# Characterization of a null mutation in *ace-1*, the gene encoding class A acetylcholinesterase in the nematode *Caenorhabditis elegans*

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Received 18 November 1994

**Abstract** **Two genes (*ace-1* and *ace-2*) encode two major classes (A and B) of acetylcholinesterase (AChE) in the nematode *Caenorhabditis elegans*. A null mutation in *ace-1* (allele *p1000*) suppresses all acetylcholinesterase activity of class A. We have identified an opal mutation TGG (W99)→TGA (Stop) as the only alteration in the mutated gene. This leads to a truncated protein (98 instead of 620 amino acids) with no enzymatic activity. The mutation also reduces the level of *ace-1* transcripts to only 10% of that in wild-type animals. This most likely results from a destabilization of mRNA containing the nonsense message. In contrast, compensation of class B by class A AChE in the null mutant strain *ace-2* takes place with unchanged *ace-1* mRNA level and enzymatic activity similar to class A AChE.**

*Key words:* Acetylcholinesterase; mRNA stability; Nematode; Null mutation; *Caenorhabditis elegans*

## 1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is an essential enzyme responsible for the hydrolysis of acetylcholine and thus for the termination of transmission at cholinergic synapses. There is only one gene coding for AChE in vertebrates [1] and insects [2]. In the nematode *Caenorhabditis elegans*, three genes (*ace-1*, *ace-2* and *ace-3*) encode three classes of AChE differing in their catalytic properties and called AChE of class A, B and C, respectively [3–5]. Classes A and B are major components that each account for approximately half the total AChE activity [3,4], whereas *ace-3* is a minor component [5] which is, however, able to maintain alive the double homozygous mutant *ace-1/ace-2* [5].

Only one mutation has been identified so far in human AChE [6] that replaces codon 322 CAC (His) by AAC (Asn). This mutation has no effect on enzyme activity [7] but the wild-type sequence is the YT1 blood group antigen and the variant with $Asn^{322}$ is the YT2 antigen [6]. A number of mutations in the locus *ace* of *Drosophila* have been reported [8]. Some are non-conditionally lethal and others are lethal only at some temperatures [9,10]. Two mutations responsible for cold- and heat-sensitive phenotypes ($Ace^{lj29}$ and $ace^{lj40}$ [10]) were shown to result from point mutations changing $Ser^{374}$ to Phe and $Pro^{75}$ to Leu, respectively [11]. These mutations most likely affect folding of the protein during maturation at the non-permissive temperature. In insects, homozygous null mutations in the AChE gene are lethal and are eliminated. In *C. elegans*, homozygous null mutations in either *ace-1* or *ace-2* have been isolated [3,4]. They have no visible phenotype because of a functional overlap of Class A and B AChEs [4]. The null mutation in *ace-1* (allele *p1000*) was originally identified by the total absence in mutants of any AChE resistant to deoxycholate (a property of class A AChE only, [3]). We report here the characterization of this null mutation and its effects on the level of *ace-1* transcripts. Levels of *ace-1* transcripts and class A AChE activity were also studied in the null mutant *ace-2* to test whether compensation of class B by class A AChE was accompanied by an over-expression of the intact gene.

## 2. Materials and methods

### 2.1. Animals and nomenclature

Wild type *C. elegans* (N2 strain) as well as *ace-1* and *ace-2* mutants were provided by the *Caenorhabditis* Genetics Center (Saint Paul, MN). They were grown on Petri dishes seeded with *E. coli* (0P 50). *ace-2* mutants [4] were used to check whether the compensation of AChE of class B by class A AChE operated via an increase in gene transcription of *ace-1*. Unless otherwise stated *ace-1* refers to the homozygous mutant, *ace-1*$^-$/*ace-1*$^-$, and *ace-2* to *ace-2*$^-$/*ace-2*$^-$. ACE-1 and ACE-2 refer to the proteins encoded by genes *ace-1* and *ace-2* (AChE of Classes A and B), respectively.

