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Cloning and expression of the *S*-adenosylmethionine decarboxylase gene of *Neurospora crassa* and processing of its product

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Abstract *S*-adenosylmethionine decarboxylase (AdoMetDC) catalyzes the formation of decarboxylated AdoMetDC, a precursor of the polyamines spermidine and spermine. The enzyme is derived from a proenzyme by autocatalytic cleavage. We report the cloning and regulation of the gene for AdoMetDC in *Neurospora crassa*, *spe-2*, and the effect of putrescine on enzyme maturation and activity. The gene was cloned from a genomic library by complementation of a *spe-2* mutant. Like other AdoMetDCs, that of *Neurospora* is derived by cleavage of a proenzyme. The deduced sequence of the *Neurospora* proenzyme (503 codons) is over 100 codons longer than any other AdoMetDC sequence available in genomic databases. The additional amino acids are found only in the AdoMetDC of another fungus, *Aspergillus nidulans*, a cDNA for which we also sequenced. Despite the conserved processing site and four acidic residues required for putrescine stimulation of human proenzyme processing, putrescine has no effect on the rate ($t_{0.5}$ ~10 min) of processing of the *Neurospora* gene product. However, putrescine is absolutely required for activity of the *Neurospora* enzyme ($K_{0.5}$ ~100 μM). The abundance of *spe-2* mRNA and enzyme activity is regulated 2- to 4-fold by spermidine.

Key words *Neurospora* · Polyamines · S-adenosylmethionine decarboxylase · Proenzyme processing

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Introduction

The polyamines spermidine and spermine are tri- and tetravalent organic cations that are essential for the normal growth of eukaryotic organisms (Tabor and Tabor 1984; Pegg 1986). The divalent polyamine putrescine, a product of the ornithine decarboxylase (ODC) reaction, is converted to spermidine and then spermine by successive aminopropyl transfer reactions. The *S*-adenosylmethionine decarboxylase (AdoMetDC) reaction produces decarboxylated *S*-adenosylmethionine, which serves as the aminopropyl donor in putrescine and spermidine aminopropyltransferase reactions (catalyzed by spermidine synthase and spermine synthase, respectively). Studies with polyamine auxotrophs of the fungi *Saccharomyces cerevisiae* and *Neurospora crassa* have demonstrated that spermidine, the major polyamine in these organisms, serves functions for which putrescine cannot substitute (Cohn et al. 1978; Whitney and Morris 1978; Pitkin and Davis 1990; Balasundaram et al. 1991). In animal cells, both spermidine and spermine are predominant (Watanabe et al. 1991), and inhibitors that block the formation of these polyamines also restrict the growth of cultured cells (Pegg et al. 1988; Kramer et al. 1989).

In eukaryotic cells, AdoMetDC is a $(\alpha\beta)_2$ dimer, each half being composed of two non-identical subunits derived by cleavage of a proenzyme precursor. The cleavage generates a pyruvate prosthetic group at the N-terminus of the α subunit, the larger, C-terminal fragment. AdoMetDC belongs to a class of enzymes in which the presence of this pyruvoyl group is essential for activity (van Poelje and Snell 1990). Studies with the yeast and human proenzymes have shown that the cleavage reaction occurs at a glutamyl-serine bond in the sequence YVLSESS (Stanley et al. 1989; Kashiwagi et al. 1990), with the pyruvoyl moiety being derived from the serine.

The activity of AdoMetDC from many eukaryotes is stimulated by putrescine (Pegg et al. 1998). In addition,

the processing of the human AdoMetDC proenzyme is also stimulated by putrescine (Kameji and Pegg 1987). These regulatory mechanisms make the synthesis of one substrate of spermidine synthase, decarboxylated *S*-adenosylmethionine, dependent on the presence of the other, putrescine. Because *S*-adenosylmethionine serves as a methyl donor, and decarboxylated *S*-adenosylmethionine cannot fulfill this role, the regulation of AdoMetDC activity by putrescine levels prevents the inappropriate decarboxylation of *S*-adenosylmethionine. By contrast, the enzyme activity of the AdoMetDC of *Escherichia coli*, in which putrescine is the predominant polyamine, is stimulated by Mg^{2+} (Wickner et al. 1970).

In *N. crassa*, as in other eukaryotes, AdoMetDC activity is regulated by cellular polyamine levels (Pitkin and Davis 1990). In this paper we report the characterization of the *spe-2* gene of *N. crassa*, which encodes an unusually large AdoMetDC, so far peculiar to filamentous fungi. We show that changes in AdoMetDC activity in response to polyamines are correlated with similar changes in the abundance of *spe-2* mRNA. Our results demonstrate that putrescine is absolutely required for the catalytic activity of *N. crassa* AdoMetDC. However, putrescine does not stimulate proenzyme processing in *N. crassa,* even though the amino acid residues required for putrescine stimulation of processing in the human gene product are conserved in the fungal protein.

Materials and methods

N. crassa strains and growth conditions

The *N. crassa* strains used in this study were IC3 (*aga*), IC1894-53 (*spe-1*, *aga*), IC2568-15 (*spe-2*, *aga*), and IC2798-15 (*spe-1*, *spe-2*, *aga*). The specific alleles used were *aga*(UM906), *spe-1*(LV10), and *spe-2*(JP100). Strain IC2798-15 was isolated from a cross between strains IC1894-53 and IC2568-15. The *aga* mutation imposes a complete deficiency for arginase (Davis et al. 1970), the enzyme required for ornithine formation when arginine is present in excess. The addition of arginine to cultures of strains carrying the *aga* mutation results in feedback inhibition of endogenous arginine synthesis, and, owing to the lack of arginase, a consequent starvation for ornithine and polyamines.

For RFLP mapping, a set of standard strains (Metzenberg et al. 1984) was obtained from the Fungal Genetics Stock Center at the Department of Microbiology, University of Kansas Medical Center, Kansas City, Kan. (FGSC strains 4411–4430).

