Identification of the copper chaperone, CUC-1, in *Caenorhabditis elegans*: tissue specific co-expression with the copper transporting ATPase, CUA-1

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Abstract A cDNA encoding a putative copper chaperone protein, CUC-1, was cloned from *Caenorhabditis elegans*. CUC-1 had the characteristic motifs of MTCXXC and KKTGK, and showed 49.3 and 39.1% sequence identity with yeast Atx1p and human HAH1, respectively. Expression of CUC-1 cDNA complemented a null *atx1* mutant, the yeast copper chaperone gene, thus demonstrating that CUC-1 is a functional copper chaperone. Studies with transgenic worms indicated that *cuc-1* and *cua-1*, which encodes the copper transporting ATPase, are expressed together in intestinal cells of adult and hypodermal cells in the larvae. *cua-1* was also expressed in pharyngeal muscle but *cuc-1* was not. These results suggest that CUC-1 and CUA-1 constitute a copper trafficking pathway similar to the yeast counterparts in intestinal and hypodermal cells, and CUA-1 may have a different function in pharyngeal muscle.

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Key words: *ATX1*; Copper chaperone; CUC-1; Menkes disease; Wilson disease; *Caenorhabditis elegans*

1. Introduction

Three independent pathways for intracellular copper ion trafficking have been shown in yeast [1–4]. One pathway involves Atx1p and Ccc2p which transport copper to a post-Golgi compartment. Cu(I) is transported into the cytosol through the plasma membrane by Ctr1p, picked up by the copper chaperone Atx1p, and carried to the Cu transporting ATPase, Ccc2p, in the post-Golgi membranes [3,4].

Ccc2p is a homologue of the human Menkes [5–7] and Wilson [8–10] disease transporters, and of the *C. elegans* CUA-1 copper ATPase [11,12]. Expression of cDNA clones coding for these ATPases complement a null mutation of the yeast *CCC2* gene [11–15]. Potential *ATX1* homologues were found in *A. thaliana*, *O. sativa* and human in the GenBank dbest database and *C. elegans* from genomic sequence databases [16]. These findings suggest that the Atx1p/Ccc2p copper trafficking pathway is also present in higher eukaryotes. Consistent with this prediction, the human homologue, HAH1, complemented a yeast Δ*atx1* mutation [17]. Northern blot analysis identified HAH1 mRNA in all human tissues and cell lines examined [17]. Interestingly, Menkes and Wilson copper ATPases had a more restricted tissue distribution [5,7–9].

In this report, we ask if the *ATX1* homologue in *C. elegans* is also ubiquitously expressed in the same manner as *HAH1* or in a restricted fashion. If a trafficking pathway similar to yeast is also found in *C. elegans*, the chaperone would be expected to have a similar distribution as the CUA-1 copper ATPase. In this study, we cloned a cDNA from *C. elegans* for the Atx1p homologue which complemented the yeast Δ*atx1* mutation. The expression pattern was similar to *cua-1* except in the pharyngeal muscle where the CUA-1 ATPase is strongly expressed and the *cuc-1* chaperone is not.

2. Materials and methods

Wild-type strain N2 and transgenic *C. elegans* were cultured as described [18]. Standard methods [19] were used for the molecular cloning and the genomic DNA preparation. The partial cDNA for *C. elegans ATX1* homologue was obtained by 5′- and 3′-RACE using primers CeATX-1 Fwd (5′-TACGTTTTCGAAATCGGCATGACA-T-3′) and CeATX-1 Rev (5′-GTAGTTGCTTGATCTCTTTTCCTG-3′), respectively, with Marathon cDNA amplification kit (Clontech). Then the second PCR amplification was carried out on these 5′- and 3′-parts of the cDNA using the adapter primers. The obtained full-length cDNA was subcloned into pBluescript II SK$^+$ and its structure was determined by sequencing. The nucleotide sequence data reported in this paper will appear in DDBJ, EMBL and GenBank nucleotide sequence databases with accession number AB017201. Northern blot analysis was carried out using a Hybond-N$^+$ nylon membrane (Amersham Life Science), and the probe labeled with random primed DNA labeling kit (Boehringer Mannheim) [19].

