Molecular and biochemical characterization of S-adenosylmethionine decarboxylase from the free-living nematode *Caenorhabditis elegans*

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S-Adenosylmethionine decarboxylase (SAMDC) is a major regulatory enzyme in the polyamine biosynthesis and is considered a potentially important drug target for the chemotherapy of proliferative and parasitic diseases. To study regulatory mechanisms which are involved in the expression of SAMDC of the free-living nematode *Caenorhabditis elegans*, we have isolated the SAMDC gene and cDNA. Genomic Southern-blot analysis suggests that the *C. elegans* SAMDC is encoded by a single-copy gene which spans 3.9 kb and consists of six exons and five introns. The first two introns are located in the 5'-untranslated region (UTR). Analyses of the 5'-flanking region of the gene revealed several consensus sequences for the binding of different

transcription factors such as CBP, AP2, cMyb, VPE2 and others. The *C. elegans* SAMDC mRNA possesses an open reading frame (ORF) which encodes a polypeptide of 368 amino acids, corresponding to a SAMDC proenzyme with a calculated molecular mass of 42 141 Da. The active form of the *C. elegans* SAMDC is a heterotetramer, consisting of two subunits of 32 and 10 kDa derived from cleavage of the pro-enzyme. The SAMDC mRNA has an unusually long 5'-UTR of 477 nucleotides. This region has a small ORF which could encode a putative peptide of 17 residues. Moreover, the *C. elegans* SAMDC mRNA is trans-spliced with the 22 nucleotides spliced leader sequence at the 5'-end.

INTRODUCTION

The polyamines spermidine and spermine play an important role in cell growth and differentiation [1–3]. The synthesis of polyamines depends on the decarboxylation of ornithine to putrescine and the subsequent attachment of aminopropyl groups to its terminal amino substituents to form spermidine and spermine, respectively. Decarboxylated S-adenosylmethionine provided by the S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) serves as the aminopropyl donor.

In mammalian cells SAMDC represents one of the regulatory and limiting steps in the biosynthesis of polyamines. It has been shown that the expression of the mammalian SAMDC is regulated on the transcriptional, translational as well as post-translational level [4–7]. Furthermore, the mammalian SAMDC depends on putrescine for the processing of the proenzyme as well as for the enzyme activity [8,9].

The control mechanisms which are involved in the regulation of the polyamine concentration in mammalian cells have been well investigated. However, much less information is available from nematodes and other lower eukaryotes. SAMDC activity has previously been reported from parasitic nematodes [10]. Furthermore, the sequence of the SAMDC gene from the human pathogenic nematode *Onchocerca volvulus* and the processing of its transcript were recently analysed [11]. In order to establish *C. elegans* as a model nematode for studies of the regulation of polyamine synthesis at the whole-organism level, we report here nematode-specific features, demonstrating *Caenorhabditis elegans* SAMDC similarities to, and differences from, SAMDC from *O. volvulus* and mammals.

EXPERIMENTAL

Preparation of genomic DNA and RNA

A wild-type strain of *C. elegans* (N2 var. Bristol) was maintained in Petri dishes at 22 °C in nematode growth medium (0.03 % NaCl/0.025 % peptone) under xenic conditions in the presence of *Escherichia coli* strain OP50. The worms were separated from the bacteria by sucrose flotation as previously described [12]. Genomic DNA was prepared using classical procedures after proteinase K digestion, phenol extraction and ethanol precipitation [13]. Total RNA was prepared from the worms by homogenization in the presence of a monophasic solution of phenol and guanidine isothiocyanate (TRIzolTM Reagent, GIBCO-BRL) as described by the manufacturer.

Isolation of *C. elegans* SAMDC cDNAs

A SAMDC PCR product was amplified on the basis of a small expressed sequence tag (EST) (355 bp) of *C. elegans* (accession no. T00887), which showed similarity to SAMDC sequences of other organisms. The isolated SAMDC genomic PCR product was labelled with [³²P]dATP (Random Primed DNA Labelling Kit; Boehringer-Mannheim) and used as a hybridization probe to screen a mixed population *C. elegans* λ-zapII cDNA library (Stratagene) using a standard plaque hybridization assay [13]. The phagemids containing the SAMDC cDNA were recovered from the λ-zapII library by the *in vivo* excision process as described by the manufacturer (Stratagene).