Growth and maintenance of *N. crassa* strains followed standard methods (Davis and de Serres 1970). Cultures were supplemented with 1 mM arginine hydrochloride, 1 mM putrescine dihydrochloride, and 1 mM spermidine trihydrochloride, where indicated.

Plasmids, cosmid DNA preparation, and *N. crassa* transformation

The Orbach/Sachs pMOcosX genomic library (Orbach and Sachs 1991) was obtained from the Fungal Genetics Stock Center as an ordered set of 50 96-well microtiter plates containing a total of 4800 individual clones. For screening by complementation, pools of 96 cosmid clones were replicated onto LB agar containing ampicillin (100 μg/ml) and grown overnight at 37 °C. Bacteria were harvested as a pool from the agar plates and cosmid DNA was isolated using a Qiagen plasmid midi kit. Cosmid DNA (5 μg) was used for transformation of *N. crassa* spheroplasts (Orbach et al. 1986). Cosmid pools that complemented strain IC2798-15 (*spe-1, spe-2, aga*) were selected by their ability to enable the recipient to use putrescine as a polyamine precursor. A single complementing plasmid was identified in an active pool, using a sib selection strategy (Vollmer and Yanofsky 1986).

Restriction fragments of *N. crassa* genomic DNA from the complementing cosmid were subcloned into pSP72 vectors (Promega): pWVC1 contains a 6-kb *Eco*RI fragment with the *spe-2* gene at the 5′ end; pWVC2 contains the same insert as pWVC1, in the opposite orientation; pSPE2 contains a *Eco*RI-*Sma*I fragment of pWVC2, in which the *spe-2* gene is located; and pSP6-SPE2 contains a *Hin*cII-*Sma*I fragment carrying the *spe-2* gene with the AdoMetDC start codon downstream of the SP6 promoter. The pSP6-SPE2 plasmid was constructed by digesting pWVC2 with *Hin*dIII at a unique restriction site, in the pSP72 multiple cloning site, between the SP6 promoter and the *Eco*RI insert. This site was filled in, to generate blunt ends, using Klenow DNA polymerase (Sambrook et al. 1989), and the linear plasmid was subsequently cut at a unique *Bam*HI site in the *spe-2* coding region. A *Hin*cII-*Bam*HI fragment containing the 5′ end of the *spe-2* coding region was directionally cloned into the *Hin*dIII + *Bam*HI-cleaved pWVC2 plasmid. Blunt-end ligation joined the filled *Hin*dIII site in the multiple cloning site to the *Hin*cII site found 26 bp upstream of the initiating ATG of the *spe-2* coding region.

Nucleic acid isolation and analysis

N. crassa genomic DNA was isolated from lyophilized mycelial powders (Bainbridge et al. 1990). Small-scale plasmid DNA preparations were obtained by the alkaline lysis method as previously described (Williams et al. 1992). Plasmid DNA for sequencing was prepared using a Qiagen plasmid midi kit, following the manufacturer's protocol. Plasmids for sequencing were generated using convenient restriction sites in pSPE2 to generate overlapping DNA fragments that were then subcloned into pSP72 vectors. DNA sequencing was carried out using SP6 and T7 primers flanking the pSP72 multiple cloning site. DNA sequencing was performed by the University of Chicago Cancer Research Center's DNA sequencing facility (http://cancer-seqbase.uchicago.edu). Chromatograms were manually inspected and edited using Chromas software (version 1.44; http://www.technelysium.com.au/chromas.html), and sequences were assembled using the SeqMan program in the DNASTAR program suite. Sequence information was analyzed using the DNASTAR program suite and the BLAST sequence similarity search tool at the National Center for Biotechnology Information, Bethesda, Md. (http://www.ncbi.nlm.nih.gov/BLAST/).

N. crassa RNA was prepared as previously described (Williams et al. 1992), with minor modifications. Wet mycelial pads were collected by filtration, frozen, lyophilized overnight, and powdered by vortexing in a tube with a spatula. Dissolution of RNA was routinely followed by a brief (2 min) centrifugation (10,000 × *g*) to remove insoluble material.

Total RNA was denatured in 10× 3-(N-morpholino) propanesulfonic acid (MOPS) buffer/37% (v/v) formaldehyde (2:15; 1 × MOPS buffer is 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) and separated by electrophoresis on denaturing (6% formaldehyde, 1 × MOPS buffer) 1% agarose gels. RNA was transferred to Nytran nylon membranes (Schleicher and Schuell) by capillary transfer using 10 × SSC (Sambrook et al. 1989). The RNA was crosslinked to the membranes by irradiation with UV light (following the manufacturer's protocol) and prehybridized in 50% deionized formamide, 5 × SSPE, 5× Denhardt's reagent, 0.1% SDS, and 0.1 mg/ml sheared salmon sperm DNA. Hybridization was carried out overnight at 42 °C using a (random primed) ^{32}P-labeled 1.8-kb *Eco*RI-*Bam*HI fragment from pSPE2 (to probe for *spe-2* mRNA), or a 1.2-kb *Sst*I fragment from p*β*T6 (for *β*-tubulin mRNA; Orbach et al. 1986). Hybridized membranes

were washed three times at room temperature for 5 min with 2 × SSPE, 0.1% SDS, and three times at 65 °C for 30 min with 0.1 × SSPE, 0.1% SDS. Signals from hybridized membranes were visualized by autoradiography, and autoradiographs were quantified by densitometry, using Photoshop software (Adobe Systems).

For reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis, 10 μg of total RNA isolated from an *aga* strain grown in the presence of arginine, and primer P2 (5′-TCCAGGCGCTTGGAACCACT-3′) were used for first-strand synthesis with Superscript RT RNase H-reverse transcriptase (Life Technologies) following the manufacturer's protocol. PCR was carried out using 10% (v/v) of the first-strand synthesis reaction or 5 ng of plasmid DNA, 1× PCR buffer (10 mM TRIS-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, Boehringer Mannheim), 0.2 mM of each dNTP, 1.0 μM of each primer, P1 (5′-TCAACAACCCCCTCAAACCC-3′) and P2, and 0.025 units/μl *Taq* DNA polymerase (Boehringer Mannheim). Reactions were carried out, in 100-μl volumes, in a MJ Research PTC-100 thermal controller for 25 cycles [2 min 95 °C (4 min for the first cycle), 2 min annealing at 55 °C, 2 min extension at 68 °C].