Yeast DNA segment (−398 to +800) containing the *ATX1* gene [20] was introduced into pRS316 [21] to generate pATX1. The Δ*atx1::LEU2* strain was constructed by deleting the *ATX1* gene of YPH499 [21] by homologous recombination using a DNA fragment in which the *ATX1* open reading frame was replaced with *LEU2*. *cuc-1* cDNA was introduced into yeast expression plasmid, pKT10 [22], to generate pKTcuc-1. pKTcuc-1-VSV was constructed by inserting the DNA sequence corresponding to Tyr-500 to Lys-511 of VSV glycoprotein [23] between the Met-1 and Thr-2 of CUC-1 by PCR. Iron-dependent growth was examined on agarose plates containing synthetic medium with 2% glucose and 25 mM Na-MES (2-(*N*-morpholino)ethanesulfonic acid), pH 6.1 [17,24]. Where indicated, 1 mM ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; Sigma), or 350 μM ferrous ammonium sulfate was added to the medium.

The 1.7-kbp genomic fragment of the *cuc-1* gene was amplified by PCR using a primer pair cucUP (5′-CGGGATCCGTAGTTGCTT-GATCTCTTTTCCTG-3′) and cucE3 (5′-CACATGCATGCTTGG-GTTCACTGGTTGAACTGCGGA-3′), digested by *Sph*I and *Bam*HI and introduced into a promoter-less GFP reporter plasmid pPD95.70 (kindly provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu). Similarly, the upstream regulatory and a part of the coding region of the *cua-1* gene was amplified by primers cuaLA10 (5′-ACGCG-TCGACTCCTCCAATTGTTGGTCCGGTTCCTGTTTCT-3′) and cuaex2.0 (5′-GTAATTGGAGCGAAGCCAGGAATTCATAGGG-

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Abbreviations: bp, base pair(s); kb, kilobase(s); kDa, kilodalton; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; VSV, vesicular stomatitis virus

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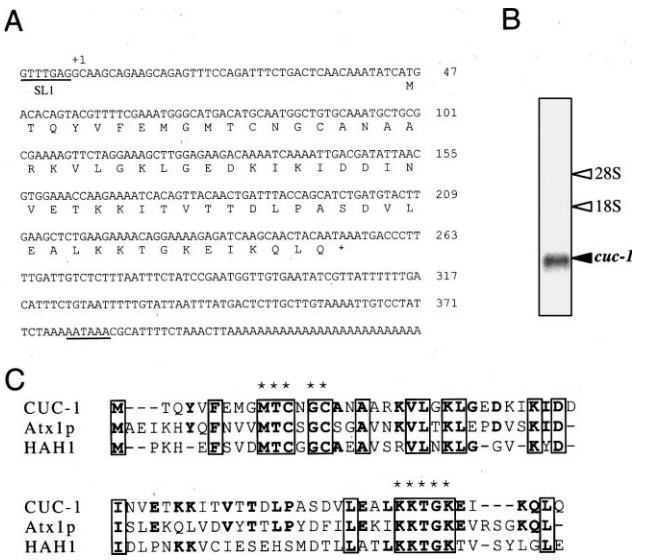

Fig. 1. cDNA structure and Northern blotting of *cuc-1*. A: Structure of the *cuc-1* cDNA and its protein. The partial spliced leader sequence (SL1) and polyadenylation signal were underlined; +1, spliced leader acceptor site. B: Northern blot analysis of total RNA from a mixed-stage *C. elegans* population using *cuc-1* cDNA as a probe. Filled and open arrowheads represent *cuc-1* transcript and ribosomal RNAs, respectively. C: Alignment of Atx1p homologues. Deduced amino acid sequence of CUC-1 was aligned with those of yeast Atx1p and human HAH1. Conserved residues among the three proteins are boxed. Residues identical to CUC-1 are in bold letters. The characteristic copper binding motif, MTCXXC, and the lysine rich sequence, KKTGK, are indicated by asterisks.

ATCCCG-3′) and introduced into the *Bam*HI and *Sal*I sites of the GFP reporter plasmid pPD95.67.

