Existence of four acetylcholinesterase genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*

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Abstract Three genes, *ace-1*, *ace-2* and *ace-3*, respectively located on chromosomes X, I and II, were reported to encode acetylcholinesterases (AChEs) of classes A, B and C in the nematode *Caenorhabditis elegans*. We have previously cloned and sequenced *ace-1* in the two related species *C. elegans* and *C. briggsae*. We report here partial sequences of *ace-2* (encoding class B) and of two other *ace* sequences located in close proximity on chromosome II in *C. elegans* and *C. briggsae*. These two sequences are provisionally named *ace-x* and *ace-y*, because it is not possible at the moment to establish which of these two genes corresponds to *ace-3*. *Ace-x* and *ace-y* are transcribed in vivo as shown by RT-PCR and they are likely to be included in a single operon.

Key words: Acetylcholinesterase; *Caenorhabditis elegans*; *Caenorhabditis briggsae*

1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a key enzyme at cholinergic synapses, including neuromuscular junctions in nematodes [1]. Whereas a single gene encodes AChE in vertebrates [2] and in the majority of insects [3], the situation is more complex in *Caenorhabditis elegans*. Initial studies clearly indicated that two kinetically distinct major classes of AChE, A and B, were present in this species. An unusual feature of classes A and B was their apparent functional overlap. Analysis of AChE-affecting mutations defined two loci, *ace-1* on chromosome X and *ace-2* on chromosome I, that corresponded to the structural genes encoding class A and class B, respectively [4–6]. Later, a third class of AChE (class C) was defined. It accounts for less than 5% of the total AChE activity and differs markedly from classes A and B: AChE of class C is characterized by its very low K_m for ACh and its high resistance to eserine, a carbamate compound which is a classical inhibitor of acetylcholinesterases [7,8]. Analysis of mutations in class C AChE led to the definition of *ace-3*, a locus on chromosome II corresponding to the structural gene of class C AChE [9]. Finally, a careful examination of class C mutants suggested the existence of a fourth class (D) of AChE that represented less than 0.1% of the total AChE activity [10]. Class D AChE showed a similar resistance to eserine as class C but differed in its K_m for ACh and its *S* value in centrifugation, was much more thermostable and did not cross-react with anti-C antibodies [10]. Since no mutation in class D AChE was isolated, the putative *ace-4* locus was neither identified nor chromosome-mapped and as a consequence class D AChE has not been further characterized.

The complete coding sequences of *ace-1* in *C. elegans* [11] and in the related nematode species *C. briggsae* [12] have been reported recently, as well as partial sequences of *ace-2* and *ace-3* in *C. elegans* [13]. We now present additional data on *ace* genes in *C. elegans* and *C. briggsae*, obtained by homology screening, indicating that four distinct genes encode AChE in both nematodes. Two of these genes are located in close proximity on chromosome II, and it is therefore not possible to decide which sequence corresponds to *ace-3*. For the moment, these two genes are named *ace-x* and *ace-y*.

2. Materials and methods

2.1. Animals

Wild type *C. elegans* (N2 strain) and *C. briggsae* (AF16 strain) as well as the *ace-1* null mutant strain (p1000) were provided by the *Caenorhabditis* Genetics Center (Saint Paul, MN, USA). Worms were grown on Petri dishes seeded with *Escherichia coli* (0P 50), as previously described [14].

2.2. Nomenclature and numbering of amino acids in AChE sequences

AChE of classes A, B, C and D refer to proteins encoded by *ace-1*, *ace-2*, *ace-3* and *ace-4* genes. The numbering of amino acids in AChE sequences is that of *Torpedo* AChE (international convention [15]). The correspondence between initial and conventional numberings can be found in [11].