Restriction fragment length polymorphism mapping

In RFLP mapping of the *spe-2* cosmid in the *N. crassa* genome (Metzenberg et al. 1984), random-primer labeling (Sambrook et al. 1989) of a *Not*I insert from the X2:B09 cosmid was used to generate probes. This probe recognized an *Eco*RI polymorphism between the Oak Ridge and Mauriceville strains. Southern analysis (Sambrook et al. 1989) of *Eco*RI-digested genomic DNA from these strains and a standard set of 18 of their progeny was carried out using the X2:B09 insert as a probe.

Extraction and assay of AdoMetDC activity

Cell extracts were prepared as described previously (Pitkin and Davis 1990). Briefly, 250–1000 ml exponential cultures of strain IC3 were harvested by filtration, and extraction was carried out at 4 °C. Wet pads were ground with sand in extraction buffer (50 mM potassium phosphate pH 7.3, 2 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride). The crude extract was subjected to centrifugation (12,500 × *g*, 15 min) and the supernatant was desalted using Sephadex G-25, which removed endogenous polyamines. Crude extracts were immediately used for AdoMetDC assays (Pitkin and Davis 1990).

In vitro synthesis and processing of AdoMetDC proenzyme

The pSP6-SPE2 plasmid, in which the coding region for the *N. crassa* AdoMetDC proenzyme lies downstream from the SP6 promoter, was used in transcription and translation reactions with the TNT SP6 Coupled Reticulocyte Lysate system (Promega) to measure AdoMetDC processing by the method of Xiong et al. (1997), who established that this preparation contains less than 1 nM putrescine. Reactions were carried out following the manufacturer's protocol, with the modifications indicated below. A 50-μl reaction contained 50% TNT rabbit reticulocyte lysate, 0.5 μg of pSP6-SPE2 plasmid DNA, 1× TNT reaction buffer, 20 pmol of [^{35}S]methionine (1000 Ci/mmol), the other 19 amino acids at 20 μM each, 40 units of RNasin, and 1 μl of TNT SP6 RNA polymerase. In reactions containing putrescine, the polyamine was added to a final concentration of 1 mM. Reactions were incubated for the indicated periods at 30 °C, and further incorporation of [^{35}S]methionine was blocked by the addition of cycloheximide to a final concentration of 200 μM. Incubation was then continued at 30 °C, and 5-μl samples were removed to 20 μl of sample buffer (Garfin 1990) and heated to 100 °C for 2 min at various times after translation was stopped. Labeled products of the translation reactions were separated by SDS-PAGE on 12% gels, fixed, dried, and exposed to autoradiographic film.

Results

Identification and mapping of a cosmid containing the *spe-2* gene

To clone the *spe-2* gene, we screened the Orbach/Sachs pMOcosX genomic library by sib-selection (Staben et al. 1989) for complementation of a strain carrying the *spe-2* mutation. Strains containing the *spe-2* JP100 allele are unable to synthesize spermidine from putrescine; however, the spermidine pool present in *spe-2* mutant cells at the time of inoculation is sufficient to allow growth for several doubling times before this polyamine pool is exhausted and growth stops (Pitkin and Davis 1990). This "leaky" growth of the *spe-2* mutant was eliminated by using a *spe-1*, *spe-2* double mutant (strain IC2798-15) lacking both ODC and AdoMetDC activities. The double mutant failed to grow in putrescine-supplemented medium, confirming its specific and absolute requirement for spermidine. The strain was used as a transformation recipient to screen the *N. crassa* genomic library for a cosmid that would enable this strain to grow on putrescine. A single complementing cosmid, X2:B09, was identified.

An approximately 30-kb *Not*I fragment of the X2:B09 cosmid was used to generate a random-primer labeled probe for RFLP mapping. An *Eco*RI polymorphism between the Oak Ridge (O) and Mauriceville (M) parental strains was detected, and recombinants from a cross between these strains were scored for this difference (Fig. 1). Comparison of the mapping data with the available RFLP maps (Nelson et al. 1998) indicates that the genomic DNA in cosmid X2:B09 segregates with markers for the loci *spe-1*, *am* and *inl*. The *spe-2* locus is located near (~20 cM) the *inl* locus on the right arm of Linkage Group V, 6 and 7 cM to the left of the *am* and *spe-1* loci, respectively (Pitkin and Davis 1990) (Fig. 1). The ability of cosmid X2:B09 to complement the *spe-2* mutation and its map localization in the genome at the same position as the *spe-2* locus strongly suggests that the cosmid contains the *spe-2* gene.

The *spe-2* gene encodes the S-adenosylmethionine decarboxylase proenzyme

A mutation at the *spe-2* locus eliminates AdoMetDC activity, and AdoMetDC activity is kinetically altered in revertants of this mutant (Pitkin and Davis 1990). This suggests that the *spe-2* locus is the structural gene for AdoMetDC. To identify a DNA fragment from cosmid X2:B09 containing the *spe-2* gene, we first tested various restriction endonucleases for their ability to destroy the complementation activity of the cosmid. Among the enzymes tested, *Bam*HI, *Cla*I, *Kpn*I, and *Xho*I eliminated the ability of the cosmid DNA to complement the *spe-2* mutation, suggesting that sites for these enzymes lay within the *spe-2* gene. Subcloning was carried out