3. Results

3.1. Cloning a cDNA for an ATX1 homologue from C. elegans

An open reading frame exhibiting strong homology to yeast copper chaperone (Atx1p) was found in the *C. elegans* genome [16,20]. The frame was covered by cosmid ZK652 from chromosome III and named *cuc-1* gene in this study. A cDNA corresponds to this locus was obtained by 5′- and 3′-RACE using a specific primer set. The cDNA structure exactly matched the 3′-portion of open reading frame ZK652.10 which was predicted by the *C. elegans* genome project [16]. However, we found that the 5′-part of the cDNA was shorter than the predicted one. The isolated cDNA contained a *trans*-spliced leader sequence (SL1) and poly(A) tail, indicating that it represents a full length message (Fig. 1A). Because spliced leaders are attached to the 5′-end of *C. elegans* transcripts and SL1 is attached only to the most upstream genes of operons [25,26], this finding suggested that the *cuc-1* gene is located immediately downstream of the promoter. Consistent with this notion, *cuc-1* mRNA was amplified by reverse tran-

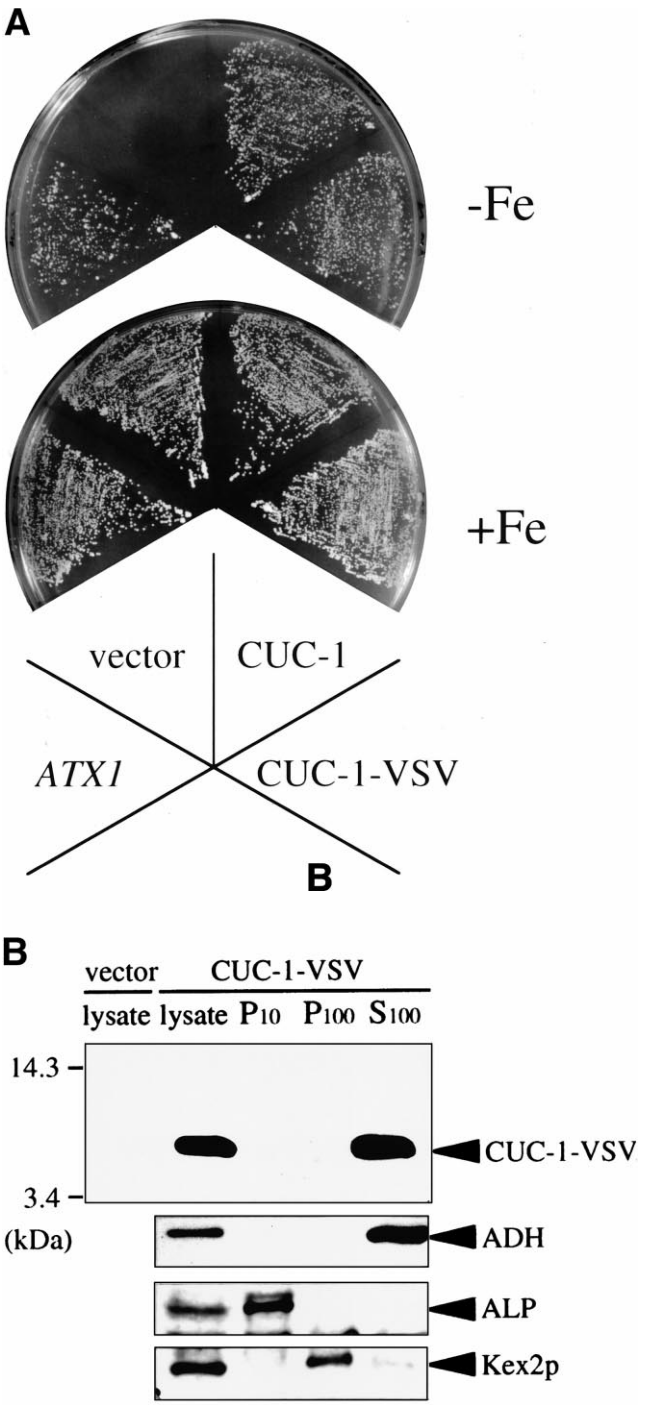

Fig. 2. Expression of CUC-1 in the yeast *atx1* null mutant. A: Complementation of Δ*atx1* by *cuc-1*. The Δ*atx1* cells harboring vector with no insert (vector), the CUC-1 expression plasmid (CUC-1), CUC-1-VSV tag expression plasmid (CUC-1-VSV), or the *ATX1* on a single copy plasmid (*ATX1*) were grown for 5 days at 30°C on minimal medium containing 1 mM ferrozine (−Fe) or 350 μM ferrous ammonium sulfate (+Fe). B: Subcellular localization of CUC-1. Cells of Δ*atx1* harboring vector with no insert and CUC-1-VSV tag expression plasmid were converted to spheroplasts and osmotically lysed. The total lysate (lysate) of the spheroplasts was centrifuged at $10\,000\times g$ to obtain low speed pellet (P_{10}). Then, the supernatant was centrifuged at $100\,000\times g$ to obtain pellet (P_{100}) and supernatant (S_{100}) fractions. Each fraction from 1.5×10^7 cells was loaded on a polyacrylamide gel in the presence of sodium dodecylsulfate. After electrophoresis, the proteins were electrotransferred to nitrocellulose and probed for VSV-tagged CUC-1 using the mouse monoclonal antibody P5D4 (1 μg/ml, Boehringer Mannheim). Organelle marker proteins alcohol dehydrogenase (ADH, cytosol), alkaline phosphatase (ALP, vacuolar membrane), and Kex2p (late Golgi) were also detected by specific antibodies. Blots were developed using the ECL detection kit (Amersham).