### 2.2. Solubilization of AChE, enzymatic assay and sucrose gradient centrifugation

Worms (N2, *ace-1* or *ace-2*) were collected from Petri dishes and rinsed twice in M9 medium [12]. Homogenization was performed twice in HSB buffer (m/v = 1/5 for the first extraction and 1/2 for the second). HSB buffer contains 10 mM Tris-HCl, pH 7.0, 1 M NaCl, 0.5% 10-oleyl-ether (Brij 96; Sigma), 0.1 mg/ml bacitracin, $7.5 \times 10^{-3}$ TIU aprotinin and 1 mM EDTA. Homogenizations were achieved through two successive high speed vortexings of 5 min in the presence of a half vol. of siliconized glass beads (400–800 $\mu$m in diameter). Homogenates were centrifuged for 1 h at 100,000 × *g*. Supernatants were assayed for AChE activity according to [13] using 1 mM acetylthiocholine as substrate. Protein content of extracts was determined by the bicinchoninic acid method following the manufacturer's instructions for use (Pierce). Sedimentation analyses were performed in 5–20% sucrose gradients in 10 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl and a cocktail of antiproteolytics as in extraction buffer and 0.5% Triton X-100. Samples (250 ml) were layered on 11 ml of gradient and centrifuged for 16 h at 40,000 rpm at 4°C in a SW41 rotor (200,000 × *g*).

### 2.3. DNA sequencing

Total RNA from the mutant strain *ace-1* were reverse transcribed using the reverse transcription kit from Pharmacia and pdN6 primers. The entire coding sequence and portions of the 5′ and 3′ non-coding regions of *ace-1* cDNA were then amplified by PCR using six pairs of synthetic oligonucleotides (see Fig. 1) deduced from the sequence of

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*SSDI* 0014-5793(94)01343-8

wild-type *ace-1* [14] and *Taq* polymerase from Promega. PCR experiments were run for 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min). PCR fragments were cloned in pGEM-T (Promega). Sequencing was performed using the dideoxynucleotide procedure of Sanger et al. [15] either with fluorescent dye primers for automated sequencing or with [α-$S^{35}$]dATP and the 2.0 Sequenase kit from USB for illustration (Fig. 3).

*2.4. Extraction of total RNA and Northern blot analysis*

Total RNA were extracted in guanidinium isothiocyanate according to [16], electrophoresed in formaldehyde-containing 1% agarose gels, and transferred to nylon membrane (Hybond N; Amersham). Membranes were prehybridized and hybridized at 42°C as described previously [14] with $10^6$ cpm/ml of a 2 kb *Pst*I fragment of *ace-1* cDNA (see Fig. 1) labeled by random priming. The same membranes were then rehybridized with a *C. elegans* actin cDNA (gift of Lisa Matthews, CRBM, Montpellier). This probe was used to calibrate the amounts of RNA loaded. Autoradiograms were densitometrically scanned with an Apple OneScanner (300 dpi) coupled to an image processing and analysis program.

Table 1
Amount of AChE activity solubilized from N2, *ace-1* and *ace-2* strains

| Strains | Extracts | AChE activity (OD/mn/ml) | Proteins (mg/ml) | Specific AChE activity (OD/mn/mg) |
|---|---|---|---|---|
| N2 | 1 | 5.6 | 10.6 | 0.53 |
| | 2 | 4.0 | 2.9 | 1.34 |
| *ace-1* | 1 | 1.0 | 4.1 | 0.24 |
| | 2 | 0.6 | 0.8 | 0.75 |
| *ace-2* | 1 | 1.5 | 5.8 | 0.26 |
| | 2 | 0.7 | 1.2 | 0.58 |

Two successive extractions in HSB buffer were performed (m/v = 1/5 for the first extract and 1/2 for the second). AChE activity (in OD/ml/min), protein content (mg/ml) and AChE specific activity (OD/min/mg) are indicated. Extracts were performed with pooled worms from three different Petri dishes for each strain.

## 3. Results

We first studied the effects of null mutations in either *ace-1* or *ace-2* on total AChE activity and molecular forms. The growth of both mutant strains was apparently similar to that of the N2 strain. After two successive homogenizations in HSB buffer each followed by 1 h centrifugation at 100,000 × *g*, specific AChE activity was estimated for each strain. Table 1 shows that each mutation roughly resulted in a reduction of 50% in AChE specific activity. These results are in good agreement with those previously reported by Culotti et al. [4] and confirm that *ace-1* and *ace-2* genes both account for approximately half of the total AChE activity. In addition it shows that a deficiency of either *ace-1* or *ace-2* does not induce over-expression of the remaining enzyme. AChE activity due to the third gene, *ace-3*, as measured in the double homozygous mutants *ace-1/ace-2*, was almost undetectable under our conditions (not shown).