2.3. PCR

Genomic DNA was prepared from a mixed stage population of *C. elegans* or *C. briggsae* according to the procedure described by Michael Koelle available on the *C. elegans* web site (http://eatworms.swmed.edu/). Total RNAs were extracted with the RNAeasy kit from Qiagen and treated with RNase-free DNase. Reverse transcription (RT) was performed at 42°C on 3 μg RNAs using the Expand Reverse Transcriptase from Boehringer and random hexanucleotides. A quarter of the RT reaction was used for PCR in 50 μl with 200 μM of each dNTP, 100 ng of each primer and 2.5 U of *Taq* polymerase (Promega). PCR reactions were performed at 94°C for 2 min followed by 40 cycles with 45 s denaturation at 94°C, 45 s annealing at 45–55°C depending on the primers used, 45 s extension at 72°C, followed by a final extension of 15 min at 72°C. 10 μl of the PCR products were analyzed on a 1% agarose gel.

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Abbreviations: AChE, acetylcholinesterase; *ace-1, ace-2, ace-3* and *ace-4*, genes encoding AChE of classes A, B, C and D

Accession numbers: *ace-1*: X75331 (*C. elegans*), U41846 (*C. briggsae*); *ace-2*: AF025378 (*C. elegans*), AF030037 (*C. briggsae*); *ace-x*: AF025379 (*C. elegans*); *ace-y*: AF039650 (*C. elegans*). Sequences of *ace-x* and *ace-y* in *C. briggsae* were released by the GSC (St. Louis, MO, USA) from phosmid G18H21.

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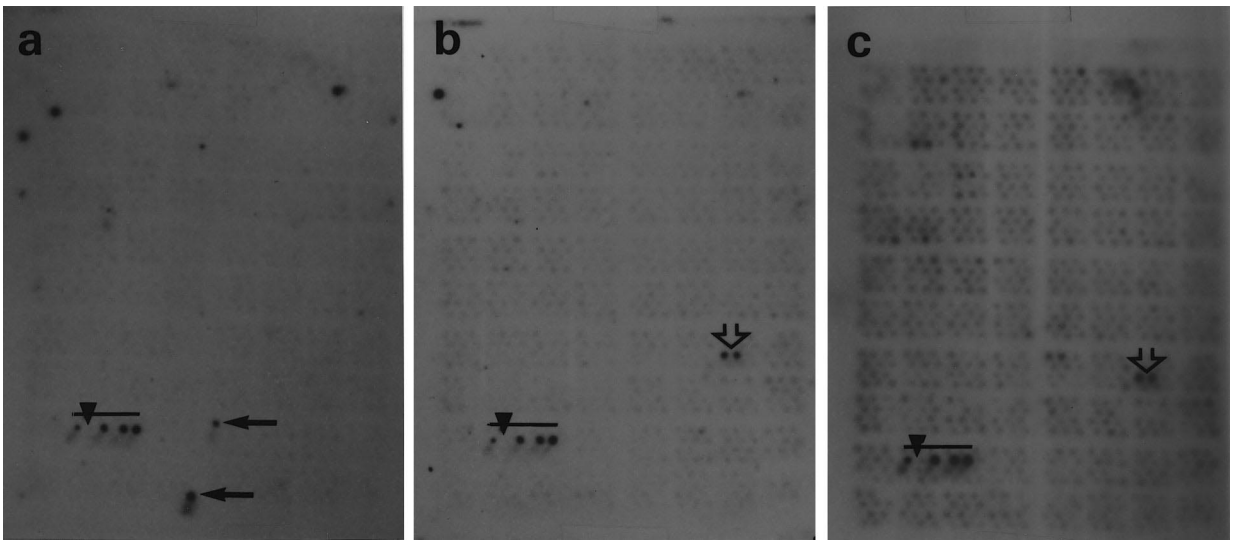

