

Four Subunit *a* Isoforms of *Caenorhabditis elegans* Vacuolar H⁺-ATPase

CELL-SPECIFIC EXPRESSION DURING DEVELOPMENT*

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We have identified four genes (*vha-5*, *vha-6*, *vha-7*, and *unc-32*) coding for vacuolar-type proton-translocating ATPase (V-ATPase) subunit *a* in *Caenorhabditis elegans*, the first example of four distinct isoforms in eukaryotes. Their products had nine putative transmembrane regions, exhibited 43–60% identity and 62–84% similarity with the bovine subunit *a1* isoform, and retained 11 amino acid residues essential for yeast V-ATPase activity (Leng, X. H., Manolson, M. F., and Forgac, M. (1998) *J. Biol. Chem.* 273, 6717–6723). The similarities, together with the results of immunoprecipitation, suggest that these isoforms are components of V-ATPase. Transgenic and immunofluorescence analyses revealed that these genes were strongly expressed in distinct cells; *vha-5* was strongly expressed in an H-shaped excretory cell, *vha-6* was strongly expressed in intestine, *vha-7* was strongly expressed in hypodermis, and *unc-32* was strongly expressed in nerve cells. Furthermore, the *vha-7* and *unc-32* genes were also expressed in the uteri of hermaphrodites. RNA interference analysis showed that the double-stranded RNA for *unc-32* caused embryonic lethality similar to that seen with other subunit genes (*vha-1*, *vha-4*, and *vha-11*) (Oka, T., and Futai, M. (2000) *J. Biol. Chem.* 275, 29556–29561). The progenies of worms injected with the *vha-5* or *vha-6* double-stranded RNA became died at a specific larval stage, whereas the *vha-7* double-stranded RNA showed no effect on development. These results suggest that V-ATPases with these isoforms generate acidic compartments essential for worm development in a cell-specific manner.

Vacuolar-type proton-translocating ATPases (V-ATPases)¹ are large complex enzymes consisting of at least 13 subunits (1). The structure and catalytic mechanism of V-ATPases are similar to those of FoF1-ATPases (2–4). They pump protons

across membranes coupled with ATP hydrolysis. Acidification caused by V-ATPases is responsible for intracellular processes such as activation of zymogen, release of ligands from receptors, degradation of macromolecules, accumulation of neurotransmitters in secretory vesicles, and sorting of nascent polypeptides (5, 6). These enzymes are also found in the plasma membranes of osteoclasts (7), renal intercalated cells (8), and epithelial cells of seminal ducts (9).

V-ATPases have a peripheral sector (V1) for ATP hydrolysis and an integral sector (Vo) for proton translocation across membranes. Vo consists of at least five different subunits (*a*, *d*, *c*, *c'*, and *c''*). Subunits *c*, *c'*, and *c''* are often called proteolipids and function mainly to form a proton pathway (4). Subunit *d* is peripherally associated with other integral subunits (1). Subunit *a* is the largest protein (116 kDa) among the V-ATPase subunits and has been reported to have organelle- or cell-specific isoforms responsible for cellular processes (7, 10–16). In yeast, two *a* isoforms (Vph1p and Stv1p) have been found in vacuolar and Golgi/endosomal membranes, respectively (10). In mammals, three *a* isoforms (*a1*, *a2*, and *a3*) exhibit cell- and tissue-specific expressions (7, 14). In particular, the *a3* isoform is highly expressed in osteoclasts and specifically localized in their plasma membranes for bone resorption (7, 17). Furthermore, mutations of the *a3* gene have been shown to cause severe osteopetrosis (17–21). However, functions of the *a1* and *a2* isoforms remain unclear.

We have focused on V-ATPase in *Caenorhabditis elegans* to elucidate the functional roles of acidic compartments in development and cellular processes. Analysis involving RNA interference indicated that V-ATPase is indispensable for early embryogenesis of the worm (22). In this study, we identified four genes (*vha-5*, *vha-6*, *vha-7*, and *unc-32*) for subunit *a* isoforms, the first example of four distinct subunit *a* isoforms in higher eukaryotes. These genes are expressed in a cell-specific manner during worm development. The *unc-32* gene is essential for embryogenesis, and *vha-5* and *vha-6* are required for larval development, whereas *vha-7* is dispensable. The results suggest that V-ATPases with these isoforms generate specific acidic compartments required for development.

EXPERIMENTAL PROCEDURES

Maintenance of Worm Strains and Preparation of Total Lysate and Membranes—The strain used in this study, wild-type Bristol N2, was cultured and maintained as described previously (23). Transgenic worms were obtained by microinjection with the selectable marker gene, *rol-6* (*su1006*) (24).

Mixed stage worms (5 g, wet weight) were suspended at 4 °C in 25 ml of 10 mM HEPES-KOH (pH 7.4) containing 0.25 M sucrose, 10 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol. The suspension was passed through a French Press (1200 kg/cm²), and the total lysate was centrifuged at 10,000 × *g* for 15 min. The supernatant was further

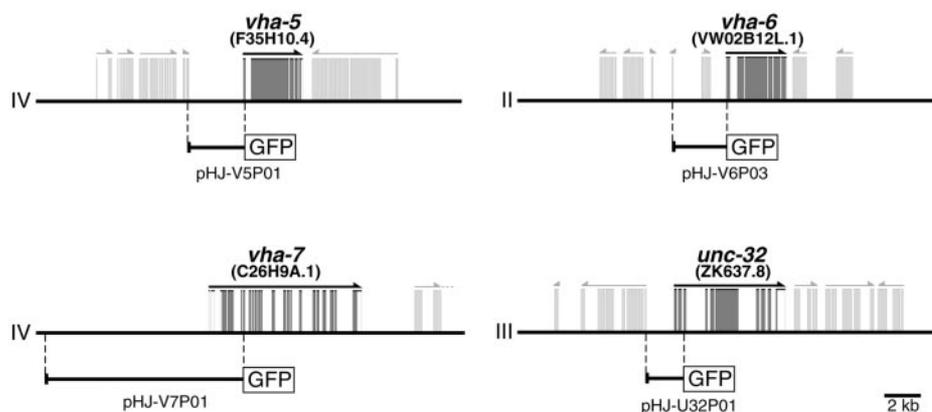
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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AB055110, AB055111, and AB055112.