The molecular forms of AChE were analyzed by sucrose gradient centrifugation. Fig. 2 shows the comparison of sedimentation profiles of AChE in N2 and *ace-1* (Fig. 2A) and between N2 and *ace-2* (Fig. 2B). This analysis shows that ACE-1 (the enzyme absent in *ace-1* mutants but responsible for AChE activity in *ace-2* mutants) was essentially composed of fast sedimenting forms. One major peak of AChE activity at 13.5 S with a shoulder at 11.5 S was observed, with a small amount of 4.5–5 S forms. ACE-2 was composed only of two peaks of slow-sedimenting forms (4.5 S and 7 S). It has been shown that for ACE-1, the 13.5 S form is a complex tetrameric structure that associates four catalytic subunits with a non catalytic element, whereas the 11.5 S peak is a (simple) homotetrameric form. For ACE-2, the 7 S and 4.5 S forms are, respectively, dimers and monomers of the catalytic subunit [17–19].

The *ace-1* null mutant was originally isolated by Johnson et al. [3] after ethylmethanesulfonate treatment (exposure to 0.1 M EMS for 4 h at 20°C). EMS treatment is known to induce mostly point mutations (GC-to-AT transitions), although small deletions can also be generated [12]. We have recently reported the complete coding sequence of the class A AChE catalytic subunit in wild-type *C. elegans* [14]. Sequencing the mutant *ace-1* showed only one point mutation that changed the codon TGG of W99 into TGA (Stop). This opal mutation was first found by automatic sequencing of PCR fragments obtained from *ace-1* mRNAs in three independent experiments and was then confirmed in genomic DNA of the mutant (one experiment). Sequencing of this region with radiolabeled dATP is shown in Fig. 3. Translation of this transcript would result in a truncated protein (98 amino acids instead of 620 [14]) devoid of enzymatic activity.

We compared the amounts of *ace-1* mRNA present in N2, *ace-1* and *ace-2* strains by Northern blot analysis (Fig. 4a). An actin probe was used for calibration (Fig. 4b). The ratio of *ace-1* to actin transcripts was calculated for N2, *ace-1* and *ace-2* strains by scanning Northern blots of three independent experiments. Fig. 4c shows that there was a significant ($P = 0.99$) decrease in *ace-1* transcripts in the *ace-1* mutant compared to N2 (reduction to 10% of control values). In contrast, amounts of *ace-1* transcripts in N2 and *ace-2* strains were not significantly different ($P = 0.95$).

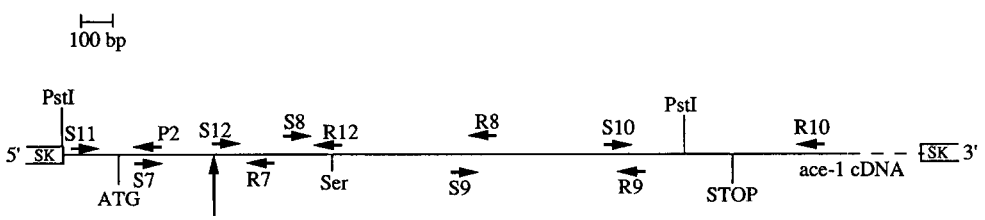


Fig. 1. Position of the 12 oligonucleotides used for PCR. Six pairs of primers were used: S11–P2, S12–R12, S7–R7, S8–R8, S9–R9 and S10–R10. The null mutation was found in S7–R7 (vertical arrow). The cDNA probe used in Northern blot analysis is the 2 kb *Pst*I fragment. SK, bluescript vector.