Fig. 1. Chromosome mapping of *ace* genes in *C. elegans*. The YAC polytene filter was hybridized at 65°C with different α-^{32}P-labelled probes as indicated. In each case, an *ace-1* probe was used as a positive control; it hybridizes with four overlapping YACs: Y45E8, Y49E3, Y50A2 and Y43C8 [11] indicated by the vertical black arrowheads in a, b and c. A second probe was added to *ace-1*: in a, a probe derived from clone 40 (initial amplified fragment of *ace-2*) hybridizes to YACs Y44E3 and Y52G11 (two contiguous YACs on chromosome I, horizontal arrows). In b, the probe is derived from clone 48 (*ace-x*): it hybridizes to YACs Y48B6 and Y59C8 of chromosome II (vertical arrow). In c, the probe is derived from the initial PCR fragment of *ace-y*: it hybridizes to the same YACs Y48B6 and Y59C8 as the clone 48 probe (vertical arrow). Note the absence of cross-hybridization between *ace-2* and *ace-x*/*ace-y* (*ace-1* does not cross-hybridize with other *ace* genes, as shown in [11]). The same YAC grid was used in a, b and c. It was dehybridized after each experiment and the absence of any remaining signal was checked by autoradiography. Other radioactive spots than those labelled were carefully checked: some of them did not correspond to any YAC colony; in c, some hybridization signals were noted on YACs. These YACs were searched by BLAST with *ace* sequences and rejected for lack of any homology.

2.4. Cloning and sequencing

PCR products were purified using the Qiaquick PCR purification kit from Qiagen and ligated into the pGEM-T vector (Promega). DNA samples suitable for sequencing were prepared with the plasmid mini kit (Qiagen). DNA sequencing was performed using the dideoxynucleotide procedure of Sanger et al. [16] using the −21M13 and RP fluorescent dye primers kits (Perkin Elmer) and analyzed in an automated sequencer (model 370A from Applied Biosystems).

2.5. Hybridization of YAC grids

Probes were labelled with α-^{32}P-dCTP using the Rediprime random labelling kit from Amersham, and purified from unincorporated nucleotides on ProbeQuant G-50 columns (Pharmacia). Hybridization was performed overnight at 65°C as in [17]. Filters were washed four times for 20 min at room temperature in 0.5% SDS, 0.5% SCP warmed initially at 50°C (SCP is 0.1 M NaCl, 40 mM Na_2HPO_4, 1 mM EDTA, pH 6.2). After extensive drying, filters were autoradiographed to Fuji X-Ray films using an intensifying screen at −70°C for 18–24 h.

2.6. Use of the GCG data base, alignments and phylogeny

Sequences of *ace* genes were compared to genomic DNA sequences released by the Genome Sequencing Center of St. Louis (MO, USA) using either *C. elegans* or *C. briggsae* BLAST servers (http://genome.wustl.edu/gsc/gschmpg.html). Alignment of the eight *ace* sequences (*ace-1*, *ace-2*, *ace-x* and *ace-y* in *C. elegans* and *C. briggsae*) was performed with the computer program CLUSTALW [18]. Sequences of esterases and cholinesterases were retrieved using ESTHER, a specialized database on cholinesterases [19] and phylogenetic trees were built using the PHYLIP package based on the Protein Parsimony program of Felsenstein [20].

3. Results

ace-1 in *C. elegans* was initially found by homology with other cholinesterase sequences using PCR [11]. In order to amplify fragments of *ace* genes other than *ace-1* by RT-PCR, we used total RNA prepared from *ace-1* null mutants of *C. elegans*. In these null mutants (allele p1000 [5]), a point mutation introduces a stop codon at the position of Trp-84 (TGG→TGA): as a consequence, nonsense *ace-1* mRNA are destabilized and their level is reduced to only 10% of that in wild-type animals [21]. Sense oligonucleotides were designed from highly conserved sequences corresponding to the peptides GSEMW(84)N (*Torpedo*) and GTEMW(84)N (mammalian AChEs) assuming that they would have a reduced tendency to anneal with the mutant *ace-1* RNA. We thus used

