A novel *trans*-spliced mRNA from *Onchocerca volvulus* encodes a functional *S*-adenosylmethionine decarboxylase

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Complete cDNA and genomic sequences encoding the *Onchocerca volvulus S*-adenosylmethionine decarboxylase (SAMDC), a key enzyme in polyamine biosynthesis, have been isolated and characterized. The deduced amino acid sequence encodes a 42 kDa proenzyme with a moderate level of sequence homology to eukaryotic SAMDCs. Enzymically active *O. volvulus* SAMDC was expressed at a high level in an *Escherichia coli* mutant strain lacking endogenous SAMDC. The recombinant enzyme was purified to homogeneity using DEAE-cellulose, methylglyoxal bis(guanylhydrazone)–Sepharose and Superdex S-200 chromatography. It was determined that the recombinant proenzyme is cleaved to produce 32 and 10 kDa subunits. The sequence of the N-terminal portion of the large subunit was determined and comparison with the sequence of the proenzyme revealed that the precise cleavage site lies between Glu^{86} and Ser^{87}. Gel-filtration experiments demonstrated that these two subunits combine to form an active heterotetramer. Comparison of the cDNA and genomic sequences revealed that the SAMDC mRNA undergoes both *cis*- and *trans*-splicing in its 5′-untranslated region (UTR). Anchored PCR on *O. volvulus* mRNA confirmed the cDNA sequence and identified two distinct *trans*-spliced products, a 22-nucleotide spliced-leader sequence and a 138 bp sequence containing the 22 nucleotide spliced-leader sequence. Genomic Southern-blot analysis suggests that the *O. volvulus* SAMDC is encoded by a single-copy gene. This gene spans 5.3 kb and is comprised of nine exons and eight introns. The first intron is located in the 5′-UTR and processing of this intron has a potential regulatory function. The 5′-flanking region of the gene contains potential transcriptional regulatory elements such as a TATA box, two CAAT boxes and AP-1-, C/EBP-, ELP-, H-APF-1-, HNF-5- and PEA3-binding sites.

INTRODUCTION

Onchocerca volvulus is a human parasitic nematode responsible for the disease onchocerciasis, which affects more than 20 million people in Africa, South and Central America and Yemen. Current chemotherapeutic treatment of this infection is not ideal, since the drugs used do not affect all of the life stages of this parasite. Attempts to identify additional drug targets for this, as well as other filarial infections, have indicated that the polyamine-biosynthesis pathway offers various possibilities and advantages. The polyamine metabolism of filarial worms exhibits novel features, such as the absence of the initial enzyme of this pathway, ornithine decarboxylase [1,2]. The other major enzyme in this pathway, *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), is therefore likely to be the key regulatory enzyme in these parasites. The SAMDC reaction product, decarboxylated *S*-adenosylmethionine, serves as the aminopropyl donor for the biosynthesis of spermidine and spermine [3,4]. These polyamines play an important role in cell growth and differentiation [3,5–8]. Spermidine and spermine are also involved in cell transformation [9–11] and cell protection from oxidative damage [12]. Therefore SAMDC is a potentially important target for chemotherapeutic agents [13]. Some SAMDC inhibitors exhibit strong therapeutic effects in proliferative [7,14–17] and parasitic diseases [18–20].

In mammalian cells, the concentration of decarboxylated *S*-adenosylmethionine is usually very low and it is the limiting component required for polyamine synthesis [7]. SAMDC has a very short half-life and its expression is regulated at multiple levels, transcriptional, translational, as well as post-translational [21,22]. One of the major mechanisms that regulates the expression of SAMDC is the modulation of translation efficiency by sequences in the 5′-untranslated region (UTR) of the mRNA [23,24]. SAMDC expression is also regulated by the polyamines themselves [24–28], and can be induced by a variety of proliferative stimuli such as hormones, growth factors and tumour promoters [3,7,8,21].

Cloning, sequencing and expression of *Escherichia coli* [29], yeast [30] and mammalian [31] SAMDC cDNAs have shown that SAMDC is synthesized as a proenzyme with no enzymic activity, which is cleaved to produce two different subunits. The pyruvate prosthetic group is generated at the N-terminus of the large subunit from the *N*-terminal serine residue [30,32].

In order to analyse the structure, activity and regulation of expression of the *O. volvulus* SAMDC, we isolated cDNAs and examined mRNA processing. We expressed the complete cDNA in a SAMDC-deficient *E. coli* strain allowing analysis of the activity and quaternary structure of the active protein. Finally, we determined the structure and sequence of the entire gene, enabling the identification of potential sequence elements involved in transcription regulation.

MATERIALS AND METHODS

Parasites

Adult *O. volvulus* worms were isolated and prepared as previously described [33]. Briefly, nodules containing the parasites were

Abbreviations used: SAMDC, *S*-adenosylmethionine decarboxylase; MGBG, methylglyoxal bis(guanylhydrazone); ORF, open reading frame; RACE, rapid amplification of cDNA ends; UTR, untranslated region; DTT, dithiothreitol.

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The nucleotide sequences reported in this paper have been submitted to the GenBank™/EMBL Data Bank with the accession numbers X95713 and X95714 for the cDNA and gene sequences respectively.

surgically isolated from Liberian patients. Worms were prepared from the nodules by microdissection and then incubated in RPMI 1640 with 0.5% collagenase to eliminate host tissue. The worms were maintained in liquid nitrogen until use.

Preparation of genomic DNA and RNA

Adult *O. volvulus* worms were recovered from liquid nitrogen, homogenized immediately in guanidinium thiocyanate solution, layered on a CsCl step gradient and the DNA and RNA were prepared as previously described [34].

Isolation of *O. volvulus* SAMDC genomic DNA fragments by PCR

Four degenerate inosine-containing primers (Biometra; see Figure 1) were designed on the basis of highly conserved regions in the sequence of yeast [30] and human [31] SAMDCs. Amplification was carried out in a total volume of 100 μl with 100 ng of *O. volvulus* genomic DNA and 10 pmol of each primer. The template was then denatured for 5 min at 95 °C followed by five early cycles (95 °C for 2 min; 40 °C for 2 min; 2.5 min ramp time and 72 °C for 2 min), followed by 30 normal cycles (95 °C for 2 min; 45 °C for 2 min; 72 °C for 2 min). After a final extension for 7.0 min at 72 °C, the reaction mixtures were cooled down to 4 °C. The amplified DNA products were resolved by 1.0% agarose gel electrophoresis and visualized using ethidium bromide. The DNA fragments were purified using Gene Clean II kit (BIO 101, Inc.) ligated into the pCRII plasmid vector (TA-Cloning System; Invitrogen) and the nucleotide sequences were determined and analysed.

DNA sequencing

The nucleotide sequence of both strands of the PCR products, cDNA and genomic DNA was determined using the dideoxy-chain-termination method on double-stranded DNA [35] using [[^{35}S]thio]dATP and Sequenase Sequencing Kit (USB; Amersham). The *O. volvulus* SAMDC cDNA sequence was also determined using the recombinant single-stranded M13 template (Stratagene). DNA and amino acid sequence analyses were carried out using DNASIS/PROSIS computer software (Hitachi).

Isolation of *O. volvulus* SAMDC cDNAs

The isolated SAMDC genomic PCR fragment (SAMPCR2) was labelled with [^{32}P]dATP (Random Primed DNA Labelling Kit; Boehringer-Mannheim) and used as a hybridization probe to screen the adult *O. volvulus* λzapII cDNA library [36] using a standard plaque hybridization assay [37]. All prehybridization, hybridization and washing steps were performed at 55 °C. The partial SAMDC cDNA fragment identified was used as a hybridization probe in a second round of screening in order to isolate larger cDNAs. Three different *O. volvulus* SAMDC cDNA clones were identified. The phagemids containing the SAMDC cDNA were recovered from the λzapII library by the *in vivo* excision process as described by the manufacturer (Stratagene). The nucleotide sequences of these clones were determined and analysed.