Abbreviations used: EST, expressed sequence tag; ORF, open reading frame; RACE, rapid amplification of cDNA ends; SAMDC, S-adenosylmethionine decarboxylase; UTR, untranslated region.

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The nucleotide sequences reported in this paper have been submitted to the GenBank[®]/EMBL/DDBJ Nucleotide Sequence Databases under the accession numbers Y12499 and Y12500 for the mRNA and the gene sequences respectively.

ggt tta att acc aac gtt tga ggt aac tto aga aag ctc ooc cgg cag ttt tct aag acc	60
aag otc tct cto aac aac gag aac ttt ttg etc cac acc gta aga ott ggg ggt gct gta	120
agg cgt tcc tgt gga ggg acc gca gct aac tda ggg taa oot tao oca gcc aaa ato cgt	180
aga gta ago gta gac cgt tct ooc gao gag tog oca oca ooc cgt oca oot tog gao act	240
tca tot ggc aga gac aac ooc acc ota act toy toe ggc gcc gta gta tto gcc gac aag	300
M G P D R K T R I L	
oat tcc oca tto cgc tga taa ooc gga ggt gta TGG GTC CCG ACC GAA AGA CCC GCA TCC	360
V L Y R R P P *	
TGC TCC TAT ACC GTC GTC CTC CCT AGC ggg toe agc taa gga gcc tat acc toe taa acc	420
M	
oat ttt caa tag aaa ato cgt oot tog aca tao oca taa tao aac oot toe ooc ata ATG	480
S A T S A T N F A V Q T H P V K A P D E	21
TCT GCC ACG TCT GCC ACC AAC TTT GCG GTT CAA AAC CAT CCG GTC AAG GCT CCG GAT GAG	540
E Y F F E G A E K L L E L W F C S S T Q	41
GAG TAC TTC TTC GAA GGT GCG GAG AAG CTT CTG GAG CTC TGG TTC TGC TCG AGC ACA CAG	600
N E T R S L R I I P R E E I D A M L D I	61
AAT GAG ACT CCG TCC CTC CGA ATC ATC CCA AGA GAG GAG ATC GAT GCA ATG CTT GAC ATC	660
A R C K I L H S K H N E S I D S Y V L S	81
GCT CCG TGT AAG ATC CTC CAC TCC AAG CAC AAC GAG TCC ATC GAC TCT TAC GTG CTC AGC	720
↓	
E S S L F I S D N R V I L K T C G T T R	101
GAG TCC TCT CTG TTC ATC TCG GAG AAC CCG GTG ATC CTC AAC AAC TGT GGC ACA ACT CCG	780
L L A C A L P V I M Q L A G A Y A G L D Q	121
TTG TCG GCT GCC CTT CCA GTG ATC ATG CAG CTA GCC GGG GCC TAC GCC GGG CTG GAT CAA	840
V Q S V Y Y S R K N F L R P D L Q P S L	141
GTG CAA TCC GTC TAC TAC TCC AGG AAG AAC TTT TCG GCG CCG GAT CTT CAA CCA TCG CTT	900
H K N F D A E V E Y L D S F F V D G H A	161
CAT AAG AAC TTT GAT GCG GAG GTG GAG TAT GTG GAT AGT TTT TTT GTG GAT GGT CAT GCG	960
Y C L G S L K Q D R W Y L Y T F H R E V	181
TAC TGT CTT GGA TCC CTC AAA CAG GAT AGA TGG TAC CTT TAC ACA TTC CAC AGA GAA GTT	1020
E F P A H K Q P D H T L E I L M S D L D	201
GAG TTC CGA GCT CAC AAG CAA CCC GAT CAC ACC TTG GAG ATC TTG ATG AGC GAT CTG GAT	1080
E E V L H K F T K D Y A V D G N D C F M	221
GAG GAG GTG CTC CAC AAG TTT ACC AAA GAC TAT GCG GTG GAT GGC AAT GAC TGC TTC ATG	1140
R A G I D K I I P A G A D V H D E L F D	241
CGA GCC GGT ATT GAC AAG ATC ATC CCG GCA GGT GCA GAT GTT CAC GAC GAG CTG TTC GAC	1200
P C G Y S M N A Y M N D T D O Y A T I H	261
CGG TGC GGC TAC AGT ATG AAC GCT TAT ATG AAT GAC ACT GAC CAA TAC GCA ACC ATC CAC	1260
V T P E K A F S F A S F E T N Q D L V C	281
GTG ACT CCC GAG AAG GCA TTC AGC TTC GCT TCC TTC GAG ACC AAC CAG GAC TTG GTC TGC	1320
L Y S Q T R K V L Q C F R P N K I L M T	301
CTG TAC TCG CAG ACT CCG AAG GTT CTT CAG TGT TTT AGA CCA AAT AAG ATC TTG ATG ACA	1380
V F A N D I S E K G K D A Q Q Q L W D R	321
GTG TTT GCG AAT GAG ATT TCG GAG AAG GGG AAG GAT GCC CAG CAG CAG TTG TCG GAG CCG	1440
E L P G Y R R T N V Q F V R L E T E T L	341
GAG CTG CCG GGG TAT AGA AGG ACC AAT GTG CAG TTT GTG AGA CTT GAG ACG GAA ACC CTA	1500
V Y A H F V R K A S T G Q D S S S S D E	361
GTG TAC GCT CAT TTC GTG CCG AAG GCC TCC ACC GGT CAA GAT TCA TCA TCG AGC GAC GAG	1560
D D G E R S D *	368
GAC GAC GGC GAA CCG TCC GAT TGA tat caa ato ttg att gta ttt ttt ttt ota oaa ato	1620
aot gtt aaa oca tgt att ttg tao taa ooc tat tag ttt ttt ott ott aat oat tga	1680
toa ota tao ooc oot toe oot aac tao ttg gtt tto tga ggg goo tto agg oca ttt tot	1740
agt oot ttg got tot ggt aot gta gto ctg tgg tot tot ggt ota oca gaa oaa ott ooc	1800
gtt gaa ggg toa aat gtt atg ota att oot tat ota tto oca oca tot ott tgt ogt ota	1860
oot ooc oot ota tot ooc oot tto ctg ttt gtt tto aag oca gac agt tao act tto ota	1920
ttg att ttt agc aat ttt agt tat ttt tot ttt gtt aga act gto tta aac gtt aat ttt	1980
tgg gog tag aat ata ttt ttt aat aaa aaa aat ttg gga aaa aaa aaa aaa a	2038