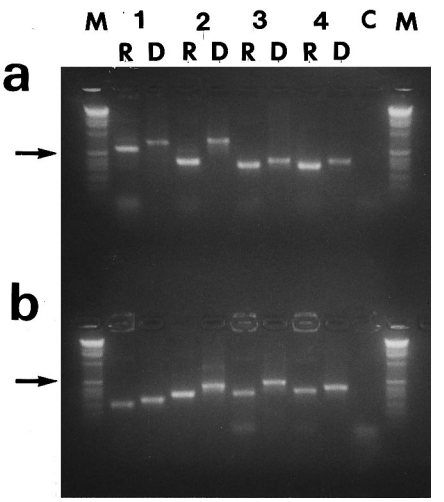

Fig. 2. Transcription of *ace* genes in vivo. Amplifications were performed either on total RNA (R) or on genomic DNA (D) from *C. elegans* (a) or *C. briggsae* (b) using primers specific of each gene *ace-1* (1), *ace-2* (2), *ace-x* (3) and *ace-y* (4). Specific primers were chosen in different regions of *ace* sequences. C: negative control is a mix of primers without template. M: molecular weight marker (1 kb ladder, BRL). Horizontal arrow: 500 bp.

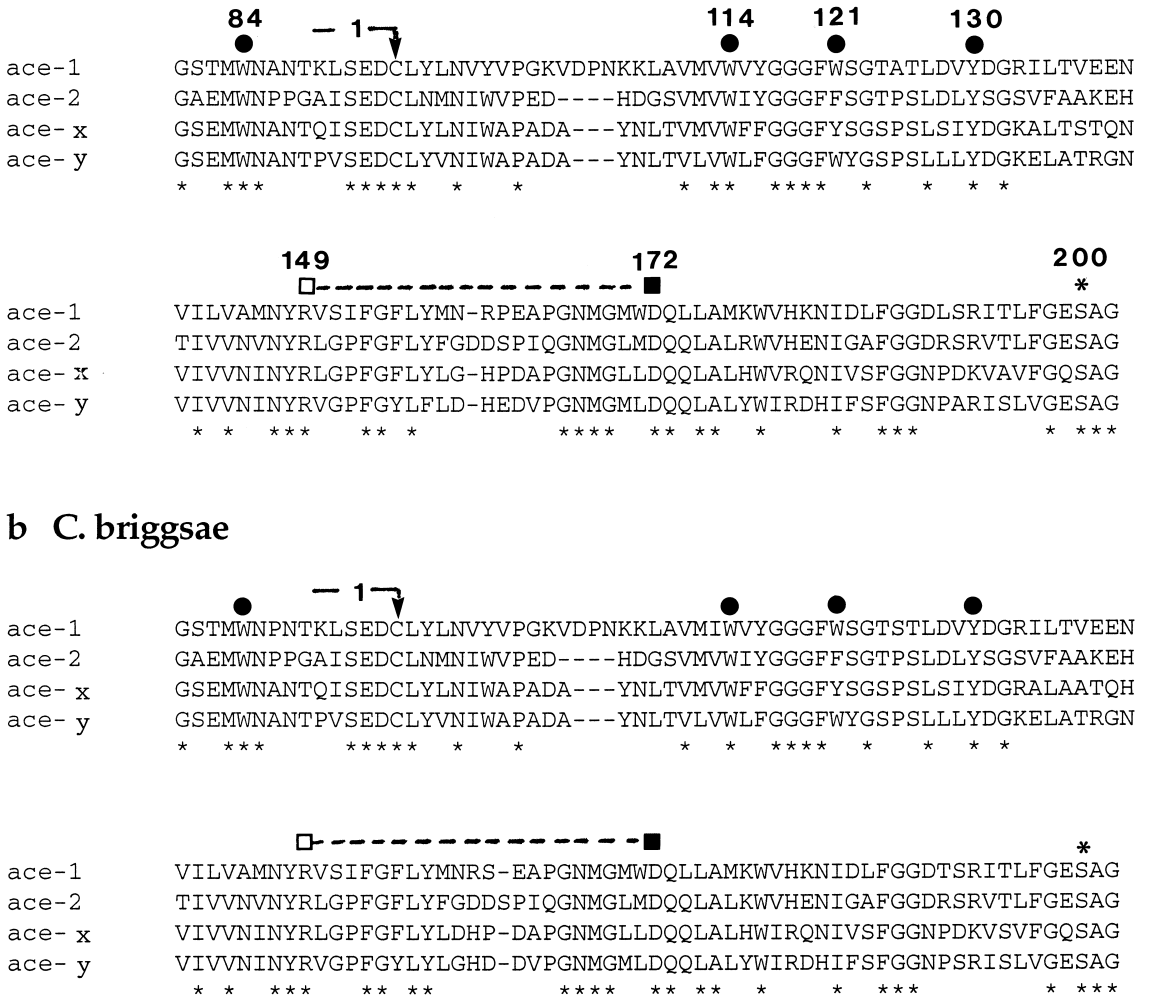

Fig. 3. Alignment of partial AChE sequences from *C. elegans* (a) and *C. briggsae* (b). The compared partial sequences extend from residues G(80) to G(202). By convention, the numbering of amino acids is that of *Torpedo* AChE [15]. It is deduced from a previous alignment between *ace-1* and *Torpedo* AChE [11]. Conserved features among all AChEs include: W(84) as the choline-binding site (●); C(94) as the second cysteine of the first disulfide bond (1); aromatic residues at position 114, 121 and 130 (●); R(149) and D(172) as potential components of the first salt bridge (open and black squares); the active serine S(200) (asterisk). Nucleotide sequences can be obtained from GenBank with the indicated accession numbers. The codon used for the active serine is TCn in all cases.

a mixture of 5′GGI ACI GA(A/G) ATG TGG AA3′ and 5′GGI (T/A)(C/G)I GA(A/G) ATG TGG3′ (I stands for inosine). The reverse oligonucleotides used were designed from the amino acid sequence FGESAG around the active serine (S200), using two different codons for serine (underlined): 5′-CC IGC IGA (C/T)TC ICC (A/G)AA-3′ and 5′-CC IGC (A/G)CT (C/T)TC ICC (A/g)AA-3′.