Expression of the *O. volvulus* SAMDC in *E. coli*

The expression plasmid pTrcHis-B (Invitrogen), which contains a *trc* (*trp-lac*) promoter, was used to express the *O. volvulus* SAMDC in *E. coli*. The coding region of the *O. volvulus* SAMDC was amplified using the sense primer 5′-GCCCATGGGC ***ATG***TCAACTTCTACTTTATCG-3′ and the antisense primer 5′-GCAAGCTT***CTA***ACGGTCGAATACTTCTTTGC-3′ which contain *Nco*I and *Hin*dIII restriction sites respectively (underlined). The PCR product was subcloned in the pCRII vector and transformed into DH5α competent cells. Recombinant plasmid DNA was isolated and digested with the restriction enzymes *Nco*I and *Hin*dIII. The resulting fragment was gel-purified and subcloned into *Nco*I–*Hin*dIII-cleaved pTrcHis-B. Recombinant expression plasmids (pTrcSAM) were partially sequenced to ensure that the insert was in the correct reading frame and were then introduced into the *E. coli* strain EWH331, which is a mutant lacking endogenous SAMDC [38]. SAMDC activity was assayed by measuring the production of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxy*-^{14}C]methionine as previously described [39].

Table 1 Purification of the *O. volvulus* SAMDC expressed in *E. coli*

The standard assay conditions including 2.0 mM putrescine and 100 μM (50 nCi) *S*-adenosylmethionine were employed.

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Purification (-fold)	Yield (%)
100000 ***g*** supernatant	155	164.5	1.05	1.0	100
DEAE-cellulose	76	128	1.68	1.6	78
MGBG–Sepharose	0.122	44	361	341	27
Superdex S-200	0.065	26	400	377	16

Purification of the recombinant *O. volvulus* SAMDC produced in *E. coli*

EWH331 cells carrying pTrcSAM were grown overnight at 37 °C in Luria–Bertani medium, the cells were then diluted 100-fold and grown until an A_{600} of 0.5 was reached. In order to induce the *trc* promoter, isopropyl β-D-thiogalactoside was added to 1.0 mM and the cells were grown for a further 3 h. The cells were harvested by centrifugation (4000 ***g***; 10 min) and sonicated in buffer A (50 mM Tris/HCl, pH 7.5, 2.0 mM putrescine, 1.0 mM dithiothreitol (DTT), 0.1 mM EDTA) and the extract was centrifuged at 100000 ***g*** for 1 h (Kontron). The supernatant was loaded on to a DEAE-cellulose column (2.6 cm × 6.0 cm), previously equilibrated with buffer A. The column was washed with buffer A and the enzyme eluted with a linear gradient of 0–1 M NaCl. The fractions in which SAMDC activity could be demonstrated were pooled and dialysed overnight against buffer A. The dialysed enzyme was loaded on to a methylglyoxal bis-(guanylhydrazone)–Sepharose (MGBG–Sepharose) column, which was prepared as previously described [40] using epoxy-activated Sepharose (Sigma). The MGBG–Sepharose column (8.0 cm × 1.6 cm) was equilibrated with buffer A. Unbound proteins were washed through with buffer A containing 0.5 M NaCl, and the SAMDC was eluted with the same buffer containing 10 μM MGBG. Fractions containing SAMDC activity were collected and concentrated to 1 ml using Centricon 10 (Amicon). SAMDC was further purified by FPLC on a Superdex S-200 column (1.6 cm × 60 cm) previously equilibrated with buffer A containing 0.1 M NaCl at a flow rate of 1 ml/min. The fractions with enzyme activity were pooled and used for further studies. The purification steps are summarized in Table 1. The purity of the protein was assessed by SDS/PAGE [41]. Protein was measured by the method of Bradford [42], with BSA as a

standard. The molecular mass of the SAMDC was determined using gel-filtration chromatography on Superdex S-200, which was previously calibrated with the gel-filtration molecular-mass marker kit (Sigma).

N-Terminal amino acid sequence of *O. volvulus* SAMDC

In order to be able to determine the N-terminal amino acid sequence of SAMDC, it was necessary to convert the pyruvate prosthetic group into alanine by reductive amination [43]. Purified SAMDC (90 μg) was incubated in 2 M ammonium acetate, pH 6.5, containing 150 mM $NaCNBH_3$, 2 mM putrescine, 1.0 mM DTT and 0.1 mM PMSF, in a total volume of 2 ml, and incubated overnight at room temperature. The inactivated protein was concentrated and washed three times with distilled water using a Centricon 10 concentrator. Protein subunits were separated by SDS/PAGE, electrotransferred to ProBlot™ membrane and stained with Coomassie Blue (Applied Biosystems). The large subunit (32 kDa) was excised and subjected to automated Edman degradation (Protein Sequencer 473A; Applied Biosystems).

Isolation of the gene encoding *O. volvulus* SAMDC

An *O. volvulus* adult worm λfixII genomic DNA library (Stratagene) was screened using the radiolabelled genomic PCR product (SAMPCR1). Five positive clones were identified. These clones were examined by Southern-blot analysis and one clone was chosen for further characterization. To identify the *O. volvulus* SAMDC gene fragments within the genomic DNA insert, the phage DNA was digested with *Eco*RI, *Eco*RV, *Sal*I and *Eco*RI–*Sal*I, resolved on a 0.8% agarose gel and analysed by the Southern transfer method [37]. The radiolabelled *O. volvulus* SAMDC cDNA (nucleotides 283–1677) was used as a probe. The positive *Eco*RI and *Eco*RV fragments were isolated, subcloned into the pBS-KS$^+$ vector (Stratagene) and the nucleotide sequence was determined.

Genomic Southern-blot analysis

O. volvulus genomic DNA (10 μg/lane) was digested with *Eco*RI and *Eco*RV, and human genomic DNA (10 μg/lane) was digested with *Eco*RI. The DNA samples were size fractionated on a 0.8% agarose gel, transferred to a nylon membrane using standard methods [37] and hybridized with the radiolabelled [^{32}P]dATP *O. volvulus* SAMDC cDNA (nucleotides 283–1677).

Identification of the 5′ end of the *O. volvulus* SAMDC mRNA by anchored PCR

To amplify the 5′ end of the SAMDC mRNA, anchored PCR was performed using the 5′-rapid amplification of cDNA ends (RACE; Gibco–BRL) as described by the manufacturer. Briefly, the first cDNA strand was synthesized using 1 μg of total *O. volvulus* RNA, SuperScript II reverse transcriptase and 2.5 pmol of a SAMDC-specific primer (SAMGSP1; double underlined in Figure 2). Subsequently, the RNA template was degraded and the single-stranded cDNA was purified using a Glass Max spin cartridge. The 3′ end of the cDNA was then tailed with dCTP using terminal transferase. The dC-tailed cDNA was amplified by PCR with the SAMGSP1 primer and with the (dG) anchored primer. To increase the specificity, two other nested PCRs using SAMGSP2 and SAMGSP3 (Figure 2) were performed. The PCR products were analysed by 1.5% agarose-gel electrophoresis. Fragments of interest were purified, ligated into the pCRII plasmid vector (TA-Cloning System) and the nucleotide sequences were determined and analysed.