Figure 1 Nucleotide and deduced amino acid sequence of the *C. elegans* SAMDC pro-enzyme

The 5'-RACE product is underlined. The polyadenylation signal sequence is underlined and in italics, and the poly(A) tail is in italics. The oligonucleotides used in the 5'-RACE procedure are double-underlined. The spliced leader sequence is boxed. The asterisk (*) indicates the stop codon. The deduced amino acid sequence in single-letter code is shown above the nucleotide sequence. The putative cleavage site is indicated by ↓. The residues that are necessary for SAMDC processing and activity are underlined.

Amplification of the 5'-end of the *C. elegans* SAMDC by 5'-rapid amplification of cDNA ends (5'-RACE)

The 5'-end of the SAMDC mRNA was determined by using the RACE (GIBCO-BRL) with total *C. elegans* RNA as described by the manufacturer. The identified RACE products were cloned, sequenced and analysed as previously described [11].

Northern- and Southern-blot analyses

For the identification of the *C. elegans* SAMDC transcript, 30 µg of total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and blotted on to a nylon membrane [13]. The membrane was then hybridized with a [³²P]dATP-labelled cDNA probe.

C. elegans genomic DNA (10 µg/lane) was digested with the restriction endonucleases *Bam*HI, *Hind*III, *Eco*RV, *Sac*I, *Xho*I and *Eco*RI, size-fractionated on a 0.8% agarose gel, transferred to a nylon membrane (Boehringer-Mannheim) using standard methods [13] and hybridized with the radiolabelled [³²P]dATP *C. elegans* SAMDC cDNA [complete open reading frame (ORF)].