With these primers, a fragment of the expected size (approximately 370 nt long) was amplified. Cloning and sequencing of this fragment revealed two different clones (40 and 48) which presented 52% and 53% identity with *ace-1* (55% identity between clones 40 and 48). We then hybridized YAC grids (kindly provided by Dr. Alan Coulson, MRC, Cambridge, UK) with probes corresponding to clones 40 and 48 and a probe corresponding to *ace-1* as a positive control. Clone 40 was shown to hybridize to YACs Y44E3 and Y52G11 (Fig. 1a) in a region of chromosome I compatible with the genetic localization of *ace-2* [5]. Clone 48 hybridized with YACs Y48B6 and Y59C8 (Fig. 1b) on chromosome II near the locus *unc-52*, the expected location of *ace-3* [6]. In both cases *ace-1* hybridized to YACs Y45E8, Y45E3, Y50A2 and Y43C8 of chromosome X (Fig. 1a,b) as previously reported [11]. We thus concluded at this moment [13] that clones 40 and 48 corresponded to *ace-2* and *ace-3*.

During the course of this study, the *C. elegans* Genome Sequencing Consortium released a sequence presenting 100% identity with clone 40. This sequence was located on YAC Y44E3 confirming our hybridization in Fig. 1b. The genomic sequence of *ace-2* in *C. elegans* was therefore available. A partial cDNA sequence of *ace-2* in *C. briggsae* was obtained by RT-PCR using primers deduced from the *ace-2* sequence in *C. elegans*.