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¹ The abbreviations used are: V-ATPase, vacuolar-type proton-translocating ATPases; dsRNA, double-stranded RNA; SL, spliced leader; PCR, polymerase chain reaction; GFP, green fluorescent protein; kb, kilobase(s); bp, base pair(s); L2, larval 2; L1, larval 1; EST, expressed sequence tag.

FIG. 1. Gene structures of the *vha-5*, *vha-6*, *vha-7*, and *unc-32* genes. The *vha-5*, *vha-6*, *vha-7*, and *unc-32* genes were mapped to chromosome IV, II, IV, and III, respectively. Closed and open boxes represent the coding and untranslated regions of these isoform genes, respectively. Arrows indicate the length and direction of the transcripts. The genomic fragment containing the indicated upstream region of each gene was fused with the GFP gene to examine expression (plasmid pHJ-V5P01, *vha-5*; plasmid pHJ-V6P03, *vha-6*; plasmid pHJ-V7P01, *vha-7*; and plasmid pHJ32P01, *unc-32*). Other putative genes and the directions for their transcription are also indicated by shaded boxes and arrows, respectively.



centrifuged at 100,000 \times g for 30 min. The precipitate was suspended in 50 mM Tris-HCl (pH 7.5) and used for membrane fraction.

Sequencing of cDNA Clones—Four *C. elegans* expressed sequence tag (EST) clones (yk458f4 (*vha-5*), yk17e5 (*vha-6*), yk287c7 (*vha-7*), and yk42b11 (*unc-32*)) were kindly provided by Y. Kohara and converted to plasmids with a Rapid Excision Kit (Stratagene). The nucleotide sequences of the clones were determined with a Dye Terminator DNA Sequencing Kit (Applied Biosystems). The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank™ data bases with the following accession numbers: (a) *vha-5*, AB055110, (b) *vha-6*, AB055111, and (c) *vha-7*, AB055112. The sequence of the *unc-32* cDNA clone exactly matches that deposited by Pujol *et al.* (Ref. 25; accession number AF320901; GenBank/DDBJ/EMBL).

PCR Amplification of the 5'-terminal Regions—Total RNA extraction and first-strand cDNA synthesis were carried out as described previously (26). PCR was performed under the following conditions: 30 s at 94 °C, 30 s at 61 °C, and 2 min at 68 °C for 30 cycles using SL primers (SL1 or SL2, equivalent to the *C. elegans* spliced leader sequences) and gene-specific primers (*vha-5*, 5'-CTCCAATTCGGTAAGAGTTCCCT-3'; *vha-6*, 5'-AGAGGTGACATGCTCCAGAAC-3'; *vha-7*, 5'-TAGAACTCCGGCGACAAACCAT-3'; and *unc-32*, 5'-CGTTCATTATGACCTCGC-GAAG-3'). The resulting PCR products were cloned, and their sequences were determined.

Construction of GFP Reporter Plasmids—To create fusion plasmids (*vha-5*::GFP, *vha-7*::GFP, and *unc-32*::GFP), genomic fragments including the upstream region and a part of the exons of each gene were subcloned from the corresponding cosmids into GFP reporter vectors (27): the 3.0-kb *BbvI* fragment from F35H10 was cloned into pPD95.69 to create the *vha-5*::GFP plasmid (pHJ-V5P01), the 10-kb *BamHI* fragment from C26H9 was cloned into pPD95.67 to create the *vha-7*::GFP plasmid (pHJ-V7P01), and the 2.0-kb *SacII/XhoI* fragment from ZK637 was cloned into pPD95.69 to create the *unc-32*::GFP plasmid (pHJU32P01). The 2.9-kb *MluI/EciI* fragment was amplified by PCR from *C. elegans* genomic DNA and cloned into pPD95.67 to create the *vha-6*::GFP plasmid (pHJ-V6P03).

Double-stranded RNA Preparation for RNA Interference—A specific segment of each cDNA was subcloned to prepare double-stranded RNA (dsRNA): a 1.1-kb cDNA fragment (Met-1 to Asn-371) was subcloned to prepare *vha-5* dsRNA, a 1.0-kb fragment (Glu-69 to Trp-405) was subcloned to prepare *vha-6* dsRNA, a 1.0-kb fragment (Ser-134 to Tyr-449) was subcloned to prepare *vha-7* dsRNA, and a 1.2-kb fragment (Gly-2 to Ala-405) was subcloned to prepare *unc-32* dsRNA. *In vitro* transcription and dsRNA injection were carried out as described previously (22).

Preparation of Antibodies against VHA-5, VHA-6, and UNC-32 and Immunostaining—A 510-bp (Met-1 to Val-170 of VHA-5), 350-bp (Glu-69 to Leu-180 of VHA-6), and 485-bp (Leu-80 to Val-240 of UNC-32) cDNA fragment was inserted into pGEX6P-2, pGEX5X-1, and pGEX4T-3 (Amersham Pharmacia Biotech), respectively. The recombinant proteins expressed in *Escherichia coli* were used to immunize rabbits, and the antisera were affinity-purified using the same recombinant proteins. Immunoblot analysis was carried out as described previously (22). Immunostaining of embryos (22) and larval and adult worms (28) was carried out as described previously. Fluorescence images were acquired with a LSM 510 confocal microscope (Carl Zeiss).