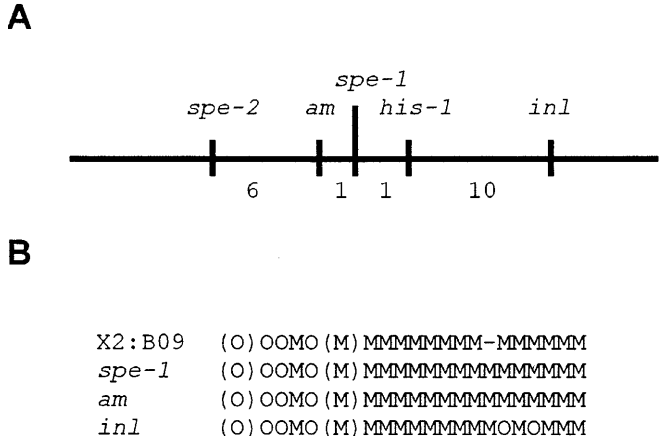

Fig. 1A, B Partial genetic map of linkage group V and RFLP mapping of cosmid X2: B09. **A** Map of the *spe-2–inl* segment of *N. crassa* linkage group V, showing the relative positions of the *spe-1* (ODC), *spe-2* (AdoMetDC), *am* (NADP-glutamate dehydrogenase), *his-1* (imidazole glycerolphosphate dehydratase) and *inl* (inositol synthase) genes. Map distances (in percent recombination) are shown *below* the line. **B** Distribution of an *Eco*RI polymorphism among progeny of a cross between Mauriceville (M) and Oak Ridge (O) parents (shown together with progeny, in *parentheses*), using probes specific for the genes involved. The distribution is shown for three of the markers shown in **A** and of the DNA insert of the X2: B09 cosmid, carrying the putative *spe-2* gene. One of the progeny (*dash*) was not probed successfully in the case of the cosmid insert

using DNA fragments generated from the X2:B09 cosmid by digestion with *Eco*RI, an enzyme that did not affect complementation activity. For sequence analysis, we subcloned a 3.6-kb *Eco*R I-*Sma* I fragment, which complemented the *spe-2* mutation, into plasmid pSPE2 (Fig. 2) and sequenced the insert.

The sequence of this 3663-bp DNA fragment (GenBank Accession No. AF151380) revealed a continuous ORF of 1512 nucleotides, encoding a 503 amino acid polypeptide with a predicted molecular weight of 54,721 Da. An examination of public databases using the BLAST search algorithm (Altschul et al. 1997) revealed that this sequence represented a reading frame with homology to the AdoMetDC proenzyme from numerous eukaryotic sources. In agreement with the results of the complementation studies, restriction sites for the endonucleases that eliminated complementing

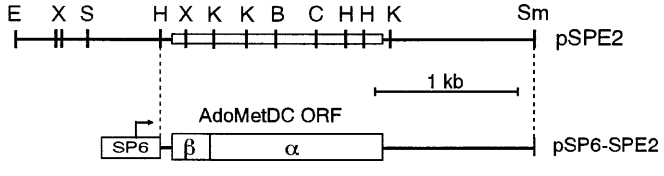

Fig. 2 Maps of inserts in plasmids pSPE2 and pSP6-SPE2. The restriction map of the 3.6-kb *Eco*R1-*Sma*I *N. crassa* genomic fragment containing the *spe-2* gene in plasmid pSPE2 is shown at the *top*, and *below* it is a diagram of the *spe-2* ORF subcloned into pSP6-SPE2. The relative positions of the AdoMetDC proenzyme ORF and the predicted α and β subunits are indicated in the *box* to the *right* of the SP6 promoter. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I

activity of the *spe-2* cosmid lay within the predicted AdoMetDC open reading frame (Fig. 2). These results, and those of the RFLP mapping studies, indicate that *spe-2* is the *N. crassa* structural gene for AdoMetDC.

The context of the initiator methionine codon in the putative *spe-2* ORF, GACTCAAAATGTCT, is similar to the consensus for sequences surrounding initiation codons of known coding regions in *N. crassa*, CNNNCA(A/C)(A/C)ATGGCT (Edelmann and Staben 1994). In addition, the presence of an in-frame stop codon, 66 nucleotides upstream of this methionine codon, and the relative position of the predicted N-terminus compared to other eukaryotic AdoMetDC sequences (see below) supports the assumption that this is the translational start codon. The coding region shows a bias for codons with C or G in the third position, typical of *N. crassa* coding regions, and this coding region is terminated by a TAA codon, the most frequently used stop codon in *N. crassa* (Edelmann and Staben 1994).

The deduced amino acid sequence of the *N. crassa* AdoMetDC proenzyme shows moderate homology to those of yeast (*S. cerevisiae*, 30% identity), and mammals (human, 28% identity), and less to that of plants (potato, 23% identity). Although overall identity is low when these AdoMetDC proenzymes are compared to that of *N. crassa*, sequence identity is highly conserved in several regions (Fig. 3). These regions include residues involved in proenzyme processing, enzyme activity, and putrescine stimulation of these processes in other eukaryotes. The *N. crassa* proenzyme contains the sequence YLLSESSMFV (aa 98–107), which is nearly identical to the sequence surrounding the site of proenzyme cleavage in AdoMetDC proenzymes from human (YVLSESSMFV; Stanley et al. 1989) and yeast (YVLSESSLFI; Kashiwagi et al. 1990). The amino acid residues corresponding to those of the human proenzyme previously shown to be required for catalytic activity (Glu8, Glu11, Ser68, Cys82, Ser229, and His243 in the human sequence), and putrescine stimulation of processing and activity (Glu11, Glu15, Arg76, Lys80, Asp174, Glu178, and Glu256) are identical or similar in the *N. crassa* AdoMetDC (Fig. 3) (Stanley and Pegg 1991; Stanley et al. 1994; Xiong et al. 1997, 1999) . The conservation of these residues suggests that proenzyme processing and the catalytic activity of the *N. crassa* enzyme might be stimulated by putrescine.

The deduced length of the *N. crassa* AdoMetDC proenzyme (503 amino acids) makes it the largest identified thus far – more than 100 residues longer than the *S. cerevisiae* proenzyme (396 amino acids). Two large segments in the *N. crassa* sequence (Ser216 to Pro244, and Ala281 to Cys306) account for most of the additional amino acids when compared to the *S. cerevisiae* proenzyme. In addition, the AdoMetDC proenzymes from *S. cerevisiae* and *N. crassa* contain N-terminal extensions when compared to those of mammalian or plant origin.