No identity was found when the *C. elegans* BLAST server was searched with the partial sequence of clone 48, indicating that this region of chromosome II had not yet been sequenced. In the *C. briggsae* BLAST server, however, we found *two* sequences that showed homology to clone 48. These two sequences were contained within a single phosmid (G18H21,

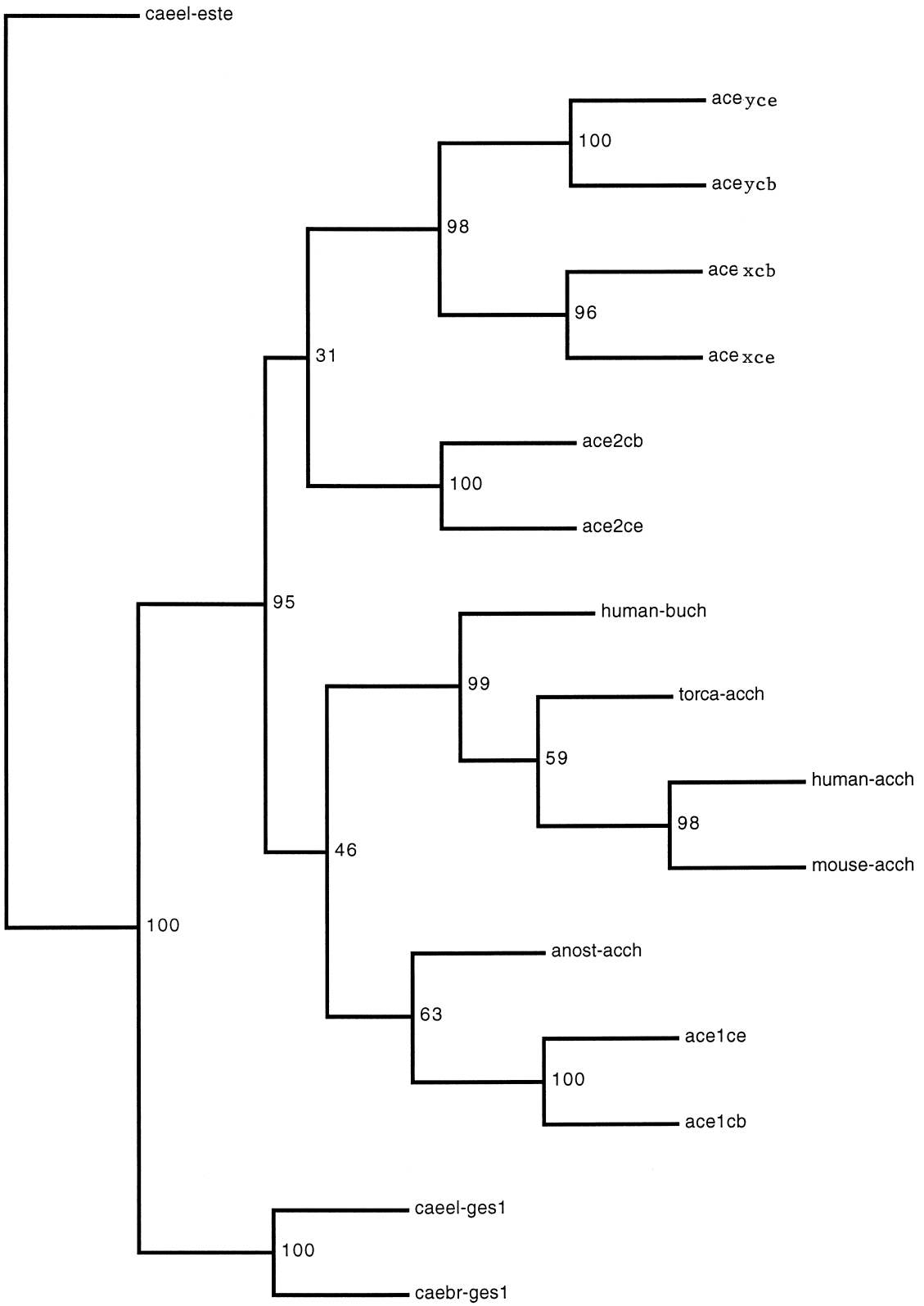

Fig. 4. Phylogenetic tree built of partial *ace* sequences in *C. elegans* and *C. briggsae*. In addition to the partial sequences (from G80 to G202) of the four *ace* genes in *C. elegans* and *C. briggsae* the tree includes homologous sequences of human AChE (human-acch, M55040) and butyrylcholinesterase (human-buch, M16541), mouse (mouse-acch, X56518) and *Torpedo* (torca-acch, X03439) AChEs, AChE from *Anopheles stephensi* (anost-acch, P56161) and the following carboxylesterases: gut esterase (*ges-1*) from *C. elegans* (caeel-ges1, M96144) and *C. briggsae* (caebr-ges1, M96145) and the *C. elegans* esterase cm06b1 (caeel-este, X66104). Accession numbers are from GenBank except for *Anopheles* (SwissProt). The tree is unrooted. Numbers at each fork show bootstrap values with 100 replicates.

Table 1
Percentages of amino acid identity and homology (top right) and nucleotide identity (bottom left) between *ace* sequences of *C. elegans* and *C. briggsae*

		C. elegans				*C. briggsae*			
		ace-1	*ace-2*	*ace-x*	*ace-y*	*ace-1*	*ace-2*	*ace-x*	*ace-y*
C. elegans	*ace-1*		54 (69)	56 (77)	52 (73)	96 (96)	–	–	–
	ace-2	57		58 (75)	53 (75)	–	99 (100)	–	–
	ace-x	58	61		73 (87)	–	–	94 (94)	74 (88)
	ace-y	57	59	70		–	–	–	86 (100)
C. briggsae	*ace-1*	79	–	–	–		55 (70)	52 (75)	52 (73)
	ace-2	–	82	–	–	57		60 (75)	56 (75)
	ace-x	–	–	81	–	58	60		74 (87)
	ace-y	–	–	–	86	59	61	69	

Percentages are calculated on the nucleotide and amino acid sequences G(80)–G(202). For amino acids, the percentage of homology (including semi-conservation) is in parentheses.