RESULTS

Identification of Four Isoforms of *C. elegans* V-ATPase Subunit *a*—We found that four putative genes (C26H9A.1, F35H10.4, VW02B12L.1, and ZK637.8) homologous to the bo-

vine V-ATPase subunit *a1* gene were present in the *C. elegans* genome (29). We determined the DNA sequences of the corresponding EST clones and found that they lacked the spliced leaders (SL1 or SL2) that are attached to the 5' end of almost all *trans*-spliced transcripts (30).

Using spliced leader and gene-specific primers, reverse transcription-PCR was carried out to obtain the 5'-terminal regions of the cDNA clones. All amplified products had the SL1 sequence exclusively, implying that the corresponding genes are localized upstream of polycistronic units because SL1 is not found in the downstream genes of the units (30, 31).

By combining the EST clones and PCR products, the entire cDNA sequences of these genes were determined. The cDNAs corresponding to C26H9A.1, F35H10.4, VW02B12L.1, and ZK637.8 comprised 3518, 2764, 2719, and 3158 bp (not including polyadenylation), respectively. Because these cDNAs exhibited high similarity with the mammalian counterparts, the corresponding genes were named *vha-5* (F35H10.4), *vha-6* (VW02B12L.1), *vha-7* (C26H9A.1), and *vha-8* (ZK637.8) coding for *C. elegans* subunit *a* isoforms (*vha*, vacuolar-type H⁺-ATPase). Pujol *et al.* (25) reported recently that *unc-32* mutations were found in the coding region of ZK637.8. Therefore, we named the corresponding gene *unc-32*.

Sequence comparison of these cDNAs with the *C. elegans* genome showed that these genes had different numbers of exons (*vha-5*, 5 exons; *vha-6*, 6 exons; *vha-7*, 22 exons; and *unc-32*, 10 exons; Fig. 1), indicating that gene duplication was not enough to generate these isoform genes.

Amino Acid Sequence Comparison of *C. elegans* V-ATPase Subunit *a* Isoforms with Other Sources—cDNA analysis indicated that the *vha-5*, *vha-6*, *vha-7*, and *unc-32* genes coded for isoforms with 873, 865, 966, and 894 amino acid residues, respectively (Fig. 2). Unexpectedly, VHA-7 (966 amino acids) was larger than the other isoforms and subunit *a* of different origins (7, 10–13, 15, 16). Forty-seven amino acid residues (Met-1 to Ser-47) seemed to have been additionally attached to the amino-terminal region of the ancestral protein (Fig. 2). The *C. elegans* isoforms exhibited 34.0–48.9% overall sequence identity with nine highly conserved transmembrane segments in their carboxyl-terminal halves (Fig. 2, I–IX). These isoforms also exhibited 43.4–60.4% identity with bovine subunit *a1*, with UNC-32 showing the highest identity (60.4%) (Table I). VHA-5, VHA-6, VHA-7, and UNC-32 are 62%, 78%, 78%, and 84% similar to bovine *a1*, respectively, as estimated by DNASTAR. Mutational analysis of yeast subunit *a* (Vph1p) revealed 11 amino acid residues important for activity, assembly, or intracellular sorting (32). These residues were completely conserved in the *C. elegans* isoforms (Fig. 2, boxes), supporting their functioning as V-ATPase subunit *a*.

RNA Interference of the *vha-5*, *vha-6*, *vha-7*, and *unc-32* Genes—It is of interest to determine whether or not the four