RESULTS

Identification of genomic SAMDC PCR products

A comparison of the amino acid sequences of yeast [30] and human [31] SAMDCs revealed some regions that exhibited a high degree of homology between these two organisms. Based upon three of these conserved regions, four different degenerate, inosine-containing primers were designed (Figure 1). A PCR using SAMSI and SAMASII primers (box I and II, Figure 1) with *O. volvulus* genomic DNA produced a 537 bp fragment (SAMPCR1). Another PCR using SAMSII and SAMASIII primers (box II and III, Figure 1) resulted in a 776 bp PCR fragment (SAMPCR2). The sequences of both PCR products (SAMPCR1 and SAMPCR2) were determined. The deduced amino acid sequences were analysed and found to have similarity to other SAMDCs.

Characterization of the λzapII *O. volvulus* SAMDC cDNA

A λzapII *O. volvulus* cDNA library was screened using the radiolabelled SAMPCR2 fragment. One positive clone was identified which contained a partial SAMDC cDNA insert. This cDNA insert was used as a hybridization probe in a second round of screening of the same library, in order to isolate longer cDNA clones. Three positive clones were identified. Only one clone was found to contain the entire SAMDC coding region. The complete nucleotide sequence of the cDNA and its deduced amino acid sequence are presented in Figure 2. The 1677 bp cDNA possesses a 1095 bp open reading frame (ORF), beginning at position 283 and ending at 1378. This ORF encodes a polypeptide of 365 amino acids which corresponds to the SAMDC proenzyme, with a calculated molecular mass of 41 890 Da. This cDNA also possesses a second ORF in the sequence 5′ to the ORF encoding the SAMDC. This upstream

Box I; Sense primer (SAMSI):
Amino acid sequence **(F E G P E K L L E)**
5′ TT$^{T}/_{C}$ GA$^{G}/_{A}$ GGI $^{C}/_{A}$CI GA$^{G}/_{A}$ AA$^{G}/_{A}$ $^{T}/_{C}$TI TTN GAA 3′

Box II; Sense primer (SAMSII):
Amino acid sequence **(I L K T C G T T)**
5′ ATI ITI AA$^{A}/_{G}$ ACI TG$^{C}/_{T}$ GGN ACN AC- 3′

Box II; Anti-sense primer (SAMASII):
Amino acid sequence **(K T C G T T)**
5′ GTN GTN CC$^{G}/_{A}$ CAN $^{G}/_{C}$TT TT- 3′

Box III; Anti-sense primer (SAMASIII):
Amino acid sequence **(F $^{T}/_{N}$ P C G Y S $^{M}/_{S}$ N)**
5′ -TT II$^{A}/_{T}$ I$^{G}/_{C}$$^{A}/_{G}$ TAI CC$^{A}/_{G}$ CAI GGI G$^{A}/_{T}$$^{A}/_{G}$ A-- 3′

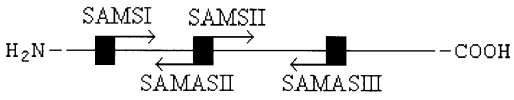

Figure 1 PCR strategy used to identify SAMDC sequences

The primers were designed on the basis of conserved motifs in yeast [30] and human [31] SAMDC sequences. All possible triplet codons encoding the conserved peptide motif are included in the degenerate oligonucleotides. In order to reduce the complexity of the primer population, inosine (I) was introduced in some positions.

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gtt tga gat att tta act tca tcc att ttc cat tat cgt tct cag cca gac taa taa gtc      60

aac tga aaa aat tct act cga gaa taa tat atg ttg agt aga atc att gct cgt ggc ata     120
                    § Trans-splicing site
gaa gga aca ata caa caa aga act gtt tta tga ata atc ctt gta aaa tta tca tta tca     180
ttc acc atc gtc gac ctc cct agt agg cct caa ttt ctt att tct gtt ccc att atc att     240

                          SAMGSP3                                  M  S  T  S  T  L    6
cac ttc taa tag gaa gca gtt aaa ttc tgt tat ttt taa agc ATG TCA ACT TCT ACT TTA     300
                                                          SAMGSP2
 S  D  G  S  L  D  E  G  T  F  A  A  E  D  V  S  G  V  I  E   26
TCG GAT GGT TCT CTT GAT GAA GGT ACT TTT GCT GCA GAA GAT GTA TCC GGT GTT ATT GAA     360

 D  Y  F  F  E  G  A  E  K  L  L  E  I  W  F  D  K  N  Q  N   46
GAT TAT TTT TTT GAA GGC GCG GAA AAA TTG CTG GAA ATA TGG TTT GAT AAA AAT CAG AAT     420
                   SAMGSP1
 G  A  T  S  L  R  N  I  P  Y  S  E  L  V  S  M  L  D  I  A   66
GGA GCT ACG TCC TTG AGA AAT ATA CCG TAT TCG GAA TTG GTT TCT ATG CTA GAT ATA GCG     480

 Q  C  R  I  L  H  S  K  S  N  E  C  M  D  S  Y  V  L  S  E   86
CAG TGC CGT ATT TTG CAT TCT AAA AGT AAT GAA TGC ATG GAT AGT TAT GTT TTG AGC GAA     540
↓ Cleavage site
 A  S  M  F  I  S  D  F  R  I  I  L  K  T  C  G  T  T  R  L
 S  S  M  F  I  S  D  F  R  I  I  L  K  T  C  G  T  T  R  L  106
AGC AGT ATG TTT ATC TCG GAT TTT CGT ATC ATT TTG AAA ACA TGT GGC ACA ACG CGA TTA     600

 L  H  A  I  E
 L  H  A  I  E  R  I  L  H  I  A  K  I  Y  C  N  M  D  N  V  126
CTT CAC GCG ATA GAA CGA ATT CTA CAT ATT GCT AAG ATA TAC TGC AAC ATG GAT AAC GTT     660

 V  S  V  F  Y  S  R  K  N  F  M  H  P  E  K  Q  P  Y  P  H  146
GTC AGC GTT TTT TAC TCA AGA AAG AAT TTT ATG CAT CCC GAG AAA CAA CCT TAT CCA CAT     720

 S  S  F  E  T  E  V  D  Y  L  E  E  H  F  A  G  G  S  A  Y  166
TCT TCG TTT GAA ACT GAA GTT GAT TAT CTT GAA GAA CAT TTC GCA GGA GGA TCT GCT TAT     780

 C  I  G  P  Q  R  Q  D  R  W  F  L  Y  T  M  V  T  P  Q  A  186
TGT ATC GGT CCA CAA AGA CAG GAT CGT TGG TTT CTC TAC ACA ATG GTT ACT CCA CAG GCT     840

 V  F  P  F  P  E  H  T  L  E  I  L  M  N  G  L  P  E  D  V  206
GTT TTT CCA TTT CCG GAA CAT ACA CTC GAA ATA CTT ATG AAT GGT TTA CCA GAA GAT GTT     900

 L  S  T  F  S  P  N  V  S  K  D  G  K  D  C  R  M  K  S  A  226
CTT AGT ACA TTC AGT CCT AAT GTA AGC AAA GAT GGG AAG GAC TGC AGA ATG AAA TCA GCA     960

 I  N  T  I  L  P  P  D  I  V  V  H  E  E  L  F  S  P  C  G  246
ATC AAC ACA ATA CTG CCA CCT GAT ATC GTC GTC CAT GAG GAA TTA TTT AGT CCA TGT GGT    1020

 Y  S  L  N  G  L  I  P  H  S  D  H  Y  I  T  I  H  V  T  P  266
TAC AGC CTG AAT GGA TTG ATT CCA CAT TCA GAT CAT TAC ATC ACG ATT CAT GTA ACA CCA    1080

 E  P  D  F  S  Y  V  S  F  E  T  N  Q  H  T  L  N  L  C  E  286
GAG CCA GAT TTT TCA TAT GTC AGT TTC GAG ACA AAT CAG CAT ACG TTG AAT TTG TGT GAG    1140