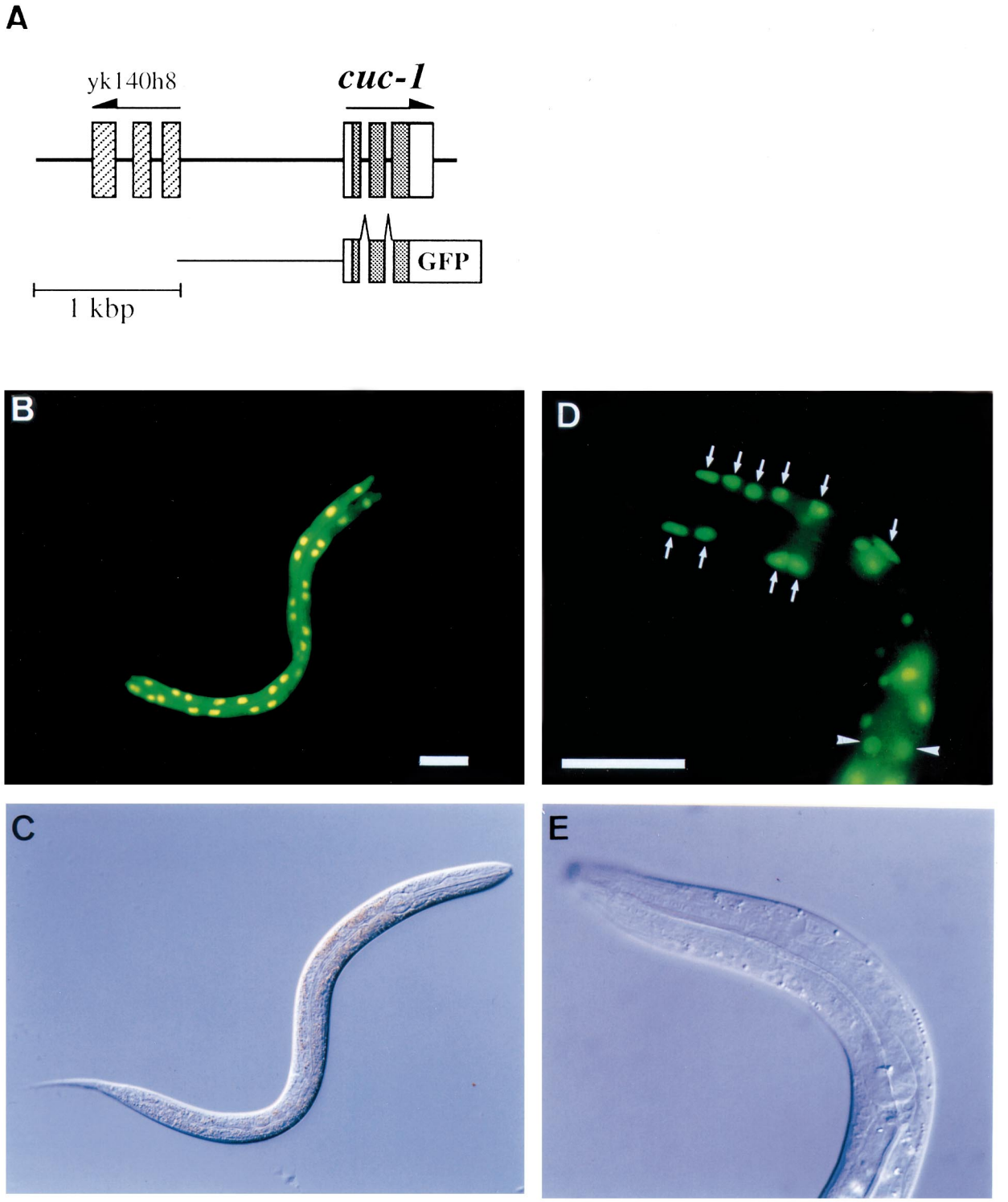

Fig. 3. Expression of *cuc-1* in intestinal and hypodermal cells. A: Structure of gene *cuc-1* and a fusion gene *cuc-1::GFP*. Filled and open boxes represent coding and untranslated regions, respectively. Hatched boxes represent exons corresponding to clone yk140h8 identified in the Expressed Sequence Tag database. Direction of transcription is indicated by arrows. A 1.7-kbp DNA fragment containing the upstream region and the *cuc-1* open reading frame was fused with a GFP reporter gene. Three transmitting lines carrying the *cuc-1::GFP* construct were established and had indistinguishable expression patterns. B: Fluorescence micrograph of a young adult worm showing intestinal expression. C: Nomarski micrograph of the same worm in B. D and E: Lateral view of anterior half of L1 larvae. Nuclei in the head corresponding to the hyp-5, hyp-6, and hyp-7 cells are indicated by the arrows. Hypodermal cell nuclei of the body region are indicated by the arrowheads. Expressions were repressed in dauer larvae (not shown). Other procedures are described in the text or in [30]. Scale bars indicate 100 μm.