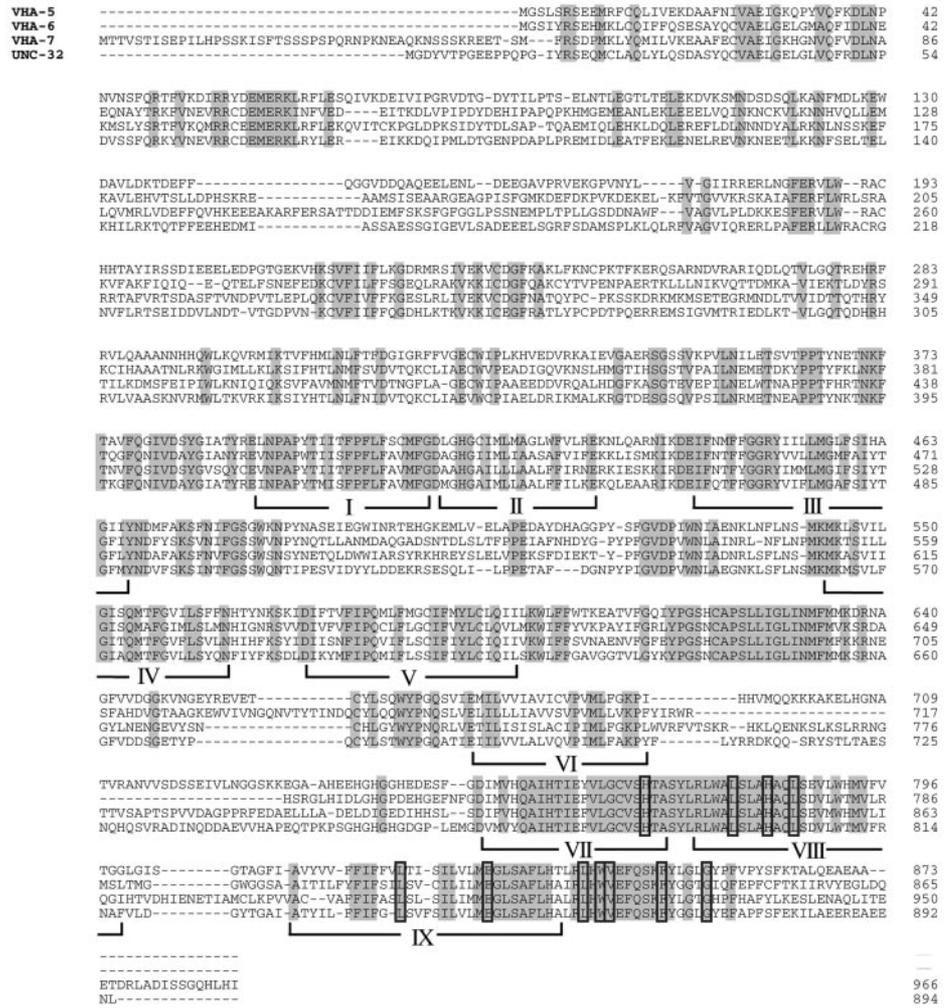


FIG. 2. Multiple alignment of *C. elegans* subunit a isoforms. The amino acid sequences of the *vha-5* (VHA-5), *vha-6* (VHA-6), *vha-7* (VHA-7), and *unc-32* (UNC-32) gene products were aligned for maximal homology. Putative transmembrane domains (I-IX) were defined by hydropathy analysis. Shaded boxes represent amino acid residues conserved in all four proteins. Open boxes indicate amino acid residues essential for the activity, assembly, or intracellular sorting of yeast V-ATPase (32).

TABLE I

Amino acid sequence comparison of the four *C. elegans* and bovine subunit a proteins

The amino acid sequences of the four *C. elegans* gene products (VHA-5, VHA-6, VHA-7, and UNC-32) and that of bovine subunit a (Bov a) (12) were aligned for maximal homology. Sequence identities (%) are shown.

Subunit a	VHA-5	VHA-6	VHA-7	UNC-32
VHA-5				
VHA-6	47.7			
VHA-7	51.5	47.9		
UNC-32	52.5	54.5	48.5	
Bov a	48.8	49.4	43.4	60.4

subunit a isoforms are expressed in the same cells and which isoforms are important for worm development. We addressed these questions by means of RNA interference, a powerful tool for silencing gene expression (33). We introduced dsRNAs of the isoform genes into adult worms to disrupt the expression of the corresponding genes in progenies. Introduction of *unc-32* dsRNA arrested almost all progenies at an embryonic stage (~100 cells) (Table II), indicating that the *unc-32* gene is required for a specific process during embryogenesis.

The progenies of worms injected with *vha-5* dsRNA were able to develop normally up to the larval 2 (L2) stage, but most of them died at this stage (Table II). In contrast, the progenies of worms injected with *vha-6* dsRNA remained at the L1 stage for several days and finally died without further development. On the other hand, *vha-7* dsRNA had no effect on the development, morphology, or behavior of the resulting progenies or the in-

TABLE II

RNA interference with the four a isoform genes

Each dsRNA (0.2 mg/ml) was injected into hermaphrodites. To exclude pre-fertilized eggs, the injected worms were transferred to new plates at 8 h after injection. They were incubated for an additional 16 h and then eggs were collected. Progenies (embryos and larvae (L1, L2, or L3)) were identified and counted at 48 or 72 h after injection, and developmental arrest was scored.

dsRNA	Injected worms	Progeny		Developmental arrest (%)
		48 h (embryo/L1)	72 h (L1/L2/L3)	
<i>vha-5</i>	14	5/268	5/248 ^a /29	87.9
<i>vha-6</i>	13	3/277	202 ^a /10/0	95.3
<i>vha-7</i>	13	5/379	2/10/352	3.3
<i>unc-32</i>	12	402 ^a /3	N.D. ^b	98.8

^a Almost all progenies had finally died at the indicated developmental stages.

^b N.D., not determined.

jected worms (Table II; data not shown). These findings indicate that V-ATPases with these subunit a isoforms may have critical functions at distinct developmental stages.

Cell-specific Expression of V-ATPase Subunit a Isoforms in *C. elegans*—We visualized the expression of these genes using GFP reporter genes. The genomic organization around these genes supports the argument that a promoter region for a polycistronic unit is present in front of the corresponding gene (Fig. 1). Based on this observation, the upstream region and a part of the exons for each isoform were inserted in front of the GFP gene.

In transgenic worms carrying the *vha-5::GFP* construct, a fluorescent signal was strongly detected in an H-shaped excre-