 Q  M  L  K  V  L  E  I  F  K  P  S  K  F  L  L  T  I  F  T  306
CAA ATG CTG AAA GTT CTA GAA ATT TTT AAA CCG AGT AAA TTT TTA TTG ACT ATT TTC ACC    1200

 N  E  L  S  N  E  G  K  K  M  Q  K  N  L  W  D  L  K  I  C  326
AAT GAA CTA TCA AAC GAA GGC AAA AAA ATG CAG AAA AAT TTG TGG GAT CTG AAA ATT TGT    1260

 G  C  R  R  T  N  L  Q  F  L  E  L  P  T  E  T  L  I  Y  V  346
GGA TGT CGT CGG ACG AAC TTG CAA TTT CTT GAA TTG CCG ACT GAA ACA CTA ATT TAT GTG    1320

 Q  F  E  R  I  K  S  A  E  Q  V  T  C  K  E  V  F  D  R  *  365
CAG TTC GAA CGA ATA AAA AGT GCG GAA CAA GTA ACG TGC AAA GAA GTA TTC GAC CGT TAG    1380
taa tga cag tta caa tgt tga gtg atg aat att tgg gat tct tca ctt cat tat gat tgg    1440
tct ttt aat tac cgg tgg ttt ggt gct gtt tct aat att ttc aag ttt ttt tct tca cac    1500
ttt ttc ttt ttt att ctc ttt cca taa act tta ttg aat aca tca ttc tca ata tga ctg    1560
gta gtc aat tgt ttg ttt att tct gta ttt tat tct ctt ttt gag ctt tgt ttt tga gaa    1620
tct ttt ttt ttt ctt ttt ctg tag cat aat aaa tag att aaa aaa aaa aaa aaa aaa         1677
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ORF encodes a small peptide of 17 residues (MNNPC KIIIIIHHRRPP). The 282 bp 5′-UTR possesses the last seven nucleotides of a consensus 5′ spliced-leader sequence (Figure 2) [44]. The cDNA possesses a 300 bp 3′-UTR, including the poly(A) tail. This region contains a typical polyadenylation signal sequence (AATAAA) located 6 bp upstream from the poly(A).

Analysis of the deduced amino acid sequence of the *O. volvulus* SAMDC cDNA

The amino acid sequences of the *O. volvulus* and *E. coli* [29] SAMDCs have almost no identity. Pairwise sequence comparisons of the *O. volvulus* SAMDC deduced amino acid sequence with that of yeast [30], human [31], *Leishmania donovani* (EMBL accession no. U20091) and *Trypanosoma brucei* (EMBL accession no. U20092) SAMDCs exhibited 42, 26, 32 and 31% identity respectively. However, there are at least five highly conserved regions which the *O. volvulus* SAMDC has in common with the other eukaryotic SAMDCs (Figure 3). The amino acid sequence from residue 81 to 90 (YVLSE↓SSMF) which surrounds the predicted proenzyme cleavage site of the *O. volvulus* sequence is identical with that surrounding the mammalian proenzyme cleavage site [32] and has a high homology to that of yeast [30]. The *O. volvulus* SAMDC sequence contains two PEST sequences (sequences rich in the amino acids P, E/D, S/T). One consists of 14 amino acids between residues 146 and 160 (HSSFETEVDYLEEH). The second consists of 18 amino acids from residues 263 to 280 (HVTPEPDFSYVSFETNQH) and exhibits about 90% sequence identity with the PEST sequence identified in the mammalian SAMDC [31]. *O. volvulus* SAMDC contains all the residues that have been described to be critical for enzyme activity as well as those necessary for the putrescine-dependent stimulation of proenzyme processing and catalytic activity (underlined in Figure 2).

Production of an enzymically active recombinant *O. volvulus* SAMDC

In order to determine whether the *O. volvulus* SAMDC cDNA clone encodes a functional protein, it was expressed in *E. coli* using the pTrcHis-B expression vector (Invitrogen). Expression was carried out in EWH 331 cells which is a mutant lacking endogenous SAMDC activity [38]. The recombinant *O. volvulus* SAMDC was expressed at high levels determined by analysis of the bacterial extract by SDS/PAGE. A large amount of the recombinant protein produced was found to be in inclusion bodies (results not shown). This inactive protein was purified from the inclusion bodies under denaturing conditions and determined to have a molecular mass of 42 kDa corresponding to the SAMDC proenzyme. The soluble active protein was also purified from bacteria in a four-step procedure (Table 1), over 350-fold to apparent homogeneity. SDS/PAGE analysis of the purified active protein revealed two protein subunits, a 32 kDa protein corresponding to the SAMDC α-subunit and a 10 kDa protein corresponding to the SAMDC β-subunit (Figure 4). The purified recombinant *O. volvulus* SAMDC has a specific activity of 400 nmol of CO_2 produced/min per mg of protein. The molecular mass of the active SAMDC, determined by Superdex S-200 gel-filtration chromatography, is approx. 84 kDa.

Proenzyme processing in the *O. volvulus* SAMDC

The active recombinant enzyme consists of two distinct subunits as shown by SDS/PAGE (Figure 4). These two subunits are typically generated from the 42 kDa proenzyme by a post-translational processing reaction. As indicated in the amino acid sequence analysis, this cleavage site sequence is conserved in the *O. volvulus* SAMDC sequence. To demonstrate that the *O. volvulus* SAMDC is processed and to identify the precise cleavage site, the N-terminal amino acid sequence of the large subunit was determined after reductive amination. The amino acid sequence obtained was compared with the deduced amino acid sequence of the *O. volvulus* SAMDC proenzyme derived from the nucleotide sequence. The amino acid sequence determined by Edman degradation corresponds exactly to residues 87 to 111 of the proenzyme sequence (Figure 2). The cleavage site in the proenzyme is therefore the amide linkage between Glu^{86} and Ser^{87}. The discrepancy at the first position in the amino acid sequence is due to the conversion of serine into pyruvate, which was then converted into alanine by the reductive amination.

Structure and sequence of the *O. volvulus* SAMDC gene

Five *O. volvulus* λfixII genomic DNA inserts were mapped by restriction enzyme cleavage analysis. A genomic DNA insert that encompassed the entire *O. volvulus* SAMDC gene was selected for further analysis. This genomic DNA insert was analysed by Southern blotting, after digestion with a variety of restriction enzymes (Figure 5, top). The positive *Eco*RI and *Eco*RV fragments were isolated and the nucleotide sequence was determined. Genomic Southern-blot analysis was performed to confirm the parasite origin of the cDNA and to determine the complexity and copy number of the SAMDC gene(s) in the *O. volvulus* genome. As shown in the bottom panel of Figure 5, the cDNA hybridized specifically with the *O. volvulus* DNA and not with the human DNA. The hybridization pattern observed with the *O. volvulus* total genomic DNA was identical with that observed for the isolated gene. The lack of additional hybridizing fragments suggests that *O. volvulus* SAMDC is encoded by a single-copy gene. The complete sequence of the gene as well as the 3′ and 5′ flanking regions are presented in Figure 6. The structural organization and the partial restriction map of the *O. volvulus* SAMDC gene is presented in Figure 7. It spans 5.3 kb of the genomic DNA. The coding region is comprised of eight exons, interrupted by seven introns. The nucleotide sequence of the *O. volvulus* SAMDC protein-coding region of the chromosomal gene was identical with that in the cDNA sequence. A comparison of the 5′-UTR of the *O. volvulus* SAMDC cDNA with the corresponding region in the gene revealed that the 5′ end of the primary mRNA transcript is interrupted by a 235-nucleotide intron (Figures 6 and 7). This intron is located 1 bp upstream of the translation-initiation codon, and it is removed from the mature mRNA. The lengths of the exons and introns and the sequences of the intron/exon junctions are listed in Table

Figure 2 Nucleotide and deduced amino acid sequence of the *O. volvulus* SAMDC proenzyme

The novel *trans*-spliced product is underlined. The *trans*-splicing location is indicated with § and the last seven nucleotides of the spliced leader are shown in an open box. The polyadenylation signal sequence is underlined and the poly(A) tail is in italics. The oligonucleotides used in the 5′-RACE procedure are double underlined. The *Eco*RI site is shown in bold type. The asterisk (*) indicates the stop codon. The deduced amino acid sequence in single-letter code is shown above the nucleotide sequence. The N-terminal amino acids of the large subunit (determined after reductive amination) are shown in italics. The cleavage site at Glu^{86}-Ser^{87} is indicated by an arrow. The residues that are necessary for SAMDC processing and activity are underlined.