Isolation of the genomic *C. elegans* SAMDC sequences

A mixed worm population λ-fixII genomic library of *C. elegans* (Stratagene) was screened using the radiolabelled cDNA. Positive clones were purified and examined by PCR and Southern-blot analysis. To identify the *C. elegans* SAMDC gene fragment within the genomic DNA insert, the phage DNA was digested with *Sac*I, *Eco*RV and *Sac*I/*Eco*RV, resolved on a 0.8% agarose gel and analysed by the Southern-transfer method [13]. The radiolabelled *C. elegans* SAMDC cDNA (complete ORF) was used as a probe. The positive *Sac*I and *Eco*RV fragments were isolated, subcloned into the pBS-KS⁺ vector (Stratagene) and the nucleotide sequence was determined.

DNA sequencing

The nucleotide sequences of both strands of the PCR products, cDNA and genomic DNA were determined by the dideoxy-chain-termination method on double-stranded DNA [13] using [³⁵S]thio-dATP and the Sequenase Sequencing Kit (USB; Amersham). In addition, the nucleotide sequence of the genomic clone was determined by the Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's (Perkin-Elmer) instructions. DNA and amino acid sequence analyses were carried out using DNASIS/PROSIS computer software (Hitachi).

Expression of the recombinant *C. elegans* SAMDC in *E. coli*

The expression plasmid pTrcHis-B (InVitrogen), which contains a *trc* (*trp-lac*) promoter, was used to express the *C. elegans* SAMDC in *E. coli*. The coding region of the *C. elegans* SAMDC was amplified with *Pfu*-DNA-polymerase (Stratagene) using the sense and the antisense primers which contain *Nco*I and *Eco*RI restriction sites respectively. The PCR product was subcloned into the pBS-KS⁺ vector and transformed into DH5α competent cells. Recombinant plasmid DNA was isolated and digested with the restriction enzymes *Nco*I and *Eco*RI. The resulting fragment was gel-purified and subcloned into *Nco*I-*Eco*RI-cleaved pTrcHis-B to produce a non-fusion protein. Recombinant expression plasmids (pTrcCeSAM) were partially sequenced to ensure that the insert was in the correct reading frame and subsequently introduced into the *E. coli* strain EWH 331, which is a mutant lacking endogenous SAMDC [14]. Recombinant enzyme was produced and purified as previously described [11]. The homogeneity of the enzyme preparation was analysed by

Tricine/SDS/PAGE (12% gel) as described in [15]. The proteins were revealed by silver staining. Enzyme activity was assayed by measuring the production of $^{14}\text{CO}_2$ from S-adenosyl-L-[carboxy- ^{14}C]methionine at 37 °C as previously described [10].

RESULTS

Characterization of the λ -zapII *C. elegans* SAMDC cDNA

A λ -zapII *C. elegans* cDNA library was screened using the radiolabelled PCR fragment. More than 30 independent SAMDC cDNA clones were identified. The largest clone (1806 bp) possesses a 1104 bp ORF, 210 bp 5'-untranslated region (UTR) and 458 bp 3'-UTR including the poly(A) tail (Figure 1). The 3'-UTR contains a typical polyadenylation signal sequence (AATAAA) located 17 bp upstream from the poly(A). The ORF encodes a polypeptide of 368 amino acids which corresponds to the SAMDC proenzyme with a calculated molecular mass of 42 141 Da. The complete sequence of the 5'-UTR was determined using a 5'-RACE experiment which resulted in the identification of an additional 267 bp. In addition, the RACE experiment identified the presence of the consensus 22-nucleotide spliced-leader sequence (SL1; gggttaattaccaagtttgag) at the 5' end of the *C. elegans* SAMDC transcript. The complete 5'-UTR of the *C. elegans* SAMDC mRNA is 477 nucleotides and therefore the full-length *C. elegans* SAMDC mRNA is 2038 nucleotides. This result has been confirmed using Northern-blot analysis, which revealed a single transcript of approx. 2.0 kb (Figure 2). Analysis of the 5'-UTR identified a small upstream ORF encoding a putative peptide of 17 residues (MGPDRKTRILVLYRRPP).