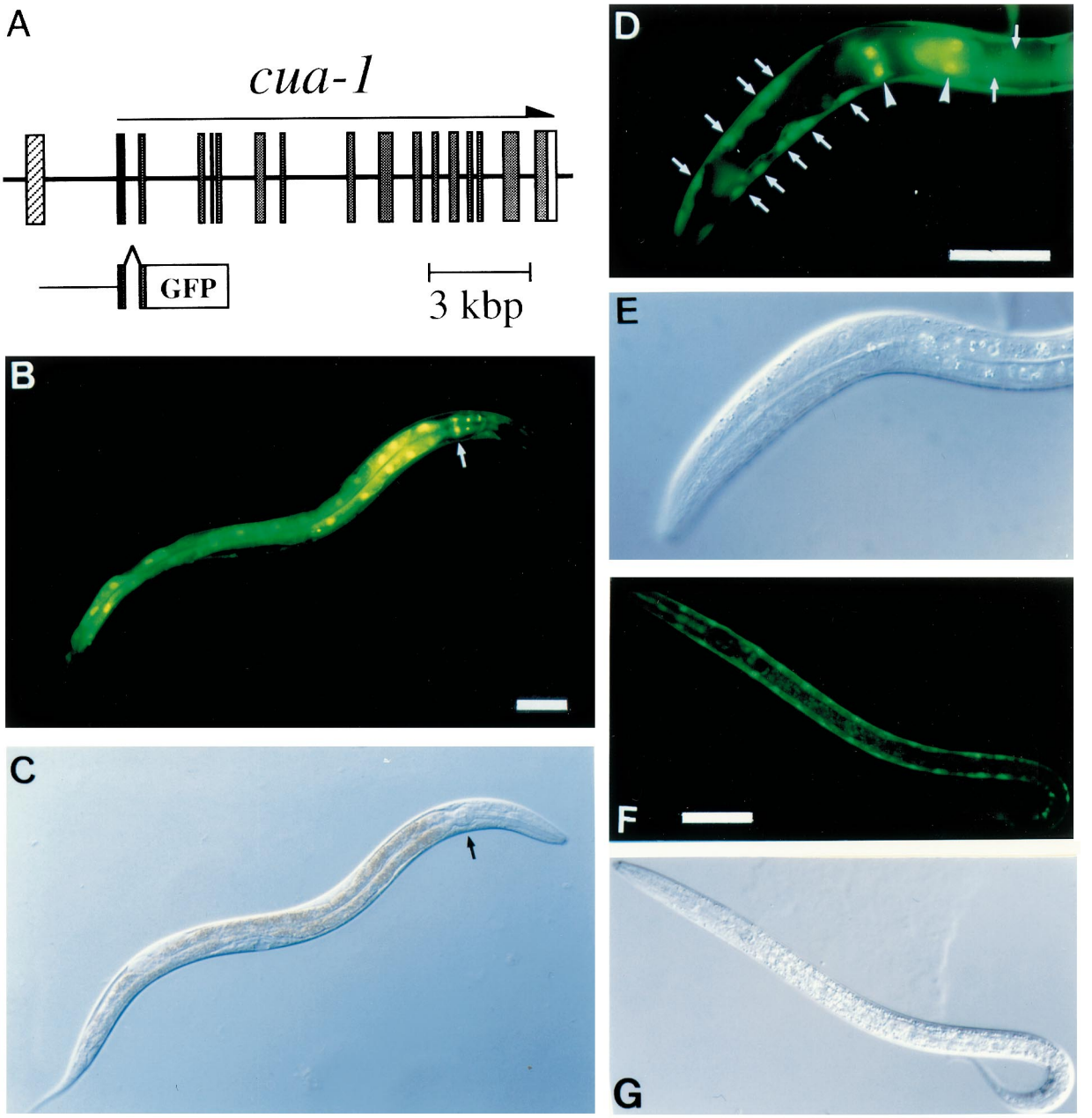

Fig. 4. Expression of *cua-1* in intestinal and hypodermal cells. A: Structure of gene *cua-1* and a fusion gene *cua-1::GFP*. Filled and open boxes represent coding and untranslated regions of *cua-1*, respectively. The hatched box indicates the last exon of the upstream gene identified in the genomic sequence. Direction of *cua-1* transcription is indicated by an arrow. A 3.0-kbp DNA fragment containing the upstream region and the first two exons of *cua-1* gene was fused with the reporter gene as indicated below. Three transmitting lines carrying the *cua-1::GFP* construct were established and had indistinguishable expression patterns. B: Fluorescence micrograph of an adult worm showing intestinal and pharyngeal expression. A pharyngeal muscle cell (pm6) is indicated by the arrow. C: Nomarski micrograph of the same field in B. D and E: Lateral view of the anterior half of the L1 larvae. Nuclei of hypodermal cells in the head and body regions are indicated by the arrows. Nuclei of the pharynx and anterior intestinal cells are indicated by the arrowheads. F and G: Ventral view of the dauer larvae. The expression of *cua-1::GFP* fusion gene is restricted to hypodermal cells in each lateral surface. Scale bars indicate 100 μm.

scription PCR using an SL1-specific primer but not by an SL2-specific primer (data not shown). A single 0.5-kb transcript was detected in Northern blots of total RNA from *C. elegans*, consistent with the size of the cDNA (Fig. 1B).