Examination of *N. crassa* expressed sequence tag (EST) databases at the University of New Mexico and

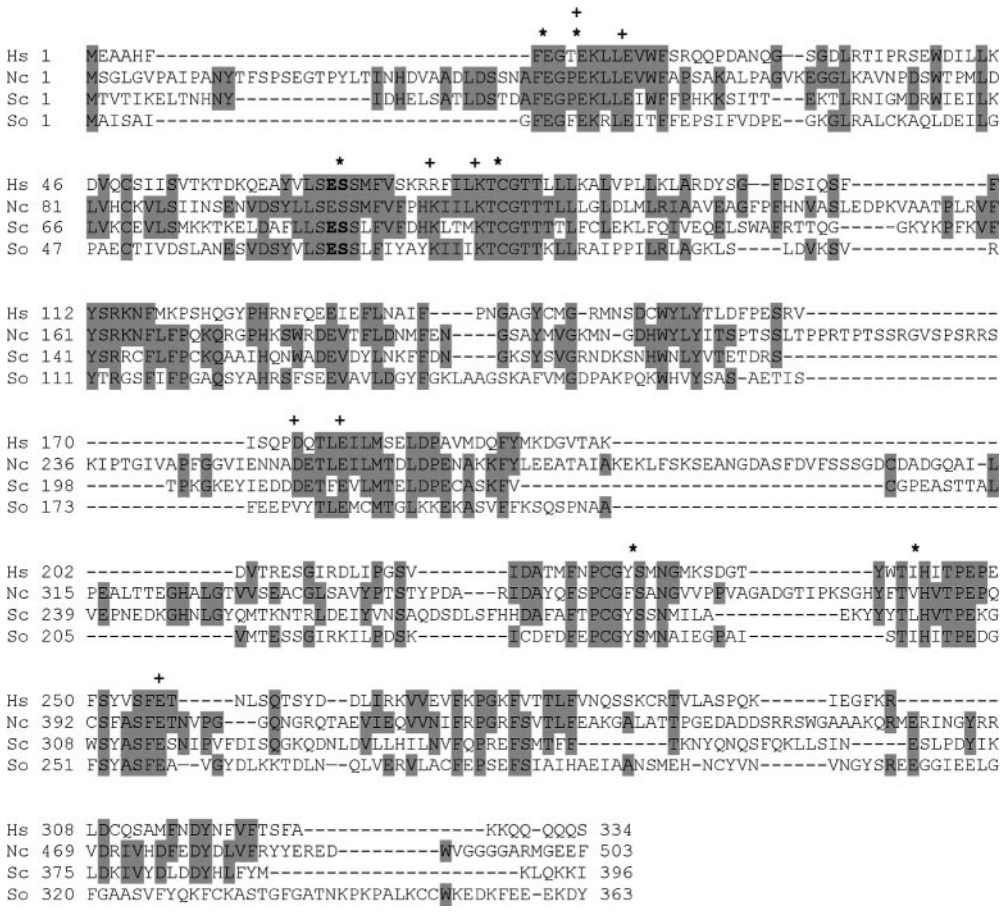

Fig. 3 Sequence alignment of AdoMetDC proenzyme sequences from human, yeast, potato and *N. crassa*. The amino acid sequences of AdoMetDC proenzymes from human (Hs, GenBank M21154), *N. crassa* (Nc), *Saccharomyces cerevisiae* (Sc, GenBank M38434), and potato (So, GenBank S74514) were aligned by the CLUSTAL method. Residues that are identical in *N. crassa* and at least one other proenzyme are *shaded*. The conserved proenzyme cleavage site in human, yeast, and potato is indicated in *bold letters*. Additional residues in the human sequence – those in the active-site pocket and those essential for catalytic activity (Glu8, Glu11, Ser68, Cys82, Ser229, and His243) are indicated by the *asterisks*. Together with Glu11, four additional residues required for putrescine stimulation of human proenzyme processing (Lys80, Asp174, Glu178, and Glu256) are indicated by *plus signs*. (see Ekstrom et al. 1999 for details)

the University of Oklahoma, using the BLAST search algorithm, failed to reveal cDNAs corresponding to the *spe-2* sequence. However, an EST database for the related fungus *Aspergillus nidulans* (Advanced Center for Genome Technology, University of Oklahoma) located a partial cDNA, m0f09a1 (GenBank Accession No. AF153765), encoding a protein sequence very similar to the C-terminal region of the *N. crassa* AdoMetDC coding region (Fig. 4). The most N-terminal conserved residue encoded by the *A. nidulans* cDNA corresponds to Gly199 of the *N. crassa* coding region. The *A. nidulans* AdoMetDC sequence shows 53% identity to the *N. crassa* sequence in the overlapping region. The insertions present in the *N. crassa* AdoMetDC coding region, relative to AdoMetDC from other eukaryotic sources, are also found in the putative AdoMetDC from *A. nidulans*. The similarity of the two enzymes with respect to the presence of additional amino acids suggests that the *N. crassa* sequence lacks introns in these positions, and that the unusually long proenzyme sequence may be characteristic of filamentous fungi.

Characterization of the *spe-2* transcript

Because the predicted coding region of the *spe-2* gene contains short amino acid sequences homologous to those of other eukaryotic AdoMetDC proenzymes throughout its entire length, and lacks signals normally associated with intron splicing in *N. crassa* genes, we assumed the coding region lacked intronic sequences. To confirm the absence of introns, primers flanking the *spe-2* coding region were used to amplify this region from the *spe-2* mRNA by RT-PCR, and from the cloned gene by conventional PCR. The amplified products were then subjected to restriction analysis with *Bam*HI and compared (Fig. 5). The results show that PCR amplification from *spe-2* cDNA and genomic DNA results in products of identical size. Digestion of the amplified genomic DNA with *Bam*HI generates fragments of 740 and 820 bp in length. Because the average *N. crassa* intron is approximately 70 bases in length (Edelmann and Staben 1994), differences in the size of the amplified cDNA due to the presence of introns would have been detectable. We conclude that the *spe-2* coding region lacks introns, and this is supported further by the similarity of the large carboxyl portion of the *N. crassa* coding region to the predicted sequence encoded by the *A. nidulans* cDNA.