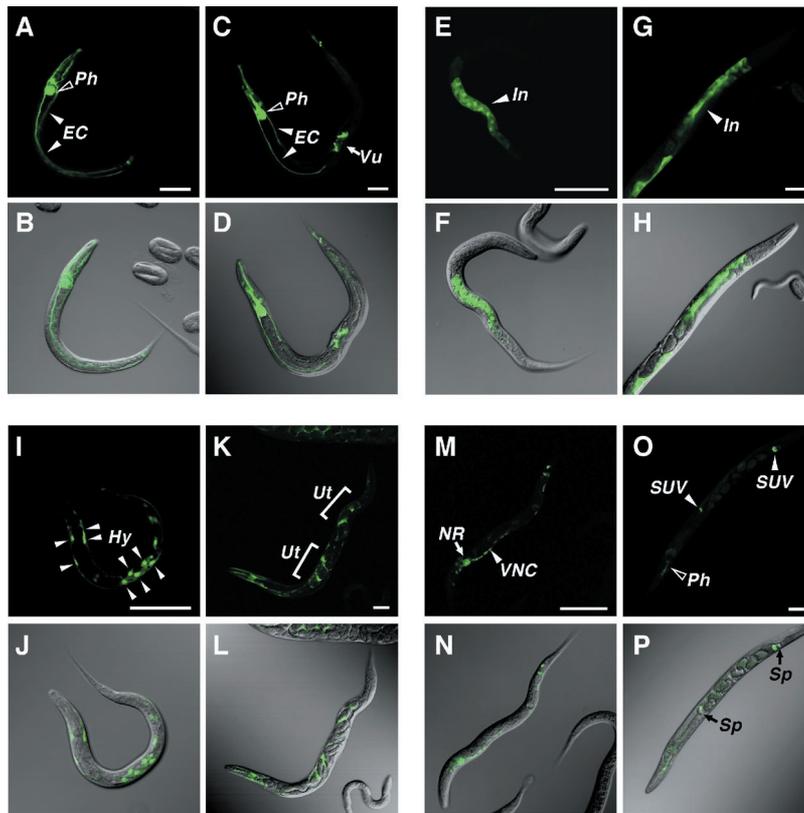


FIG. 3. Expression of the GFP reporter gene in transgenic worms. Transgenic worms carrying different expression plasmids were fixed with 4% paraformaldehyde in phosphate-buffered saline. Fluorescence confocal (A, C, E, G, I, K, M, and O) and merged images with phase contrast (B, D, F, H, J, L, N, and P) were acquired. A–D, L2 (A and B) and adult (C and D) worms carrying the *vha-5*::GFP plasmid (pHJ-V5P01). The GFP signal was detectable in an H-shaped excretory cell (A and C, *white arrowheads*) and in the pharynx (A and C, *open arrowheads*). In adult worms, the GFP fusion gene was also expressed in the hypodermis around the vulva (C, *arrow*). E–H, L1 (E and F) and adult (G and H) worms harboring the *vha-6*::GFP plasmid (pHJ-V6P03). The *vha-6*::GFP fusion gene was expressed exclusively in intestinal cells (E and G, *arrowheads*). I–L, L1 (I and J) and adult (K and L) worms carrying the *vha-7*::GFP plasmid (pHJ-V7P01). The *vha-7* promoter region was highly active in hypodermal cells. Nuclei (I, *arrowheads*) of the hypodermal cells were clearly detectable because the GFP fusion had a nuclear localization signal. The GFP signal was also detectable in the uteri of adult worms but not in embryos. M–P, L1 (M and N) and adult (O and P) worms harboring the *unc-32*::GFP plasmid (pHJ-U³²P01). In L1 larvae, the *unc-32*::GFP gene was highly expressed in the nerve ring (M, *arrow*) and the ventral nerve cord (M, *arrowhead*). The signal was clearly observed in the spermathecal-uterine valve, which is located between the uterus and the spermatheca (O, *arrowhead*). Ph, pharynx; EC, H-shaped excretory cell canal; Vu, vulva; In, intestine; Hy, hypodermal cell; Ut, uterus; NR, nerve ring; VNC, ventral nerve cord; SUV, spermathecal-uterine valve; Sp, spermatheca. Scale bars, 50 μ m.

tory cell (*white arrowheads*) and the pharynx (*open arrowheads*) at the L2 (Fig. 3, A and B) and adult (Fig. 3, C and D) stages. The H-shaped excretory cells strongly express genes for the 16-kDa proteolipid (*vha-1*, *vha-2*, and *vha-3*), the 23-kDa proteolipid (*vha-4*), and C (*vha-11*) subunits of V-ATPase (22, 26, 34). In addition to these cells, the *vha-5*::GFP fusion was found in hypodermal cells around the vulvae of adult worms (Fig. 3C, *arrow*).

The *vha-6*::GFP transgenic worms showed signals exclusively in their intestinal cells between the L1 (Fig. 3, E and F) and adult (Fig. 3, G and H) stages. The *vha-7*::GFP construct was highly expressed in hypodermal cells (Fig. 3, I, *arrowheads*, and J) and the uterus (Fig. 3, K and L). In transgenic L1 larvae (Fig. 3, M and N), the *unc-32*::GFP signals were predominantly observed in their nerve ring and ventral nerve cord, consistent with the report that the *unc-32* (*e189*) mutation causes uncoordinated movement (35). In the transgenic adult worms, the signal was detected in vulvae and spermathecal-uterine valves (Fig. 3, O and P). Weak signals were also detected in the pharynx (Fig. 3, O and P). These results indicated that subunit *a* isoform genes are expressed specifically in distinct cells and that their expression patterns do not overlap.

Immunodetection of the *vha-5* and *vha-6* Gene Products—Reporter gene analysis revealed that the four isoforms were expressed in distinctly different cells. Thus, it became of interest to determine their intracellular localization during devel-

opment. Of these genes, the *vha-5* and *vha-6* genes were essential for larval development, whereas the *unc-32* (Table II), proteolipid, and C subunit genes (22) were required for embryogenesis. We focused on VHA-5 and VHA-6 because analysis of their locations may provide clues with regard to acidic compartments or cells required for larval development. Affinity-purified antibodies against VHA-5 clearly recognized a single band (106 kDa) for a lysate of a mixed-stage population (Fig. 4A, *lane 1*). Anti-VHA-6 antibodies bound specifically to a 100-kDa band exhibiting slight smearing (~ 106 kDa) (Fig. 4A, *lane 2*). These molecular masses were larger than the calculated molecular weights (99,311, VHA-5; 98,538, VHA-6), suggesting that VHA-5 and VHA-6 were posttranslationally modified. Because bovine subunit *a* is known to be an N-linked glycosylated protein (36), the two isoforms may also be N-linked glycosylated.