The cDNA contained an open reading frame of 207 bp coding a protein of 69 amino acid residues with a predicted molecular weight of 7587. The deduced amino acid sequence showed 49.3 and 39.1% identity to yeast Atx1p [20] and human HAH1 [27], respectively. The characteristic copper binding (MTCXXC) and lysine-rich (KKTGK) motifs were conserved in each of the sequences (Fig. 1C).

3.2. *Complementation of yeast atx1 null mutation by cuc-1 cDNA*

The *cuc-1* cDNA was introduced into a yeast Δ*atx1* mutant which does not grow on iron-depleted medium [3]. As shown in Fig. 2A, expression of *cuc-1* complemented the null mutation, suggesting that CUC-1 has a similar function to Atx1p.

To enable immunochemical detection of CUC-1, the VSV glycoprotein epitope tag was introduced immediately after the *cuc-1* initiation codon. Introduction of the epitope tag did not disrupt CUC-1 function because the tagged construct still complemented the Δ*atx1* mutant (Fig. 2A). In immunoblots of cell lysates of the strain using the anti-epitope tag antibody, we detected a single band with a relative mobility equal to 7.0 kDa (Fig. 2B, lysate). The mobility was slightly faster than the predicted molecular weight of 8908 with the tag; however, Atx1p also migrates faster than expected on gel electrophoresis [3]. CUC-1 protein was co-fractionated with alcohol dehydrogenase (ADH) in the supernatant after centrifugation at $100\,000 \times g$. This result indicates that the CUC-1 is localized in the cytosol, as is Atx1p (Fig. 2B, and [3]).