Northern analysis (Fig. 6) indicates that *spe-2* mRNA is expressed as a single 2.0-kb transcript. Cells grown in minimal or spermidine-supplemented medium have little *spe-2* mRNA. Starvation for polyamines, brought about by growth of an *aga* (arginaseless)

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N. crassa   199   GKMNGDHWYLYITSPTSSLTPPRTPTSSRGVSPSRRSKIPTGIVAPFG-GVIE
A. nidulans 1     GKMNGEHWYLYLTEPYTSLTPPATPTADSDDEVTQTKFIQLPDRSDLGMGPQD

N. crassa   251   NNADETLEILMTDLDPENAKKFYLEEATAIAKEKLFSKSEANGDASFDVFSSS
A. nidulans 54    EASDETLEVLMTDLDEESAKQFYLDHATSVA-EKRYSNFEKDDHV--DVFSNG

N. crassa   304   GD------CDADGQAILPEALTTEGHALGTVVSEACGLSAVYPTSTYPDARID
A. nidulans 104   SDLEVDDVSSSQGSGILPAELTTEGHALGTVVSESCGLSDVYPKGKFPDSRID

N. crassa   351   AYQFSPCGFSANGVVPPVAGADGTIPKSGHYFTVHVTPEPQCSFASFETNVPG
A. nidulans 157   AYLFTPCGFSANGVIPSPDGGKGT-----HYFTVHVTPEPHCSYASFETNVPH

N. crassa   404   GQNGRQTAEVIEQVVNIFRPGRFSVTLFEAKGALATTPGEDADDSRRSWGAAA
A. nidulans 205   SRNGQTSAGIIQQVVNIFKPGRFTVTVFEAKPGVDGEWDADKEARYIERQAAR

N. crassa   457   K-QRMERINGYRRVDRIVHDFEDYDLVFRYYEREDWVGGGGARMGEEF
A. nidulans 258   RVSKGENVEGYKRVDRIVHDLHGYDLVFRYYERLDWKGGA-PRLGEEVIA
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Fig. 4 Comparison of the predicted amino acid sequences of AdoMetDC from *N. crassa* and an *A. nidulans* cDNA. The CLUSTAL method was used to align amino acids 199–503 of the predicted *N. crassa* AdoMetDC proenzyme and a conceptual translation of an ORF from the partial *A. nidulans* cDNA m0f09a1 (GenBank Accession No. AF153765). The predicted partial amino acid sequence of the *A. nidulans* proenzyme is numbered from the most N-terminal residue encoded by the cDNA. Amino acids which are identical in both predicted coding sequences are *highlighted*. The two sequence insertions found in *N. crassa* that are absent in most other eukaryotes are *overlined*

mutant on arginine, or growth of a *spe-1* mutant in the absence of polyamine supplementation, leads to a 2- to 4-fold increase in the abundance of *spe-2* mRNA, similar to the changes in *N. crassa* AdoMetDC activity detected under the same conditions (Pitkin and Davis 1990). Depletion of cellular polyamines also causes an increase in the abundance of ODC mRNA, encoded by the *spe-1* gene of *N. crassa* (Williams et al. 1992; Pitkin et al. 1994). Inspection of the regions of the *spe-2* and *spe-1* genes upstream of their coding regions, however, fails to reveal significant homology.

An ORF capable of encoding a 27-residue peptide lies 509 nucleotides upstream of the AdoMetDC coding region. Because upstream ORFs (uORFs) are regulatory features of mammalian AdoMetDC genes (Ruan et al. 1996), we wished to determine whether this potential uORF resided within the *spe-2* transcribed region. Northern analysis was carried out using a *Sac*I-*Eco*RV probe that overlaps the uORF. This probe failed to detect any transcript, indicating that this upstream region was not part of the *spe-2* mRNA.

Stimulation of *N. crassa* AdoMetDC activity by putrescine

The activity of AdoMetDC from the fungi *S. cerevisiae* and *A. nidulans* (Poso et al. 1975; Stevens et al. 1976), the trypanosomes *Trypanosoma brucei* and *T. cruzi* (Tekwani et al. 1992; Persson et al. 1998), the nema-

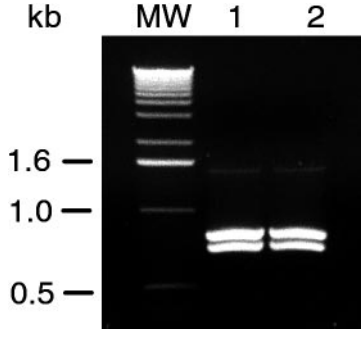

Fig. 5 Confirmation of the absence of introns from the coding region of the *spe-2* gene. Reverse transcription of total *N. crassa* DNA was carried out using primer P2 (see Materials and Methods), followed by PCR amplification of the *spe-2* coding region using primers P1 and P2. No product appeared if reverse transcriptase was omitted in the first step (not shown). As a control, the PCR product of the *spe-2* sequence in pSPE2 was also made. The products were digested with *Bam*H1 and compared after agarose gel electrophoresis. Lane 1, PCR product of total RNA after reverse transcription; lane 2, PCR product of the plasmid insert of pSPE-2, lane MW, ladder of molecular weight markers