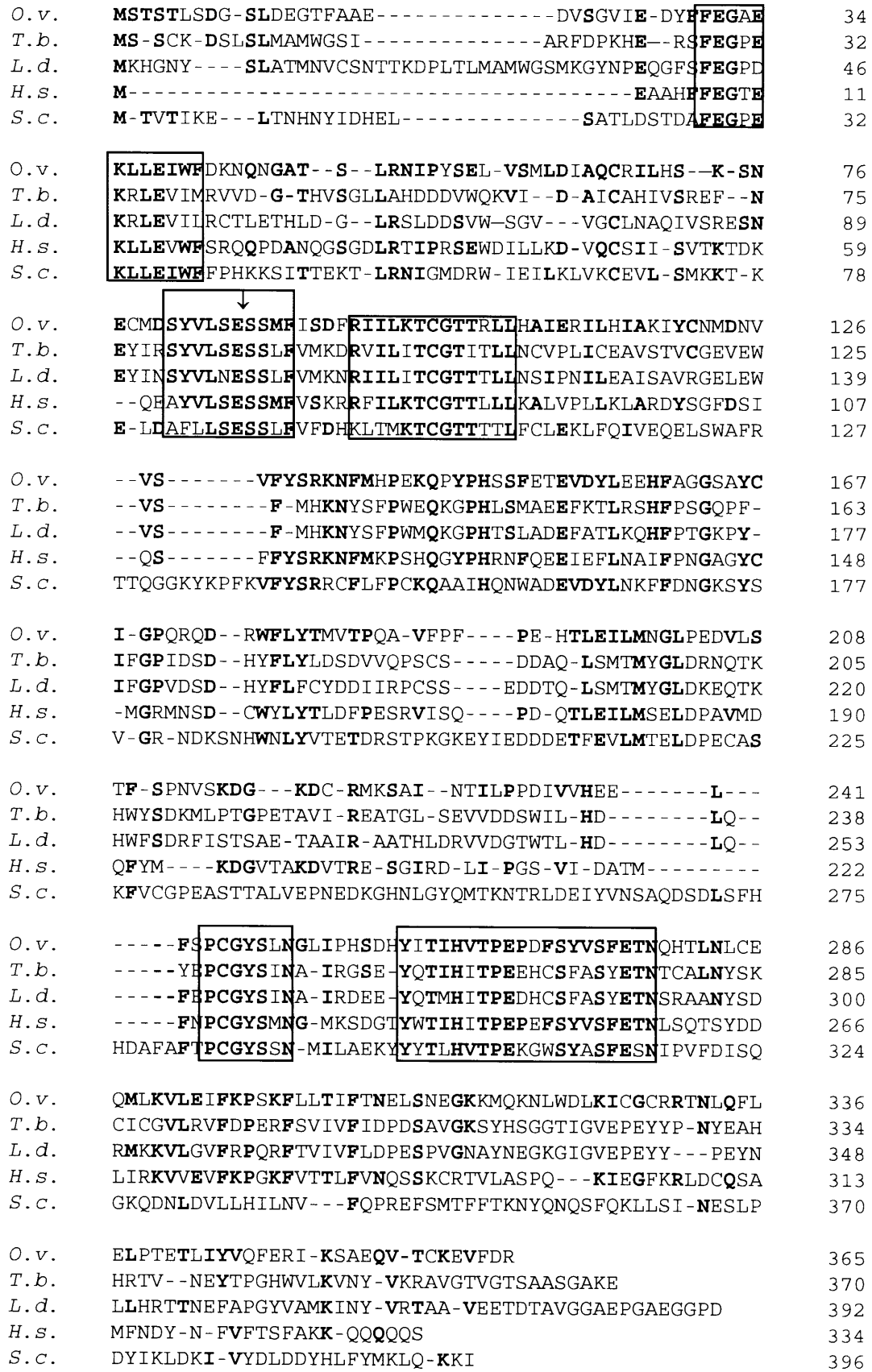

Figure 3 For caption see facing page

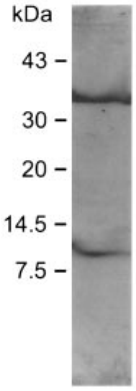

Figure 4 Analysis of the purified active *O. volvulus* SAMDC by SDS/PAGE

The recombinant *O. volvulus* SAMDC was purified as described in the text and analysed by Tricine/SDS/PAGE (16% gel) [41] and the proteins were visualized by silver staining. Sizes of the molecular mass standards are shown on the left.

2. An examination of the intron/exon boundaries of the *O. volvulus* SAMDC gene revealed conserved 5′ gt---ag 3′ intron splicing site junctions. The *O. volvulus* SAMDC gene was found to be AT-rich (70%). The 3′ region of the *O. volvulus* SAMDC gene contains three potential polyadenylation signal sequences (AATAAA; Figure 6). The first polyadenylation signal, which is found 270 bp downstream from the termination of the SAMDC protein-coding sequence, corresponds to the polyadenylation signal sequence indicated in the isolated cDNA clone (underlined).

Identification of two distinct *trans*-spliced *O. volvulus* SAMDC mRNAs

The *O. volvulus* SAMDC cDNA clone possesses a 282 bp 5′-UTR which begins with 138 bp (Figure 2) that are not encoded by the gene sequence (Figures 6 and 7). In order to identify the origin of this sequence and to examine further the sequence at the 5′ end of the *O. volvulus* SAMDC mRNA, we performed anchored PCR (5′-RACE). Two distinct fragments were produced and their nucleotide sequences were determined. One of these RACE products confirmed the cDNA sequence, indicating *trans*-splicing of a 138 bp RNA with the complete consensus 22-nucleotide spliced-leader sequence (ggtttaattacccaagtttgag) at the 5′ end (Figure 7). The second RACE product possessed the 22-nucleotide spliced-leader sequence spliced at the same site as the 138 bp RNA, 142 nucleotides before the ATG.