3.3. Expression patterns of cuc-1 and cua-1

Yeast Atx1p and Ccc2p together constitute a cytosolic copper trafficking pathway to the post-Golgi compartment [3,4]. We previously identified the *C. elegans* Cu-ATPase CUA-1 which is a functional homologue of Ccc2p [11]. To determine the expression pattern of *C. elegans* CUC-1 and CUA-1, fusion genes were constructed by inserting genomic fragments containing the *cuc-1* and *cua-1* 5′ regulatory region into a GFP expression vector (Figs. 3A and 4A). The *cuc-1::GFP* fusion gene was strongly expressed in the intestine of the adult transgenic worm (Fig. 3B,C). On the other hand, high expression levels in the L1 larval stage were found in the hypodermal cells of the head and body regions (Fig. 3D,E).

The *cua-1::GFP* showed essentially the same expression patterns as *cuc-1::GFP* in adult and larval stages. In addition, *cua-1::GFP* signals were also prominent in the pharyngeal muscle cells in the terminal bulb (pm6) (Fig. 4B,C, arrow). While *cuc-1::GFP* was expressed in most intestinal cells (Fig. 3B), *cua-1::GFP* showed stronger expression in the anterior portion (Fig. 4B). Finally, expression of both genes were repressed in the intestinal cells of the dauer larvae (Fig. 4F,G): *cua-1* was predominantly expressed in hypodermal cells (Fig. 4F,G), whereas *cuc-1* expression was not observed (data not shown).

4. Discussion

We have identified CUC-1, a copper chaperone protein expressed in *C. elegans*. CUC-1 showed significant sequence similarities with yeast Atx1p [20] and human HAH1 [27], and complemented yeast Δ*atx1* mutation. These findings strongly suggest that the CUC-1 has similar functions as Atx1p and that a copper trafficking pathway is present in higher eukaryotes which is similar to yeast. In yeast, the *ATX1* gene expression is regulated by iron concentration [3,28]. In contrast, the amount of the *cuc-1* transcript did not change regardless of the presence of iron or copper in the growth medium (T.W. and M.F., unpublished observation). Similarly, the amount of *HAH1* transcript in human cell lines is not altered by copper ion concentration [27].

Expression of *cuc-1* in *C. elegans* was tissue and developmental-stage specific. GFP fusions showed expression primarily in the hypodermal cells of L1 larval stage and in intestinal cells of the adult worm. Transgenic studies suggested that *cua-1* and *cuc-1* have similar expression patterns, which is consistent with a model that CUC-1 copper chaperone transfers copper to the CUA-1 ATPase for transport into the post-Golgi compartment. The intestinal expression of both genes may be essential for copper ion intake. The expression of both genes was repressed in the intestine when the L1 worm passes into the dauer larval stage. This is reasonable because the dauer worms are dormant and do not take food [29]. The hypodermal cells in the larval stage may transport copper to support copper-containing enzymes that carry out collagen cross-linking in the extracellular space. Cell specific expression of *cuc-1* is different from the human homologue, *HAH1*, which is expressed in all tissues and cell lines tested [26].

We note that the expression patterns of the two genes were slightly but significantly different. *cua-1::GFP* was expressed in pharyngeal muscle, whereas no *cuc-1::GFP* signal was detectable. The CUA-1 copper ATPase may function in muscle differently from intestinal or hypodermal cells and, clearly, the regulation of expression is also different. In this regard, the 5′ flanking sequences of *cuc-1* and *cua-1* do not share any sequence motifs that may indicate a common transcriptional regulation.