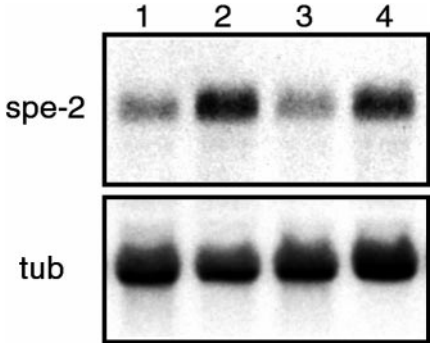

Fig. 6 Effects of cellular polyamine status on *spe-2* mRNA abundance. Total RNA was isolated from cultures of strain IC3 (*aga*) and the ODC-deficient strain IC1894-53 (*spe-1*, *aga*), and subjected to Northern analysis using a 0.7-kb *Xho*I-*Bam*HI fragment of pSP2 (*spe-2*, *above*), or a 1.2-kb *Sac*I fragment of the *N. crassa* β-tubulin gene of pβT6 (*tub*, *below*) as probes. Lanes: 1, *aga* strain grown with 1 mM spermidine; 2, *aga* strain grown with 1 mM arginine (polyamine depleted); 3, *spe-1*, *aga* strain grown with 1 mM spermidine; 4, *spe-1*, *aga* grown with 1 mM arginine (severe polyamine depeletion)

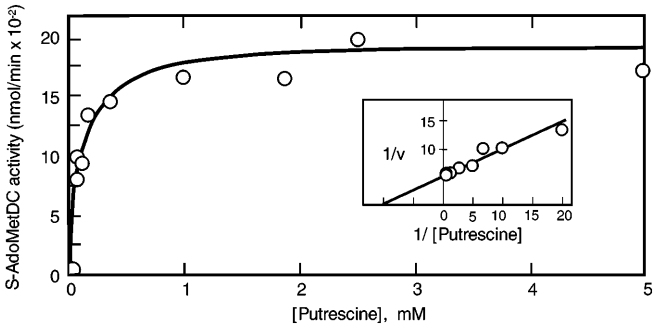

Fig. 7 Putrescine stimulation of the AdoMetDC reaction. The graph shows a plot of enzyme activity against putrescine concentration. *Inset*: Lineweaver-Burk plot of the same data. The $K_{0.5}$ for putrescine, derived from the X-intercept, is approximately 100 μM

todes *Ascaris suum* and *Onchocerca volvulus* (Rathaur et al. 1988), and mammalian cells (Pegg and Williams-Ashman 1969) is stimulated by putrescine. We measured the effects of putrescine (up to 5 mM) on *N. crassa* AdoMetDC activity in desalted crude extracts (Fig. 7). The results show that putrescine is absolutely required for *N. crassa* AdoMetDC activity in vitro, with the $K_{0.5}$ being approximately 0.1 mM.

Processing of the *N. crassa* AdoMetDC proenzyme

In the *N. crassa* AdoMetDC coding region, both the amino acid sequences surrounding the glutamyl-serine bond – the site of processing in the yeast and human AdoMetDC proenzymes – and the amino acid residues required for stimulation by putrescine of processing of the human proenzyme, which lie elsewhere in the polypeptide, are conserved. This suggests that the *N. crassa* proenzyme is processed and that putrescine might stimulate processing. In this study we used a coupled in vitro transcription/translation system to confirm that the *N. crassa* AdoMetDC enzyme is synthesized as a proenzyme and to determine the requirements, if any, for post-translational processing of the proenzyme. We placed the *spe-2* ORF downstream of the SP6 promoter in plasmid pSP6-SPE2 and synthesized the RNA and the encoded protein using the TNT SP6 assay system. We detected products corresponding to the proenzyme (predicted molecular weight 55 kDa) and the processed α subunit (44 kDa). We failed to detect the smaller (11 kDa) β subunit, perhaps due to its small size and the fact that it contains only two methionine residues.

Following a 30-min synthesis period, the majority of the *N. crassa* AdoMetDC appeared as the processed α subunit (Fig. 8A), and by 1 h after translation was inhibited with cycloheximide, almost all the proenzyme had disappeared. The *N. crassa* proenzyme, therefore, appears to be rapidly and autocatalytically processed to the mature form. The use of a shorter (10 min) synthesis period (Fig. 8B) revealed that approximately half the proenzyme was converted to the processed

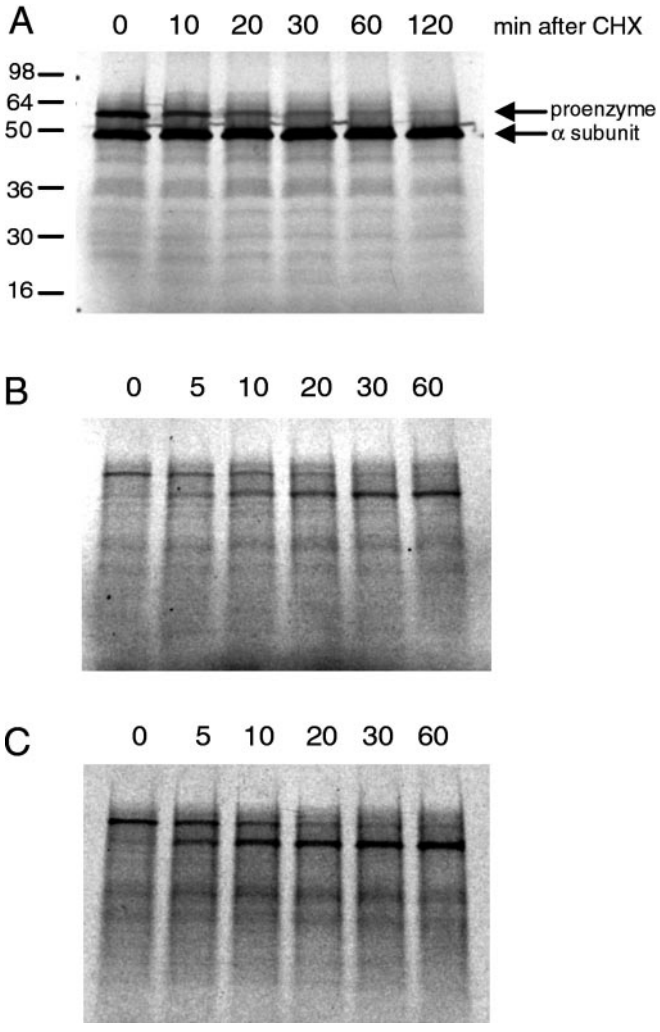

Fig. 8A–C In vitro processing of the *N. crassa* AdoMetDC proenzyme. Using pSP6-SPE2 as a template, a coupled transcription-translation reaction was allowed to proceed for 30 min (**A**) or 10 min (**B**, **C**) before terminating the reaction with 200 μg/ml cycloheximide. Samples were withdrawn periodically after termination for 60 or 120 min (*top scale*). The samples were subjected to SDS gel electrophoresis and visualized by autoradiography. Reactions were carried out in the absence (**A**, **B**) or presence (**C**) of 1 mM putrescine. The relative positions of molecular weight markers (in kDa) are indicated on the *left*. The small β subunit liberated in the processing reaction is not visible