Analysis of the deduced amino acid sequence of the *C. elegans* SAMDC cDNA

Pairwise sequence comparisons of the deduced amino acid sequence of the *C. elegans* SAMDC with that of *O. volvulus* [11] and those of other eukaryotic SAMDCs exhibited a moderate degree of similarity (31–52%). The *C. elegans* SAMDC sequence contains a putative PEST sequence (sequences rich in the amino acids P, E/D, S/T; [16]) consisting of 27 amino acids from residues 260 to 289 (HVTPEKAFSFSFETNQDLVCLYSQTR). In addition, the *C. elegans* SAMDC contains all the

Figure 2 Northern-blot analysis of RNA from *C. elegans*

Total RNA (30 μg) from *C. elegans* was fractionated on a 1%-agarose gel containing formaldehyde, blotted on to a nylon membrane and hybridized with a [^{32}P]dATP-labelled *C. elegans* SAMDC cDNA.

Figure 3 Analysis of the purified active *C. elegans* SAMDC by SDS/PAGE

(A) Tricine/SDS/PAGE analysis of the purified recombinant *C. elegans* SAMDC (0.2 μg /lane). The proteins were revealed by silver staining. Sizes of the molecular-mass standards are shown on the left. (B) Western-blot analysis of the recombinant *C. elegans* SAMDC using the antibody raised against the *O. volvulus* SAMDC [11].

Figure 4 Analysis of the isolated *C. elegans* SAMDC gene structure by Southern-blot analysis

The purified *C. elegans* SAMDC-lambda fixII phage DNA was digested with *SacI* (lane 1), *EcoRV* (lane 2), *SacI/EcoRV* (lane 3), size-fractionated on a 0.8%-agarose gel, transferred on to a nylon membrane and hybridized with a [^{32}P]dATP-labelled *C. elegans* SAMDC cDNA. The corresponding sizes (in kb) of the hybridizing fragments are indicated on the left.

residues that were demonstrated in previous studies to be critical for enzyme activity, as well as those necessary for the putrescine-dependent stimulation of proenzyme processing and catalytic activity [9,17,18] (Figure 1).

Characterization of recombinant *C. elegans* SAMDC

SDS/PAGE analysis of the purified recombinant protein revealed two protein bands: a 32 kDa protein corresponding to the SAMDC α -subunit and a 10 kDa protein corresponding to the SAMDC β -subunit (Figure 3A). In Western-blot analysis antibodies raised against the recombinant *O. volvulus* SAMDC [11] cross-reacted with the large subunit, but not with the small subunit of the *C. elegans* SAMDC (Figure 3B). The molecular mass of the active SAMDC was calculated by Superdex S-200

underlined and in italics. Nucleotide position 1 is assigned to the *trans*-splicing site (↓), and negative numbers refer to the 5'-flanking sequence. The asterisk (*) indicates the stop codon.

To identify potential transcription-factor-binding sites in this sequence, a computer search was performed to identify known transcription-factor-binding consensus sequences [19]. The *C. elegans* SAMDC promoter region lacks an obvious TATA box, but two potential CAAT boxes, one AP2, cMyb, ELP, PPAR and other transcription-factor-binding sites were identified. Furthermore, a VPE2 motif (CTGATAA; position -143 to -136) was found on the complementary strand [20].

DISCUSSION

The key attributes of the free-living nematode *C. elegans* as a general experimental system are ease of cultivation in the laboratory, short life span, small genome size and suitability for genetic analysis. In order to evaluate its potential as a model for parasitic nematodes and for addressing the regulation of polyamine levels in lower eukaryotes, we have analysed and compared genomic and cDNA sequences, as well as the SAMDC protein from *C. elegans*, with recent data from the human pathogenic filaria *O. volvulus* and those reported from mammalian and other eukaryotic SAMDCs.