Identification of potential transcriptional regulatory elements in the *O. volvulus* SAMDC gene

The nucleotide sequence of the 5′-flanking region of *O. volvulus* SAMDC gene was determined for the region 1476 bp before the *trans*-splicing site of the mRNA transcript (Figure 6). To identify

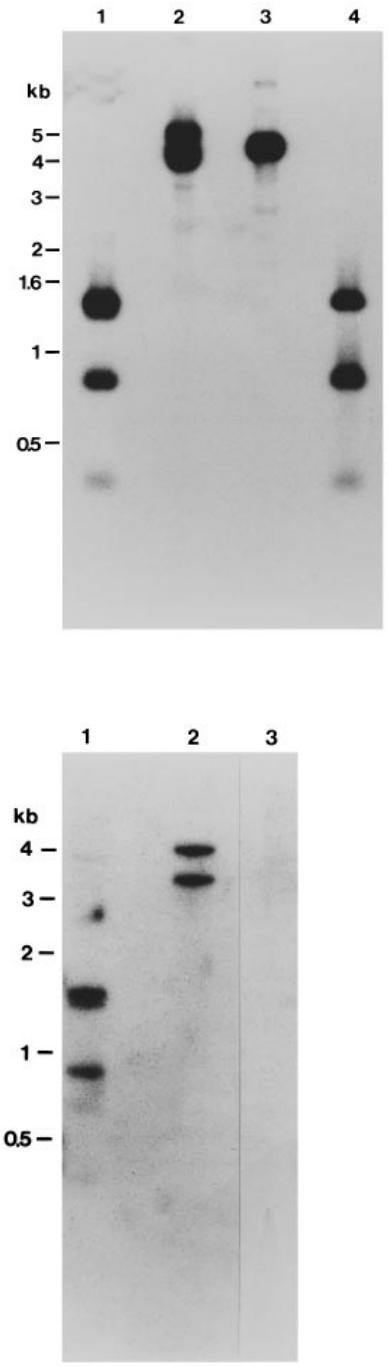

Figure 5 Examination of the *O. volvulus* SAMDC gene structure by Southern-blot analysis

Top, the purified *O. volvulus* SAMDC-λfixII phage DNA was digested with *Eco*RI (lane 1), *Eco*RV (lane 2), *Sal*I (lane 3) or *Eco*RI–*Sal*I (lane 4). The corresponding sizes of the hybridizing fragments are indicated on the left (kb). Bottom, total *O. volvulus* genomic DNA (10 μg/lane) was digested with *Eco*RI (lane 1) and *Eco*RV (lane 2), and 10 μg of human genomic DNA was digested with *Eco*RI (lane 3). The corresponding lengths of the hybridizing fragments are indicated on the left (kb).

Figure 3 Comparison of the deduced amino acid sequences of the SAMDCs from *O. volvulus* (*O.v.*), *T. brucei* (T.b.) (EMBL accession no. U20092), *L. donovani* (L.d.) (EMBL accession no. U20091), human (H.s.) [31] and yeast (S.c.) [30]

The amino acid residues in common with the *O. volvulus* sequence are shown in bold type. Gaps (–) were introduced into the sequences to maximize homology and to compensate for the different chain lengths. Regions of primary sequence homology are shown in boxes. The site of proenzyme processing is indicated by an arrow.