Immunoprecipitation of a Isoforms with Other V-ATPase Subunits—The membrane fraction of a mixed-stage population was incubated with octylglucoside, the solubilized fraction was treated with antibodies against each isoform, and 12% polyacrylamide gel electrophoresis was performed on the immunoprecipitate in the presence of sodium dodecyl sulfate. As shown by immunoblot (Fig. 4B), the immunoprecipitate obtained with antibodies against VHA-5, VHA-6, and UNC-32 contained subunit C of the membrane extrinsic V1 sector. These results, together with high similarities to the bovine $\alpha 1$ isoform, suggest that the VHA-5, VHA-6, and UNC-32 isoforms were as-

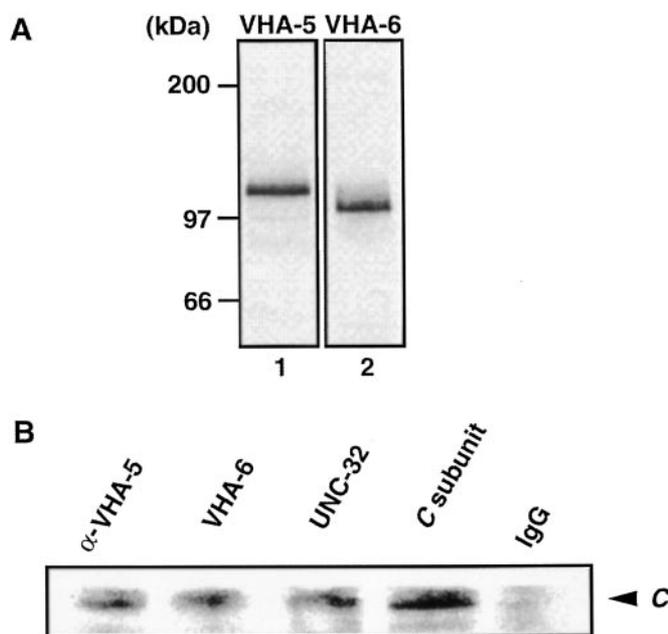


FIG. 4. Association of subunit *a* isoform with V1 subunit. *A*, immunoblot of VHA-5 and VHA-6. *C. elegans* total lysate (30 μ g) of a mixed-stage population was subjected to polyacrylamide-gel electrophoresis and blotted onto a nitrocellulose membrane. The membrane was incubated with affinity-purified antibodies against VHA-5 (lane 1) or VHA-6 (lane 2). Immunodetection was carried out using alkaline phosphatase-conjugated antibodies, 5-bromo-4-chloro-3-indolyl phosphate, and 4-nitro blue tetrazolium chloride. VHA-5 and VHA-6 were recognized as 106-kDa and 100–106-kDa bands, respectively. *B*, association of VHA-5, VHA-6, and UNC-32 with the V1 subunit of V-ATPase. Membranes (500 μ g of protein) of a mixed-stage population were incubated at 4 °C for 2 h in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2% octylglucoside, and 200 mM NaCl. The solubilized fraction (100 μ g of protein) was incubated for 1 h with antibodies against VHA-5, VHA-6, or UNC-32. The immunoprecipitate was subjected to 12% polyacrylamide gel electrophoresis and blotted onto a nitrocellulose paper. The position of C subunit is indicated by an arrowhead. The paper was incubated with antibodies against C subunit. The solubilized fraction precipitated with antibodies against C subunit (C subunit) and treated with preimmune IgG (IgG) are shown as controls. The other immunoprecipitation method was as described previously (4).

sembled with other V-ATPase subunits. Thus, immunohistochemical studies using the antibodies may indicate the location of V-ATPases with individual isoforms.

Localization of VHA-5 in Embryos, Larvae, and Adults—VHA-5 was detectable in a dot-like compartment in every embryonic cell (Fig. 5, *A*, *B*, and *E*, arrowheads). The compartment was found exclusively around the nucleus and observed during embryogenesis (4-cell stage, Fig. 5*A*; 8-cell stage, Fig. 5, *B* and *C*; and ~100-cell stage, Fig. 5, *E* and *F*). A cup-shaped structure was often observed around nuclei instead of the dot-like compartment (Fig. 5*D*, arrows). On the other hand, VHA-5 expression changed dramatically at the larval stage (Fig. 5*G*) and became dominant in an H-shaped excretory cell (Fig. 5, *H* and *I*), consistent with the results for transgenic worms carrying the *vha-5::GFP* fusion gene. VHA-5 expression was not observed in the pharynx, where the *vha-5::GFP* reporter was active, indicating that the expression may be due to an increased copy number of the reporter plasmid.

Distribution of VHA-6 in Embryos and the Intestine—VHA-6 was preferentially localized in dot-like structures in P2 cells of 4-cell stage embryos (Fig. 6, *A*, arrow, and *B*). Only single cells continued to express VHA-6 during further development (Fig. 6*C*, arrows). After the cleavage stage (~300 cells), when intestinal cells appear, VHA-6 was detected in two distinct cell types (Fig. 6*D*, arrows). These two cell types were aligned on both lateral sides at the comma stage (Fig. 6*E*, arrows). The expres-

sion pattern of VHA-6 was similar to the cell-specific distribution of P granules, which are passed onto a germ line cell lineage from P2 cells (37).

After the comma stage in embryos, expression of VHA-6 changed. In 2-fold embryos, VHA-6 was limited to the intestine (Fig. 6*F*, arrowheads). Such intestinal expression was also observed in larvae (L1, Fig. 6*G*; L3, Fig. 6*H*) and adults (data not shown). The *C. elegans* intestine comprises a tube formed from 20 cells with microvilli on their apical surface (38). VHA-6 was clearly detectable on the apical surface of intestinal cells, forming two lines along the worm body (Fig. 6, *G* and *I*, arrowheads). Furthermore, VHA-6 was clearly found at the junction between the pharynx and the intestine (Fig. 6, *H* and *I*, arrows). These results indicate that VHA-6 on the apical surface of intestinal cells may function by taking up nutrients through microvilli.