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References

[1] Culotta, V.C., Klomp, L.W.J., Strain, J., Casareno, R.L.B., Krems, B. and Gitlin, J.D. (1997) J. Biol. Chem. 272, 23469–23472.
[2] Glerum, D.M., Shtanko, A. and Tzagoloff, A. (1996) J. Biol. Chem. 271, 14504–14509.
[3] Lin, S.J., Pufahl, R.A., Dancis, A., O'Halloran, T.V. and Culotta, V.C. (1997) J. Biol. Chem. 272, 9215–9220.
[4] Pufahl, R.A., Singer, C.P., Peariso, K.L., Lin, S., Schmidt, P.J., Fahrni, C.J., Culotta, V.C. and Penner Hahn, J.E. (1997) Science 278, 853–856.
[5] Chelly, J., Tumer, Z., Tonnesen, T., Petterson, A., Ishikawa-Brush, Y., Tommerup, N., Horn, N. and Monaco, A.P. (1993) Nat. Genet. 3, 14–19.
[6] Mercer, J.F., Livingston, J., Hall, B., Paynter, J.A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D. and Glover, T.W. (1993) Nat. Genet. 3, 20–25.
[7] Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993) Nat. Genet. 3, 7–13.
[8] Bull, P.C., Thomas, G.R., Rommens, J.M., Forbes, J.R. and Cox, D.W. (1993) Nat. Genet. 5, 327–337.
[9] Tanzi, R.E., Petrukhin, K., Chernov, I., Pellequer, J.L., Wasco, W., Ross, B., Romano, D.M., Parano, E., Pavone, L., Brzustowicz, L.M., Devote, M., Peppercorn, J., Bush, A.I., Sternlieb, I., Pirastu, M., Gusella, J.F., Evgrafov, O., Penchaszadeh, G.K., Honig, B., Edelman, I.S., Soares, M.B., Scheinberg, I.H. and Gilliam, T.C. (1993) Nat. Genet. 5, 344–350.
[10] Yamaguchi, Y., Heiny, M.E. and Gitlin, J.D. (1993) Biochem. Biophys. Res. Commun. 197, 271–277.
[11] Sambongi, Y., Wakabayashi, T., Yoshimizu, T., Omote, H., Oka, T. and Futai, M. (1997) J. Biochem. 121, 1169–1175.
[12] Yoshimizu, T., Omote, H., Wakabayashi, T., Sambongi, Y. and Futai, M. (1998) Biosci. Biotechnol. Biochem. 62, 1258–1260.
[13] Iida, M., Terada, K., Sambongi, Y., Wakabayashi, T., Miura, N., Koyama, K., Futai, M. and Sugiyama, T. (1998) FEBS Lett. 428, 281–285.
[14] Payne, A.S. and Gitlin, J.D. (1998) J. Biol. Chem. 273, 3765–3770.
[15] Hung, I.H., Suzuki, M., Yamaguchi, Y., Yuan, D.S., Klausner, R.D. and Gitlin, J.D. (1997) J. Biol. Chem. 272, 21461–21466.
[16] Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J.,

Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johnston, L., Jones, M., Kershow, J., Kirsten, J., Laister, N., Latreille, P., Lightning, J., Lloyd, C., McMurray, A., Mortimore, B., O'Callaghan, M., Parsons, J., Percy, C., Rifken, L., Roopra, A., Saunders, D., Shownkeen, R., Smaldon, N., Smith, A., Sonnhammer, E., Staden, R., Sulston, J., Thierry-Mieg, J., Thomas, K., Vaudin, M., Vaughan, K., Waterston, R., Watson, A., Weinstock, L., Wilkinson-Sproat, J. and Wohldman, P. (1994) Nature 368, 32–38.

[17] Hung, I.H., Casareno, R.L., Labesse, G., Mathews, F.S. and Gitlin, J.D. (1998) J. Biol. Chem. 273, 1749–1754.

[18] Sulston, J. and Hodgkin, J. (1988) in: The Nematode *Caenorhabditis elegans* (Woods, W.B., Ed.) pp. 587–606, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning. A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[20] Lin, S.J. and Culotta, V.C. (1995) Proc. Natl. Acad. Sci. USA 92, 3784–3788.

[21] Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.

[22] Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K. and Toh-e, A. (1990) Mol. Cell. Biol. 10, 4303–4313.

[23] Kreis, T.E. (1986) EMBO J. 5, 931–941.

[24] Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D. and Dancis, A. (1996) Science 271, 1552–1557.

[25] Spieth, J., Brooke, G., Kuersten, S., Lea, K. and Blumenthal, T. (1993) Cell 73, 521–532.

[26] Zorio, D.A., Cheng, N.N., Blumenthal, T. and Spieth, J. (1994) Nature 372, 270–272.

[27] Klomp, L.W.J., Lin, S.J., Yuan, D.S., Klausner, R.D., Culotta, V.C. and Gitlin, J.D. (1997) J. Biol. Chem. 272, 9221–9226.

[28] Yamaguchi-Iwai, Y., Stearman, R., Dancis, A. and Klausner, R.D. (1996) EMBO J. 15, 3377–3384.

[29] Riddle, D.L. (1988) in: The Nematode *Caenorhabditis elegans* (Woods, W.B., Ed.) pp. 393–414, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[30] Mello, C.C., Kramer, J.M., Stinchcomb, D. and Ambros, V. (1991) EMBO J. 10, 3959–3970.