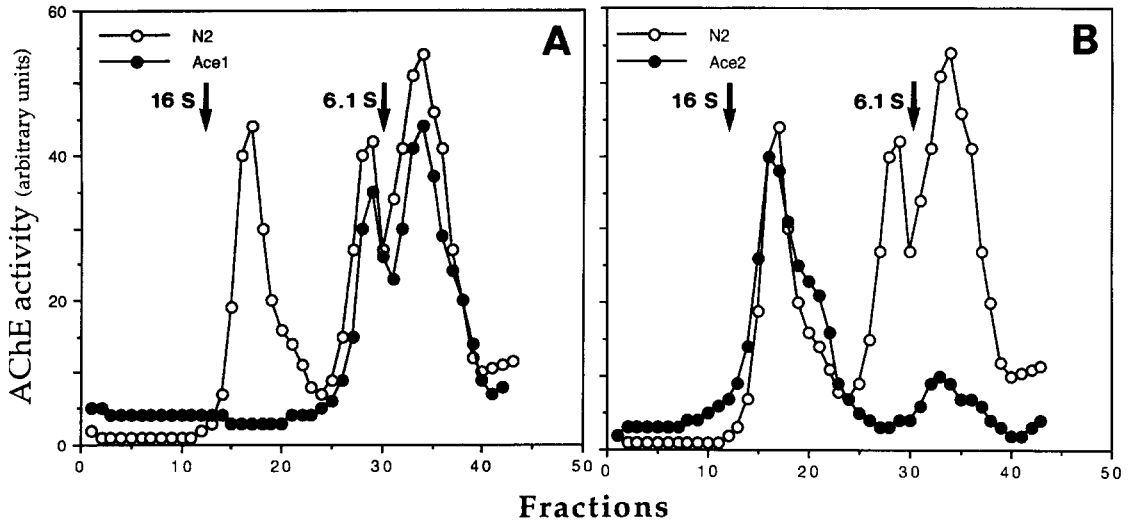


Fig. 2. Sucrose gradient analysis of AChE molecular forms in N2 (○), *ace-1* and *ace-2* strains (●). N2/*ace-1* and N2/*ace-2* comparisons are shown in A and B for clarity. Worm extraction was performed in HSB buffer and centrifugation in HST buffer. Vertical arrows show positions of alkaline phosphatase (6.1 S) and β-galactosidase (16 S).

## 4. Discussion

We report that the allele *p1000* responsible for a null mutation in the *ace-1* gene of *C. elegans* [3] corresponds to a G→A transition at the third base in codon W99. The transition generates a stop codon (TGG→TGA, opal mutation) and the translation of the resulting transcript leads to a truncated protein of 98 amino acids with no enzymatic activity. This explains the total lack of class A AChE in *ace-1* mutants.

It should be noted that besides the present opal transition TGG→TGA (Stop), any other point mutation in the codon TGG (W99) should also have led to an inactive AChE. W99 (that corresponds to W84 and W86 in *Torpedo* and human AChEs, respectively [14]) is essential for enzymatic activity since it corresponds to the binding site of the choline moiety of acetylcholine. Directed mutagenesis of W86 in human AChE (that replaces W by A or E) resulted in no activity [20,21].

We observed that the level of *ace-1* mRNAs bearing the premature stop codon was reduced to 10% of that in N2 strain. This reduction most likely results from a reduced stability of nonsense messengers, as shown originally in yeast [22,23]. RNA stability depends both upon *cis*- and *trans*-acting factors [23,24]. Such *trans*-acting factors are in the process of being identified in *C. elegans*: a series of six genes (*smg* genes) are necessary to encode a surveillance system which is responsible for the rapid degradation of messengers with a premature translational termination [25]. For example, a stop codon introduced early in the coding sequence of *unc-54*, the gene encoding a myosin heavy chain in *C. elegans*, leads to a rapid degradation of transcripts in *smg*$^+$ animals but not in *smg*$^-$ ones [26]. Products of *smg* genes are probably responsible for the reduced stability of *ace-1* transcripts in the mutant.