i.e. in close proximity on the same chromosome). Using GeneFinder, two ORFs were deduced from this genomic sequence. One of these two sequences presented 94% identity (at the amino acid level) with clone 48 of *C. elegans* and was clearly the homologue of clone 48 in *C. briggsae*. The other sequence had only 74% identity with clone 48 of *C. elegans* but presented the characteristic features of cholinesterases such as W84, the catalytic triad residues (S200, E327 and H440), conservation of aromatic residues lining the catalytic gorge and the three intrachain disulfide bonds, in conserved positions. This sequence was neither *ace-1* nor *ace-2* of *C. briggsae* that we had already sequenced ([12], and see above). This sequence thus corresponds to a fourth *ace* gene. Using specific primers deduced from this new *ace* sequence of *C. briggsae*, we amplified, by RT-PCR, a fragment of 300 bp in *C. elegans*, which presented 86% identity (100% homology) with the homologous sequence in *C. briggsae*. In addition, it hybridized to the same YACs (Y48B6 and Y59C8) as clone 48 (Fig. 1c). Thus two *ace* genes lie in close proximity on chromosome II in *C. elegans* and in *C. briggsae*. Due to this proximity it is not possible at the moment to decide which of these two sequences corresponds to *ace-3*. Therefore in this paper these two genes are referred to as *ace-x* (upstream gene, initially clone 48) and *ace-y* (downstream gene).

Fig. 2 shows amplification of the four *ace* genes in *C. elegans* and *C. briggsae* with specific primers on either genomic DNA (control) or total RNA by RT-PCR. This showed that all *ace* genes are transcribed in vivo in both *Caenorhabditis* species.

Fig. 3 shows an alignment of the eight peptide sequences deduced from the four *ace* genes in *C. elegans* and *C. briggsae*. Table 1 indicates the percentages of identity between all sequences (at the amino acid and nucleotide levels).