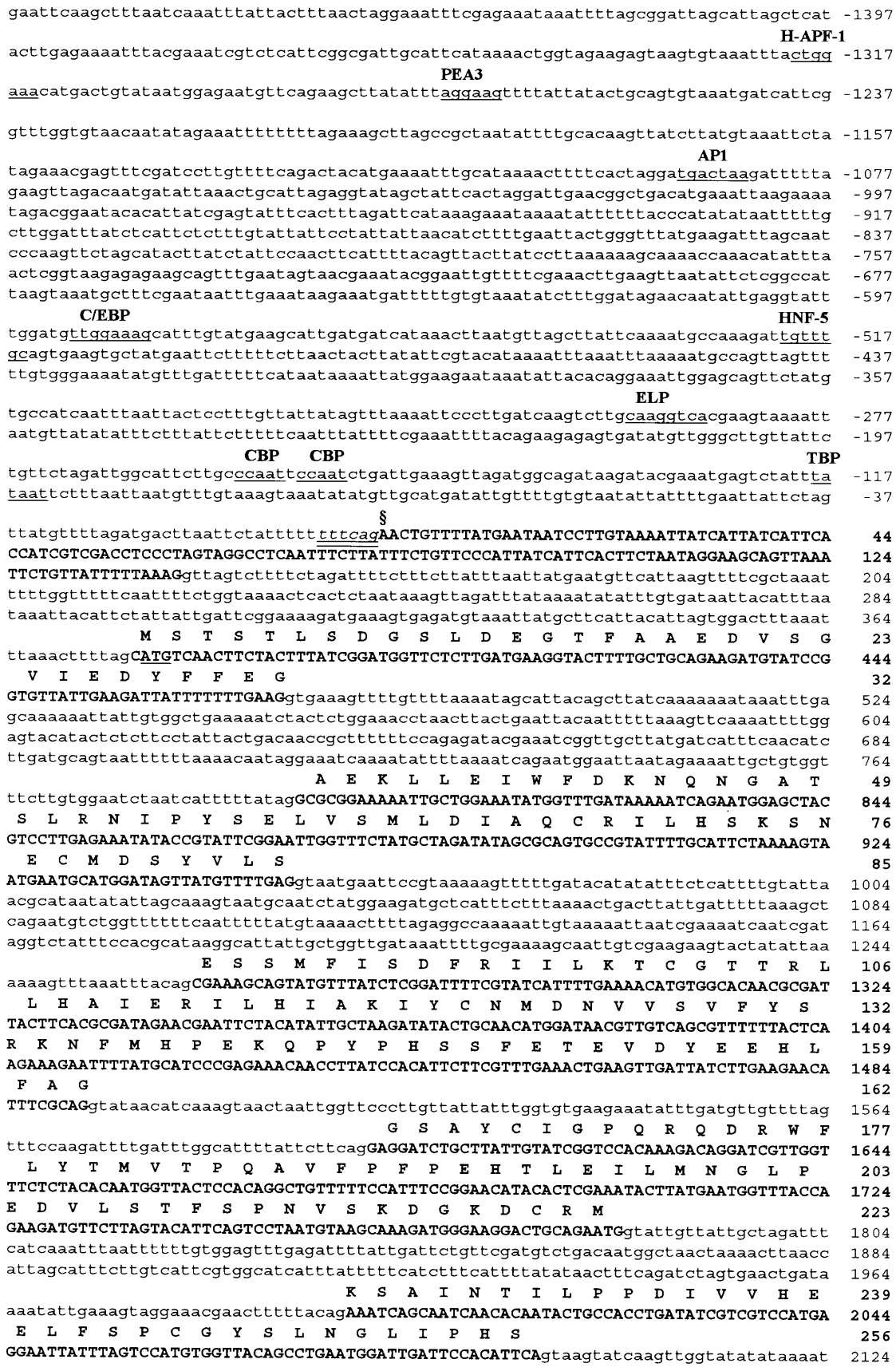

Figure 6 For caption see facing page

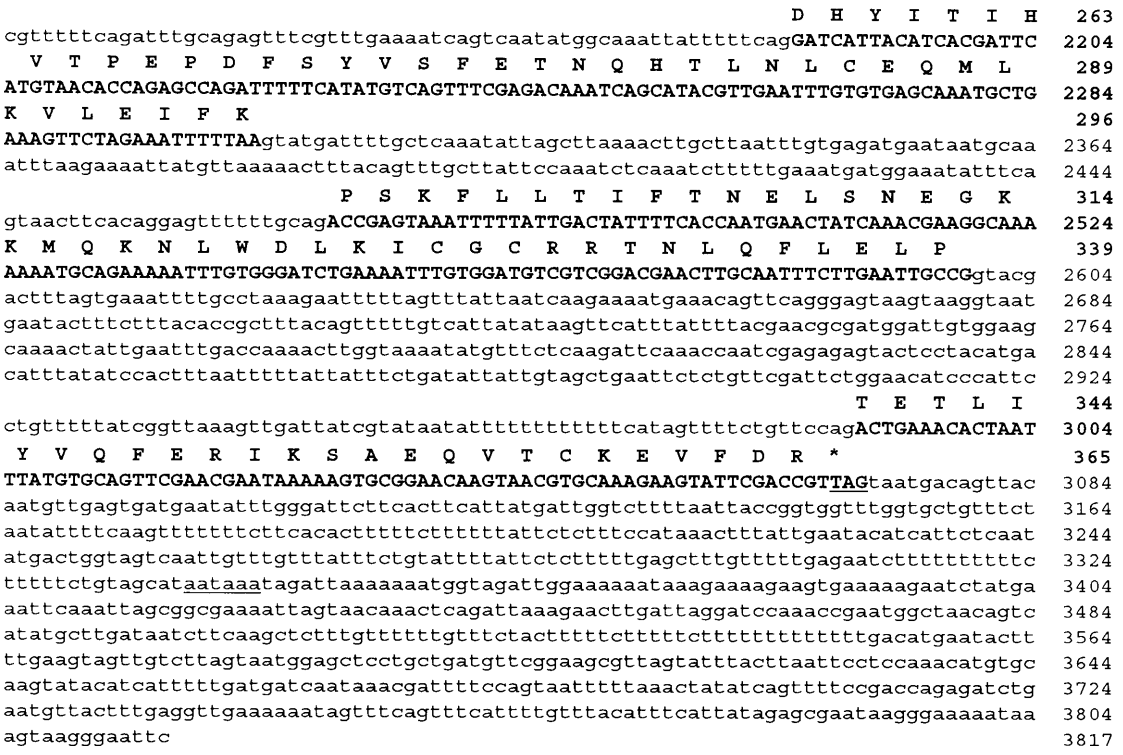

Figure 6 Nucleotide sequence of the *O. volvulus* SAMDC gene

Exonic and intronic sequences are specified by capital and lowercase letters respectively. The lengths of the intronic and exonic sequences are given in Table 2. The polyadenylation signal sequence is underlined and in italics. The *trans*-splicing site is double 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. The potential regulatory elements are underlined and labelled: AP1, activator protein; CBP, CAAT-binding protein; C/EBP, CAAT/enhancer-binding protein; ELP, embryonal long terminal binding protein; H-APF-1, hepatocyte-specific nuclear protein; HNF-5, hepatocyte nuclear factor 5; PEA3, polyomavirus enhancer A-binding protein 3; TBP, TATA-binding protein.

potential transcription-factor-binding sites in this sequence, a computer search was performed to identify known transcription-factor-binding-consensus sequences. A potential TATA box, two CAAT boxes, Ap1, C/EBP, PEA3 and other transcription-factor-binding sites were identified (Figure 6).

DISCUSSION

In order to analyse the structure, activity and regulation of SAMDC expression in helminths, cDNA and genomic sequences were isolated from *O. volvulus* libraries. The amino acid sequence of the proenzyme of *O. volvulus* SAMDC has almost no identity with the *E. coli* SAMDC [29]. Pairwise comparison of the proenzyme of *O. volvulus* SAMDC with that of other eukaryotic organisms shows a moderate degree of identity. In spite of the low degree of identity between the *O. volvulus* SAMDC sequence and the sequences of other eukaryotic SAMDCs, there are some highly conserved regions (Figure 3). For example, the sequence (RIIL**K**T**C**GTT) located in the N-terminal portion of the β-subunit of the processed enzyme (residues 95 and 104 in the proenzyme sequence) is highly conserved. This sequence contains Lys^{99} and Cys^{101}, which have been shown to be essential for SAMDC proenzyme processing and activity [45]. The amino acid sequence of the *O. volvulus* SAMDC small subunit contains a highly conserved region (FF**E**GA**E**KLL**E**) located between residues 29 and 41. This region contains three glutamic acid residues (Glu^{31}, Glu^{34}, Glu^{37}) that are located in the same context in other eukaryotic SAMDCs. The first two in this sequence are essential for catalytic activity [45]. Glutamic acid residues (Glu^{34}, Glu^{196}, Glu^{276}) are found in equivalent positions to those of human SAMDC and are critical residues in the putrescine stimulation of the SAMDC proenzyme processing [45,46]. It is well known that the SAMDC protein has a rapid turnover in many organisms. It has been suggested that certain stretches of amino acids described as PEST sequences are important in the degradation of proteins with a rapid turnover [47]. The sequence of *O. volvulus* SAMDC contains two such putative PEST sequences. Whether they are responsible for the short half-life of *O. volvulus* SAMDC remains to be determined.

The expression of a functionally active *O. volvulus* SAMDC in *E. coli* EWH331 cells, which lack endogenous SAMDC activity [38], provides a convenient method for obtaining large amounts of active *O. volvulus* enzyme for further analysis. It is noteworthy that the specific SAMDC activity in the bacterial extract expressing recombinant enzyme was 1.05 nmol/min per mg of protein, which is about 250000-fold more than that measured in the *O. volvulus* extract [39]. The specific activity of purified *O. volvulus* SAMDC from *E. coli* was 400 nmol/min per mg of protein.

Although the prokaryotic and eukaryotic SAMDC enzymes catalyse the same reaction, they differ in subunit size, holoenzyme size and specific requirements for activity. The activity of the prokaryotic enzyme is activated by Mg^{2+}, whereas that of the

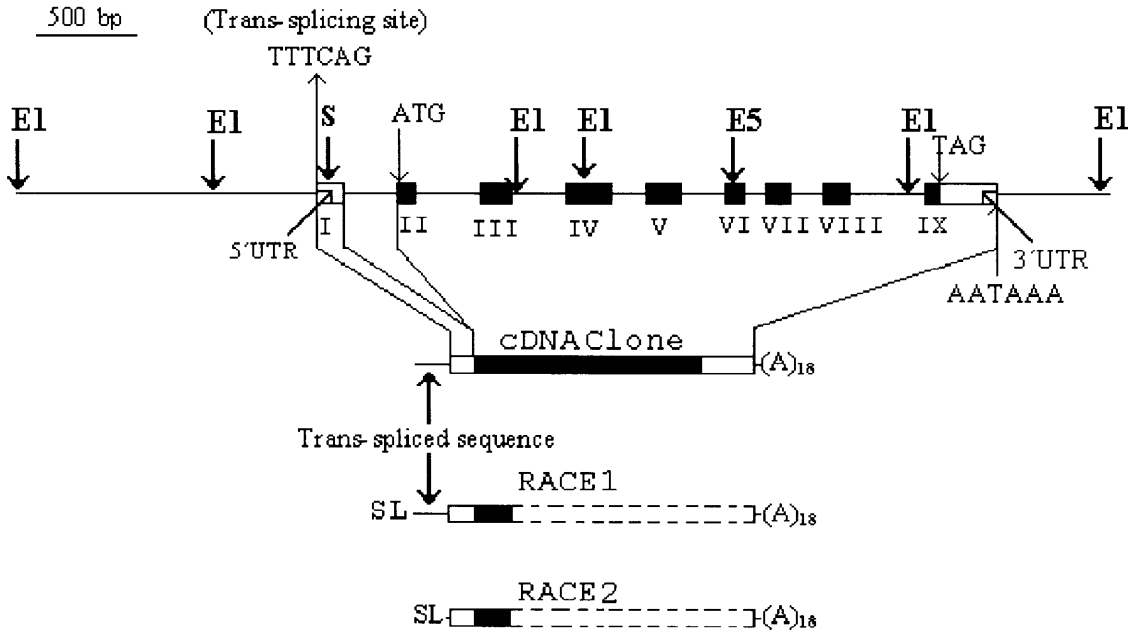

Figure 7 Organization of the *O. volvulus* SAMDC gene

The arrangement of exons (boxes and Roman numerals) and introns (solid lines) is depicted. The protein-coding region is identified by the solid boxes, and the 5′- and 3′-UTRs are shown as open boxes. The location of *Eco*RI (E1), *Eco*RV (E5) and *Sal*I (S) restriction enzyme sites in the gene are also shown. The position of the polyadenylation signal sequence and the *trans*-splicing sequence are also indicated. The lower portion of the Figure is a schematic illustration of the *O. volvulus* SAMDC cDNA and the products of the 5′-RACE experiment, indicating their relationship to the gene.