In the *ace-2* mutant (lacking class B AChE) both the level of *ace-1* transcripts (Fig. 4) and ACE-1 activity (Table 1) were not

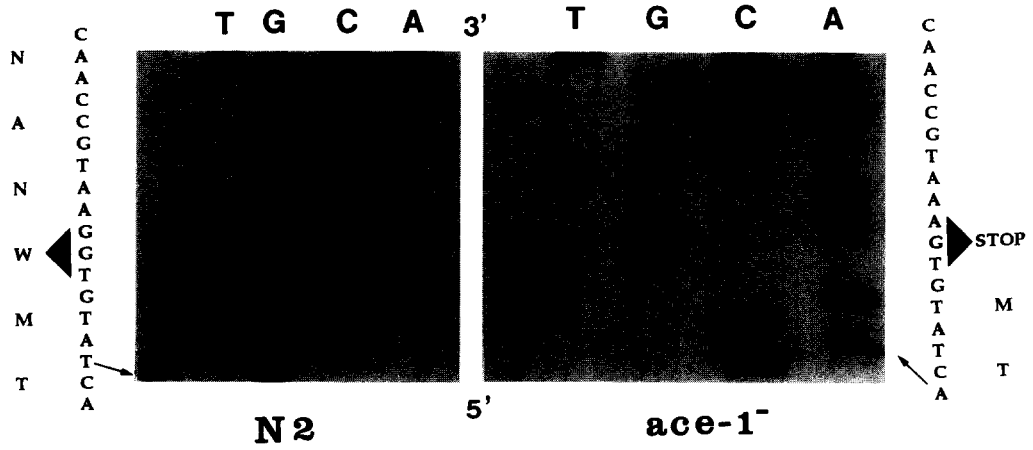


Fig. 3. Autoradiograms of sequencing gels in the region of W99 of ACE-1. N2, wild-type animals; the sequence shown corresponds to T(97)–N(102). *ace-1*$^-$, homozygous mutant. The sequences shown are identical except for the mutation G→A (arrow in *ace-1* indicates the new A, arrow in N2 shows the original G). This transition introduces a premature stop codon, TGA, in place of the TGG codon of W99 in ACE-1. 10 μg of plasmid and 1 pmol of primer were used for each sequencing reaction.

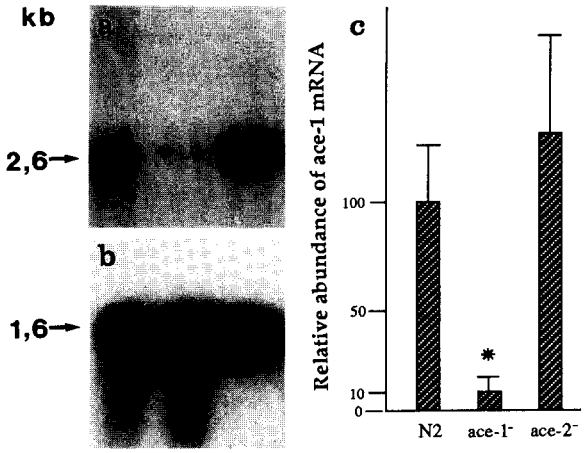


Fig. 4. Northern blot analysis of *ace-1* transcripts in N2, *ace-1* and *ace-2* mutants. (a) Hybridization of total RNA with an *ace-1* cDNA probe (*PstI*–*PstI* fragment of 2 kb, see Fig. 1). Only one transcript of 2.6 kb is detected. (b) Rehybridization of the same blot with an actin probe. The single transcript at 1.6 kb is used for calibration of amounts of RNA loaded. (c) Results of densitometric scans of three independent Northern blot analyses. Vertical bars indicate standard deviations (S.D.). The ratio of *ace-1* to actin signals were calculated for each strain in each experiment. Means and S.D. are calculated from three experiments. The asterisk indicates that the difference is significant ($P = 0.99$).

significantly increased compared to N2. Thus the functional compensation of class B by class A AChE does not require the over-expression of *ace-1*. This also tends to indicate that the normal amount of AChE in wild-type *C. elegans* largely exceeds functional requirements. It is interesting to relate this conclusion to a similar situation observed in *Drosophila* where it is possible to rescue null mutants in the *ace* locus by transfection of a minigene that produces only 20–30% of the AChE activity found in wild-type flies [27].

*Acknowledgements:* V.T. was supported by a grant from the European Commission (Program Human Capital and Mobility, network Multidisciplinary approaches of cholinesterase functions).

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