A phylogenetic tree was built using the eight partial *ace* sequences in *Caenorhabditis* and homologous sequences from insect and vertebrate AChEs and *Caenorhabditis* carboxylesterases (Fig. 4). The tree clearly shows that *ace-2*, *ace-x* and *ace-y* are closely related, with *ace-x* and *ace-y* originating from the most recent duplication before speciation of *C. elegans* and *C. briggsae*. In contrast, *ace-1* appears as different from the other *ace* genes in *Caenorhabditis* as it is from insect or vertebrate AChE genes.

4. Discussion

We report here that four distinct *ace* genes are present and transcribed in the nematodes *C. elegans* and *C. briggsae*.

ace-1 (located on chromosome X) was shown to direct in vitro the synthesis of an enzyme with properties similar to class A AChE [11] and an opal mutation (W84Z) was identified in the null mutant *ace-1*$^-$ [21]. *Ace-2* (the unique site of hybridization on chromosome I) is an excellent candidate for being the structural gene encoding class B AChE.

ace-3 was genetically defined in the literature as the gene governing class C AChE and was mapped to chromosome II [9]. Thus, during the present work the first sequence found that hybridized to chromosome II (clone 48) was called *ace-3* because we did not suspect that it could be closely linked to another *ace* gene. The close proximity of two *ace* genes on chromosome II does not permit us to decide, at the moment, which of the two genes directs the synthesis of class C AChE (and thus should be called *ace-3* in reference to [9]). For this reason we suggest calling the upstream gene in the tandem on chromosome II *ace-x*, and the downstream gene *ace-y*. One of these genes encodes class C AChE. We may suppose that the fourth gene encodes the AChE of class D which was defined by Stern [10]. Expression of the cloned *ace* genes in Sf9 cells infected by recombinant baculoviruses, as well as the identification of the gene affected in the null mutant *ace-3*$^-$ [9], will finally establish the exact correspondence of *ace-x* and *ace-y* with *ace-3* and *ace-4*.

We are currently investigating the genomic organization of *ace-x* and *ace-y* on chromosome II. In *C. elegans* and *C. briggsae* respectively, we found that the stop codon of *ace-x* was located only 356 and 372 bp upstream of the potential initiator ATG of *ace-y*. This suggests that *ace-x* and *ace-y* could belong to a polycistronic unit (or operon). In *C. elegans* several examples of operons are known in which a single promoter region controls the coordinated expression of several structural genes [22].

The phylogenetic tree of Fig. 4 shows that *ace-2*, *ace-x* and *ace-y* are closely related and that *ace-1* probably diverged from this group early during evolution. Brenner [23] noted that the active serine of AChEs was encoded by the codons AGy in vertebrate AChEs but by the codon TCn in *Drosophila* AChE and that these two codons cannot interconvert by a single mutation. In all four *ace* genes of *C. elegans* and *C. briggsae* the active serine is encoded by TCn, and this renders unlikely a direct relationship between one of the nematode *ace* genes and vertebrate AChE as could be suggested by Fig. 4.

It is interesting to note that *ace-1* encodes a hydrophilic subunit that can associate to other structural (non-catalytic) proteins in a manner resembling that of the T subunits of vertebrate AChEs [2,11]. On the contrary, preliminary obser-

vations indicate that the C-termini of AChE of class B and AChE of class C are clearly hydrophobic. This C-terminal hydrophobic sequence is replaced during post-translational maturation to a glycolipid moiety in AChE of class B [24] as in *Drosophila* AChE [25] and the H subunits of vertebrate AChEs [2]. However no clear consensus sequence is found in the C-termini of H subunits of mammalian, *Torpedo*, *Drosophila* and *Caenorhabditis* AChEs suggesting that glypiation could have appeared several times during evolution.

The comparison of *ace-x* and *ace-y* sequences shows that the duplication that led to the two genes took place long before the divergence between of *C. elegans* and *C. briggsae*. It will be interesting to test whether the fourth *ace* gene also exists in other nematodes, especially in species with an agronomic impact.

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