DISCUSSION

We have identified four genes (*vha-5*, *vha-6*, *vha-7*, and *unc-32*) encoding subunit *a* isoforms of *C. elegans* V-ATPase. These gene products exhibit high identity and completely retain 11 amino acid residues essential for the activity, assembly, or intracellular sorting of yeast V-ATPase (32). We have found no homologues in the *C. elegans* genome except for these genes. These results indicate that this is the first example of four distinct isoforms of V-ATPase subunit *a* being identified in higher eukaryotes. Pujol *et al.* (25) reported that the *unc-32* gene gives rise to six different transcripts through alternative splicing. However, no alternatively spliced transcript for *vha-5*, *vha-6*, or *vha-7* has been found in the EST data base.² Subunit *a* isoforms have been identified in human (11–13), mouse (7, 14), cow (15), chicken (16), and yeast (10). We recently found a fourth isoform ($\alpha 4$) of mouse subunit *a*, similar to the case of *C. elegans*,³ implying that at least four *a* isoforms are required in higher eukaryotic cells.

Transgenic analysis involving the GFP reporter gene showed that the four isoforms were expressed in a cell-specific manner. The *vha-5*, *vha-6*, *vha-7*, and *unc-32* promoters were highly active in the H-shaped excretory cells, intestine, hypodermis, and nerve cells, respectively. It is noteworthy that the *vha-7* and *unc-32* genes were strongly expressed in the uteri of hermaphrodites. Thus, the two gene products may have maternal roles in embryonic development.

VHA-5 was expressed predominantly in H-shaped excretory cells in adult worms. The excretory cells are thought to function in osmoregulation and the excretion of toxic and metabolic waste (39–42). We found that the cells preferentially express the genes for the 16-kDa proteolipid (*vha-1*, *vha-2*, and *vha-3*), the 23-kDa proteolipid (*vha-4*), and the C (*vha-11*) subunits of V-ATPase (22, 26, 34). These findings suggest that VHA-5 may be a critical isoform of V-ATPase with regard to its function in the H-shaped excretory cells. Furthermore, RNA interference analysis of the *vha-5* gene showed that the progenies of worms injected with *vha-5* dsRNA died specifically at the L2 stage. These results imply that further development of L2 larvae requires excretory functions driven by V-ATPase with the VHA-5 isoform.

V-ATPases localized on plasma membranes are known to generate an acidic environment outside the cells. They are found in osteoclasts (7), renal intercalated cells (8), epithelial cells of seminal ducts (9), and the bladder (43). Toyomura *et al.* (7) have clearly shown that the mouse subunit $\alpha 3$ isoform plays important roles in determining the V-ATPase localization in the plasma membrane. We have shown that VHA-6 is distrib-

² T. Oka and M. Futai, unpublished data.

³ T. Oka and M. Futai, manuscript in preparation.

FIG. 5. Immunostaining of VHA-5. Embryos at different cleavage stages (A, 4 cells; B, 8 cells; D, 16 cells; and E, ~100 cells) and adult worms (G and H) were fixed and then incubated with the affinity-purified antibodies against VHA-5. A–F, in embryos, VHA-5 was significantly observed on a dot-like compartment (A, B, and E, arrowheads) in every cell. Cup-shaped structures (D, arrows) were often detectable instead of dot-like structures (focused on the three fluorescent cells). C and F are phase-contrast images of B and E, respectively. The dark area in each cell in embryos represents the nucleus (E, open arrowhead). G–I, in adult worms, VHA-5 was strongly expressed in an H-shaped excretory cell and was detectable in its cell body (G, arrowheads) and canals (H, arrow). I is a phase-contrast image of H. Scale bars, 20 μ m (A, B, D, and E) or 50 μ m (G and H).

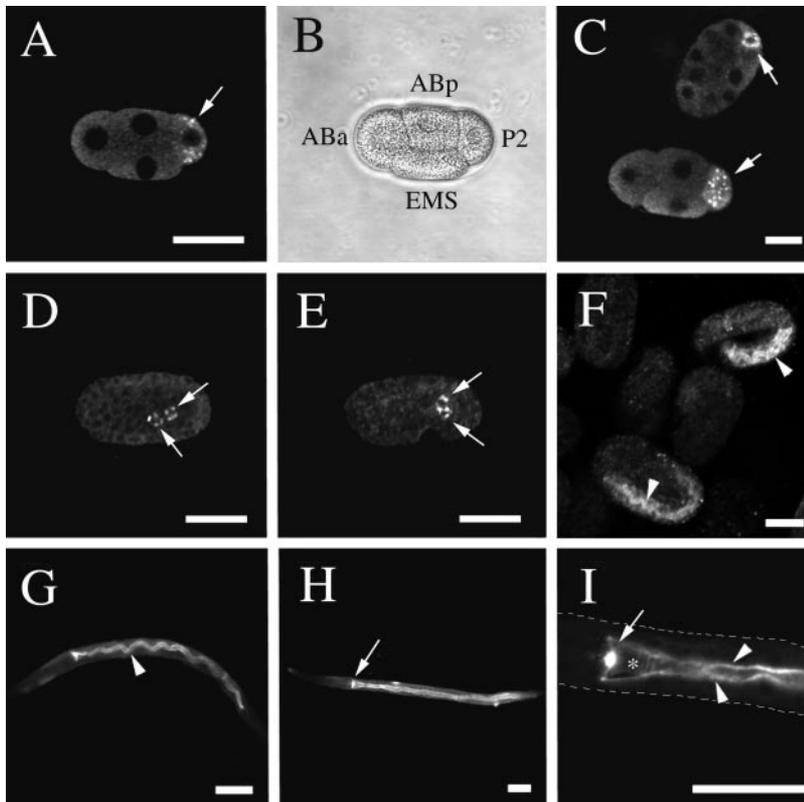
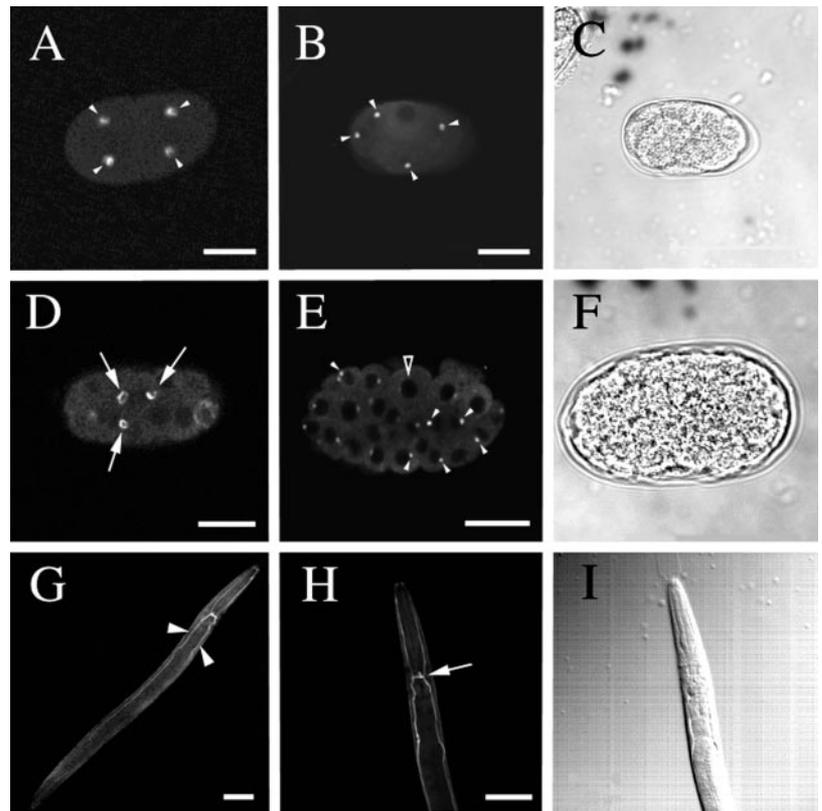