Table 2 Sizes of exons and introns and splice junction sequences in the *O. volvulus* SAMDC gene

Upper- and lower-case letters represent nucleotides in the exons and introns respectively. The start (ATG) and stop (TAG) codons are indicated in bold type. Size of exons does not include the 5′- and 3′-flanking regions. The first intron is located in the 5′-UTR, 1 bp before the translation start codon ATG.

Exon		Exon/intron junction sequences				
No.	Size (bp)	5′-Donor site		3′-Acceptor site		Intron size (bp)
1.	142	ATTTTTAAAG	gttagtcttt	aaacttttag	C**ATG**TCAACT	235
2.	94	TTTTTTGAAG	gtgaaagttt	atttttatag	GCGCGGAAAA	321
3.	160	ATGTTTTGAG	gtaatgaatt	aaatttacag	CGAAAGCAGT	310
4.	230	CATTTCGCAG	gtataacatc	tattcttcag	GAGGATCTGC	107
5.	185	CTGCAGAATG	gtattgttat	ctttttacag	AAATCAGCAA	213
6.	99	TCCACATTCA	gtaagtatca	tatttttcag	GATCATTACA	89
7.	119	AAATTTTTAA	gtatgatttt	ttttttgcag	ACCGAGTAAA	165
8.	130	TGAATTGCCG	gtacgacttt	tctgttccag	ACTGAAACAC	391
9.	81	CGAACCT**TAG**				

eukaryotic enzyme is stimulated by putrescine. We have found that *O. volvulus* SAMDC expressed in *E. coli* is activated more than 10-fold by putresine (results not shown). It has been reported that certain glutamic acid residues (Glu^{196} and Glu^{276} in the *O. volvulus* SAMDC proenzyme sequence) are critical for putrescine stimulation of SAMDC catalytic activity [46]. This activation could occur in a manner similar to that of the mammalian enzyme.

All previous studies on SAMDCs from prokaryotic and eukaryotic organisms have demonstrated that SAMDC is synthesized as a proenzyme, which is post-translationally processed into two subunits that form the active enzyme. SDS/PAGE analysis of the purified *O. volvulus* enzyme revealed two proteins of 32 and 10 kDa (Figure 4), indicating that recombinant *O. volvulus* SAMDC is processed in a similar manner. In order to identify the precise cleavage site in the proenzyme, the sequence of the N-terminus of the large subunit was determined. A comparison with the deduced amino acid sequence of the proenzyme showed that the cleavage site of the proenzyme is the peptide linkage between Glu^{86} and Ser^{87}. The location and sequence (YVLSE↓SSMF) of the cleavage site is consistent with those described in other eukaryotic SAMDCs [30,32,48]. Molecular-mass determination of the active SAMDC using gel-filtration chromatography showed that it is as an 84 kDa protein, indicating that the active form of the enzyme is a heterotetramer.

An *O. volvulus* genomic DNA Southern blot, using *O. volvulus* SAMDC cDNA as the probe, resulted in a hybridization pattern consistent with that of the isolated *O. volvulus* SAMDC gene (Figure 5). Unlike in humans [49,50] and rat [51,52], which have more than one SAMDC gene (functional and pseudo genes) per haploid genome, our results suggest that *O. volvulus* SAMDC is encoded by a single-copy gene. Yeast SAMDC is encoded by a single uninterrupted exon, whereas the human and rat genes have nine and eight exons respectively. The *O. volvulus* SAMDC gene spans 5.3 kb, and contains nine exons and eight introns. The first intron is located in the 5′-UTR, 1 bp upstream of the ATG (Figure 6). The *O. volvulus* SAMDC gene possesses the consensus 5′-GT and 3′-AG intron/exon boundaries. Interestingly, the intron III–exon IV boundary in the *O. volvulus* SAMDC gene, as well as intron II–exon III in human and rat SAMDC genes, is located adjacent to the glutamic acid residue that flanks the cleavage site in the proenzyme (Figure 6).

The importance of the 5′-UTR in the modulation of translational efficiency of SAMDC and other mRNAs is well documented [24]. The *O. volvulus* SAMDC cDNA clone has a 282 bp 5′-UTR which undergoes both *cis*- and *trans*-splicing processes. The first 138 bp of the 5′-UTR sequence was not present in the genomic sequence of *O. volvulus* SAMDC, indicating that this is a *trans*-spliced sequence. The 5′-RACE experiment confirmed that there are at least two different *trans*-spliced mRNA products (Figure 7). The *trans*-splicing site in the precursor RNA, used to produce both mature mRNA products, possesses a consensus *trans*-splicing junction (TTTCAG) [53], which is located 377 bp before the ATG in the *O. volvulus* SAMDC gene. One RACE product confirmed the presence of the 138 bp *trans*-spliced sequence found in the cDNA, with a 22-nucleotide spliced-leader sequence at its 5′ end, indicating that this is not an artifact of cDNA library construction. Such a *trans*-spliced product has not been previously described. It may, however, be an inaccurately *trans*-spliced spliced-leader RNA, which, rather than being spliced after the 22-nucleotide leader sequence, was *trans*-spliced at a site further downstream. However, this sequence was not found to be homologous to any known spliced-leader gene sequence. The second RACE product contained a fragment that was *trans*-spliced at the same position in the *O. volvulus SAMDC* sequence, but with just the 22 bp spliced-leader sequence.

The 5′ end of the *O. volvulus* SAMDC primary mRNA transcript is interrupted by an intron located 1 bp upstream of the initiation codon (Figures 6 and 7). This intron is removed by normal *cis*-splicing processes from the mature mRNA. The processing of this intron has potential regulatory functions. This phenomenon has been reported for the plant *Catharanthus roseus* [48] and potato [54] SAMDC genes. In this region, *O. volvulus* SAMDC has a small upstream ORF which could encode a small peptide of 17 residues. The presence of this upstream ORF in the 5′-UTR is interesting, because it suggests the possibility of translational regulation of *O. volvulus* SAMDC, as described for mammalian SAMDC [24,55–57]. It has been reported that the peptide encoded by the small 5′-ORF has a negative effect on the production of SAMDC at the translational level [24]. Hill and Morris [55–57] have also demonstrated that the 5′-UTR exerts a cell-specific suppression of translation, which is due to a hexapeptide (MAGDIS) encoded by the small upstream ORF. *C. roseus* [48] SAMDC has an upstream ORF that could encode a polypeptide of 51 residues. However, there are no similarities between the peptide that is encoded by the upstream ORF of *O. volvulus* SAMDC and that of the mammalian and plant SAMDCs, and it remains to be determined whether the putative peptide is important in the regulation of *O. volvulus* SAMDC.

SAMDC is a ubiquitous essential enzyme that is very highly regulated and which responds to a wide variety of stimuli affecting cell growth and differentiation. Very little is currently known about the mechanisms that regulate transcription of the SAMDC gene. In order to begin to study some factors that might regulate the transcription of SAMDC in *O. volvulus*, we analysed the 5′-flanking region of the gene. It was not possible to determine the actual transcription start site because of the *trans*-splicing of the 5′ end of the SAMDC mRNA. Therefore we determined and analysed the sequence of 1476 bp of the gene before the *trans*-splicing site of the mRNA transcript (Figure 6). The putative promoter region of the *O. volvulus* SAMDC gene contains a TATA box and two CAAT boxes, but no GC box. In addition, potential binding sites for AP1, C/EBP, PEA3 and other transcription factors were identified. It has been suggested that PEA3 functions in the activation of cellular enhancers during development and differentiation [58]. It remains to be determined whether these sites are indeed recognized by transcription factors from *O. volvulus* and whether they function *in vivo* as *cis*-regulatory elements.

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