Southern-blot analyses suggest that the SAMDC gene occurs in the *C. elegans* genome as a single-copy gene, a result consistent with recent data from *O. volvulus* [11] and various other organisms [21–23]. In contrast, the SAMDC genes of the human [24,25], rat [26,27] and mouse [28] were found to be multiple-copy genes. Structural and sequence analyses of the SAMDC genes of both nematodes reveal differences in size and exon/intron organization (Figure 7). The *C. elegans* gene is 3.9 kb in size and consists of six exons and five introns, whereas the *O. volvulus* SAMDC gene spans 5.3 kb and consists of nine exons and eight introns [11]. Of interest is the occurrence of introns in the 5'-UTR of the *C. elegans* SAMDC gene. Similar results have recently been found for *O. volvulus* [11] and reported for the SAMDC genes of plants [23,29]. Furthermore, sequence analyses of the 5'-flanking region of the *C. elegans* SAMDC gene reveal various putative transcription-factor-binding motifs. Of interest is the VPE2 motif, which is known from *C. elegans* to regulate vitellogenin gene expression [20]. Primary reporter-gene experiments using Chinese-hamster ovary cells showed that the *C. elegans* SAMDC promoter region is capable of driving promoter activity (results not shown).

The cDNA of the *C. elegans* SAMDC contains a 1104 bp ORF encoding a polypeptide of 368 amino acids with a calculated molecular mass of 42141 Da. This is similar to 41890 Da of the *O. volvulus* SAMDC [11] and slightly larger than the 38.3 kDa of the mammalian enzyme [8,30]. A comparison of the deduced amino acid sequence of the SAMDC cDNAs shows only 52% identity between the sequences of the free-living *C. elegans* and the parasitic nematode *O. volvulus*. The degree of identity of the nematode sequences with those of other eukaryotes is even lower. However, within the amino acid sequences of SAMDCs

from various eukaryotic organisms there are some highly conserved amino acids and regions. The amino acid sequences of the SAMDC of *C. elegans*, like that of *O. volvulus* [11], contain all those amino acids which have been reported to be essential for catalytic activity and for proenzyme processing of the mammalian enzyme [9,17,18]. Like all other SAMDCs, the recombinant *C. elegans* SAMDC underwent autocatalytic cleavage. Enzymically active *C. elegans* SAMDC was overexpressed in a *E. coli* mutant strain deficient in SAMDC. The activity of the recombinant enzyme was strongly stimulated by putrescine (results not shown). Such a stimulatory effect of putrescine on nematode SAMDC has been reported for the native enzyme of *Ascaris suum* [10] and has recently been confirmed for the recombinant SAMDC of *O. volvulus* [11].

Remarkable is the extremely long 5' untranslated sequence of 477 nucleotides of the *C. elegans* SAMDC mRNA, a finding that has also been reported for SAMDC transcripts of *O. volvulus* and other eukaryotes. The occurrence of an ORF in the 5'-UTR of the *C. elegans* transcript is also of interest [31]. Such an ORF has also been reported for the SAMDC of *O. volvulus* [11]. The ORF of both nematode SAMDC transcripts consists of 51 bp and may encode a peptide of 17 amino acids. The identity of these putative peptides from *C. elegans* and *O. volvulus* is 47%, with identical context of the last four amino acids. ORFs in the 5'-UTR of SAMDC transcripts have been reported and shown to affect the translational rate of SAMDCs [7,29,32–34]. Neither the hexapeptide resulting from the upstream ORF of mammalian SAMDC nor the 51-amino-acid ORF of plant SAMDC transcripts show similarity to the ORF of the 5'-UTR of the SAMDC transcript of both nematodes. The 5'-RACE experiment showed that the *C. elegans* SAMDC mRNA is *trans*-spliced at its 5' end. *Trans*-splicing of mRNAs is quite common for nematode mRNA processing [35] and has also been reported for the SAMDC of *O. volvulus* [11].

In conclusion, the results discussed here clearly demonstrate a high degree of similarity between the nematode SAMDCs, which indicates that free-living *C. elegans* is suitable as an experimental model for the investigation of the polyamine metabolism of parasitic nematodes.

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Figure 7 Comparison of *C. elegans* and *O. volvulus* SAMDC gene organization

The exons are depicted by boxes, with the closed and open boxes referring to the protein-coding region and untranslated sequences respectively.

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