FIG. 6. Immunostaining of VHA-6. Embryos at different cleavage stages (A, 4 cells; C, 4 and 32 cells; and D, ~200 cells), ones at the comma stage (E), 2-fold embryos (F), L1 larvae (G), and adult worms (H and I) were fixed and then incubated with the affinity-purified anti-VHA-6 antibodies. A–C, in early embryos, VHA-6 was clearly detectable on dot-like structures (arrows) in a germ line cell lineage. B is a phase-contrast image of A: ABa, ABp, EMS, and P2 cells were identified by their morphology. D, after the cleavage stage (~200 cells), VHA-6 was expressed in two kinds of cells (arrows). E, at the comma stage, the two types of cells highly expressing VHA-6 (arrows) were aligned on both lateral sides. F, in 2-fold embryos, VHA-6 was strongly observed on the luminal sides (arrowheads) of the intestine. G, in L1 larvae, VHA-6 was strongly expressed on the apical surface of intestinal cells (arrowhead). H and I, in addition to intestinal cells, VHA-6 was also detectable at the junction between the pharynx and the intestine (arrows) of adult worms. I is a higher magnification of H. Dashed lines indicate the edges of the worm body. The area denoted by an asterisk represents the intestinal lumen of the worm. Scale bars, 20 μ m (A, C, D, E, and F) or 50 μ m (G–I).

uted specifically on the apical surface of intestinal cells in larval and adult worms. The amino-terminal region (1–140 amino acid residues) of VHA-6 was sufficient for localization on the apical surface (data not shown). We speculate that VHA-6 is a key subunit for the sorting of V-ATPase to the plasma membrane to acidify the intestinal lumen.

RNA interference analysis showed that *vha-6* dsRNA ar-

rested larval development at the L1 stage. The L1 larvae did not die immediately but remained alive for up to a week. This phenotype is similar to that of L1 larvae starved after hatching. The L1 larvae of the *vha-6* dsRNA-injected worms took up a fluorescence dye, DiO, into their intestinal lumen (data not shown), indicating that *vha-6* dsRNA did not affect the food intake ability of the worms. Thus, it is reasonable to assume

that defective proton transport into the intestinal lumen due to the lack of VHA-6 arrested worm development at the L1 stage. These results suggest that luminal proton transport by V-ATPase with the VHA-6 isoform may be essential for the digestion of food or uptake through microvilli. In this regard, V-ATPase localized on the plasma membrane in the larval midgut of the tobacco hornworm, *Manduca sexta*, is important to generate a proton gradient (44).

In contrast to the intestinal expression during postembryonic development, VHA-6 was found predominantly in P2 cells in 4-cell embryos. The expression continued to a germ line cell lineage (P3, P4, Z2, and Z3 cells) until the comma stage. Introduction of *vha-6* dsRNA abolished this embryonic expression (data not shown). Although Z2 and Z3 cells finally proliferate to germ cells (45), VHA-6 was not observed in the gonads or spermathecia of adult worms. It should be noted that the expression of VHA-5 and VHA-6 changed dramatically between the embryonic and larval stages. As described above, expression of VHA-6 was observed in germ line cells during the embryonic stage but was observed in intestinal cells in larvae and adults. This suggests the requirement of different acidic compartments during development.

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Addendum—During preparation of this manuscript, Pujol *et al.* (25) reported that the *unc-32* mutations have been mapped within an open reading frame of the ZK637.8 gene, which we originally named *vha-8*. They have deposited the cDNA sequences of six alternatively spliced transcripts of the *unc-32* gene in the DNA data bases. We have used the name *unc-32* throughout this study. Pujol *et al.* (25) showed the presence of subunit *a* isoform transcripts by *in situ* hybridization, which is essentially consistent with our results with regard to GFP fusion genes and immunostaining.

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**Four Subunit *a* Isoforms of *Caenorhabditis elegans* Vacuolar H⁺-ATPase:
CELL-SPECIFIC EXPRESSION DURING DEVELOPMENT**
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