDNA clone. These results indicate that the vha-2 gene has two transcripts with different lengths. A cDNA clone corresponding exactly to the shorter sequence product was obtained, confirming that vha-2 is transcribed as two different forms.

The amounts of the vha-1 and vha-2 mRNA isolated were roughly the same and strengthened the notion that these two genes are transcribed as a single polycistronic unit. The amount of vha-4 mRNA was 5–10-fold lower than those of vha-1 and vha-2, which is a similar situation to yeast where the VMA16 protein is found in relatively low abundance (12).

Preferential Expression of the Three vha Genes in an Excretory Cell of C. elegans—The V-ATPase gene is expected to be a housekeeping gene because every cell has vacuole-related organelles. In addition, V-ATPase is strongly expressed in specific tissues (28–30). To determine in which C. elegans cells the vha genes were most strongly expressed, three translational fusion genes, vha-1::GFP, vha-2::GFP, and vha-4::GFP, were constructed by inserting upstream sequences plus the first two codons of the vha genes into GFP vectors. Although the GFP fusion protein contains a nuclear localization signal, the protein is known to leak to the cytoplasm. All three GFP fusion genes were strongly expressed in the large mononuclear cell with bilateral excretory canals extending along the length of the body (Fig. 5, A–E). The cell body forming a bridge between the two lateral canals is positioned on the ventral epidermal ridge slightly posterior of the nerve ring. This cell is called the H-shaped excretory cell and is believed to function in toxin and metabolic waste excretion and osmoregulation (31–34). In addition to the excretory cell, the three fusion proteins were detected in cells of the rectum (Fig. 5, B, C, and F) and a pair of cells with parallel orientation posterior to the anus (Fig. 5, C and G). No signals were detected without the control regions of the vha genes. In fact, the three fusion proteins were observed to have indistinguishable expression patterns. These results indicate that the vha promoters are strongly active in the H-shaped excretory cell, rectum, and a pair of cells posterior to the anus and imply that C.
**DISCUSSION**

We have identified three distinct *C. elegans* genes that putatively encode the V-ATPase proteolipid. All were transcriptionally active and were expressed in cells involved in excretion. The *vha-1* and *vha-2* genes form a polycistronic unit on chromosome III. The proteins coded by the two genes share 60% identity to each other and are highly similar to the 16-kDa proteolipids of yeast (19, 20, 35), *Manduca sexta* (13), *Drosophila melanogaster* (14), mouse (15), cow (17), and human (36). In yeast, VMA3 and VMA11 encode different 16-kDa proteolipids, both of which are essential for V-ATPase activity (19, 20, 35). Since *vha-1* and *vha-2* gene products share equal similarities with the VMA3 and VMA11 proteins, we could not draw definite conclusions about the correspondence of the two yeast genes to the *C. elegans* counterparts. Despite their similarities, *vha-1* and *vha-2* could not restore the negative growth of the VMA3 or VMA11 null mutants,6 which were conditionally lethal on neutral pH plates (35). A third proteolipid gene, *vha-4*, was found on chromosome II and codes for a 23-kDa proteolipid homologous to the yeast VMA16 protein, suggesting that the *vha-4* is a functional counterpart of VMA16.

The discovery of the two genes, *vha-1* and *vha-2*, for the 16-kDa proteolipid and *vha-4* gene for the 23-kDa proteolipid is the first example in higher eukaryotes of three distinct V-ATPase proteolipid genes. In the lower eukaryote *S. cerevisiae*, all three proteolipid genes (VMA3, VMA11, and VMA16) are essential for function. An important question is whether the three *C. elegans vha* gene products are all required for V-ATPase function. In this regard, the GFP reporter gene experiments found that the three proteolipids have identical expression patterns, strongly suggesting that all three isoforms are necessary (Fig. 5). One is cautioned that each proteolipid may be used in the Vo sectors of different organelles.

6 T. Oka, R. Yamamoto, and M. Futai, unpublished observation.
the overproduction of the GFP fusion proteins because transgenic animals were carrying the expression plasmid as an extrachromosomal array. This did not appear to affect the tissue-specific expression because the same expression patterns were observed for the GFP fusions under control of the upstream regions of three different vha genes. It is reasonable to assume from these results that the signals of the GFP fusion proteins are closely related to the distribution of C. elegans V-ATPase. Interestingly, the C. elegans P-glycoprotein, Pgp-3, was predominantly expressed in the H-shaped excretory cell (34). Furthermore, the ppg-3 deletion mutant was sensitive to both colchicine and chloroquine (34), suggesting that the P-glycoprotein functions in exporting toxic compounds. Because of the similar distribution of the vha genes, we suggest that the proton electrochemical potential generated by V-ATPase may also be required for exporting toxic or metabolic wastes.

The mechanisms and functions of V-ATPase have been investigated extensively and are well understood at the molecular level (3, 38); however, the roles of the enzyme in development and behavior of higher eukaryotes still remain uncertain. C. elegans is a model organism suitable for such problems using genetic approaches. The present study is the initial step in elucidating the functional roles of V-ATPase and acidic organelles in development and behavior.

Acknowledgments—We thank Dr. Andy Fire for the GFP reporter plasmids, Dr. Alan Coulson for the F20B6, R10E11, and T01H3 cosmid clones, Dr. James Kramer for plasmid pRF4, Drs. Shouhei Mitani and Kiyoshi Kita for wild-type C. elegans and T01H3 cosmid clones, Dr. James Kramer for plasmid reporter plasmids, Dr. Alan Coulson for the F20B6, R10E11, and R10E11.2 corresponding to vha-1 and vha-2, respectively, were entered in the DNA data bases.

Addendum—During preparation of this manuscript, the previous prediction of R10E11.2 (21) was revised, and the two genes named R10E11.8 and R10E11.2 corresponding to vha-1 and vha-2, respectively, were entered in the DNA data bases.

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