form 10 min after its synthesis. By 30 min after inhibition of translation, virtually all the proenzyme was processed, with a corresponding increase in the intensity of the band representing the mature α subunit. This is rapid compared to the human AdoMetDC: only 15% of this precursor is converted to the mature enzyme after 30 min in the absence of putrescine (Xiong et al. 1997). A previous study (Xiong et al. 1997) found that the TNT reticulocyte lysate, used in these experiments, contained insufficient putrescine (< 1 nM) to stimulate the processing of human AdoMetDC. Therefore, the rapid processing of the *N. crassa* proenzyme is unlikely to be caused by putrescine in the

reaction mixture. Addition of 1 mM putrescine to the reaction failed to increase the rate of processing (Fig. 8C). The processing of the *N. crassa* proenzyme was also unaffected by the addition of 1 mM $MgCl_2$ to the reactions (result not shown). These results suggest that the conservation of acidic amino acids, required for putrescine stimulation of processing in the human enzyme, is insufficient to impart this requirement to the *N. crassa* proenzyme.

Discussion

Two noteworthy aspects of AdoMetDC of *N. crassa* have come to light in this work. First, a comparison of the structural gene sequence with other AdoMetDC genes and cDNAs reveals that it has a much longer amino acid sequence than any known to date. Initially, this suggested that introns might interrupt the *N. crassa* sequence. However, while a *N. crassa* cDNA was not retrieved and sequenced, the RT-PCR product and its restriction fragments did not differ in size from the corresponding genomic sequence. More interesting was the finding, occasioned by a successful search of the *A. nidulans* EST database, that the C-terminal region of AdoMetDC from this related filamentous fungus was quite similar in length to that of *N. crassa* AdoMetDC. The corresponding sequence of yeast (*S. cerevisiae*) differs from those of the two filamentous fungi. So far, the latter form a distinct and restricted class of AdoMetDCs.

Second, the processing of the *N. crassa* proenzyme appears to differ from that in mammals. Although cDNAs encoding the AdoMetDC proenzyme have been isolated from many organisms, the processing step and its stimulation by cellular cations has been characterized in only a few cases. The amino acid residues surrounding the site of processing are highly conserved in eukaryotes. The site of cleavage has been determined definitively for the proenzymes from human (Stanley et al. 1989), yeast (Kashiwagi et al. 1990), and potato (Xiong et al. 1997): processing occurs at a glutamyl-serine bond. It is therefore likely that processing of the *N. crassa* proenzyme occurs between the corresponding amino acid residues, Glu102 and Ser103. Although we have not demonstrated this directly, the size of the α subunit liberated in the in vitro processing reaction is consistent with this assumption.

Human AdoMetDC activity is activated by putrescine, but the activity of potato AdoMetDC, unlike that of *N. crassa*, is not (Xiong et al. 1997). Studies by Pegg and his collaborators have shown that putrescine both increases the rate of processing of the human AdoMetDC proenzyme, and stimulates the catalytic activity of the mature enzyme (Pegg et al. 1998). They also found that mutation of amino acid residues distal to the processing site of the human proenzyme could slow or prevent cleavage of the proenzyme (Stanley and Pegg 1991; Stanley et al. 1994; Xiong et al. 1997). These mutations occurred within highly conserved regions, and at least four acidic residues (Glu11, Asp174, Glu178, and Glu256) were essential for stimulation of processing by putrescine (Stanley et al. 1994; Xiong et al. 1997). These acidic residues are conserved in the *N. crassa* proenzyme, as Glu44, Asp254, Glu258, and Glu398. The potato AdoMetDC proenzyme lacks the aspartic acid corresponding to the conserved residues in human (Asp-174) and *N. crassa* (Asp-254), and maximal rates of processing of the plant proenzyme do not require putrescine (Xiong et al. 1997). Based on a model in which the divalent putrescine bridges acidic residues in the proenzyme to bring about or stabilize a conformational change, Xiong et al. (1999) speculated that the absence of this aspartic acid might explain the lack of putrescine-stimulated processing in the potato proenzyme, described in previous work (Xiong et al. 1997). Despite the conservation of all amino acid residues required for putrescine stimulation of processing in human AdoMetDC in the *N. crassa* gene product, putrescine has no effect on the processing of the latter. It is noteworthy that insertions occur in the *N. crassa* sequence between the acidic amino acid residues required for putrescine stimulation of processing relative to the human sequence. Glu44 of the *N. crassa* proenzyme is separated from Asp254 and Glu258 by approximately 45 more residues than are the corresponding residues of the human proenzyme. Similarly, Asp254 and Glu258 are about 60 residues further away from Glu398 in the primary sequence than the corresponding human residues. In addition, the *N. crassa* proenzyme (and that of *S. cerevisiae*) has an extended N-terminal region when compared to the human AdoMetDC. These differences may render ionic interactions between putrescine and the acidic residues unnecessary for optimal rates of processing.

The potato proenzyme and the *N. crassa* proenzyme are processed much more rapidly than the human proenzyme in vitro (Xiong et al. 1997). The mature α subunit appears almost immediately upon synthesis of the proenzyme in the case of potato, and the $t_{0.5}$ of the *N. crassa* proenzyme is about 10 min. Putrescine stimulation of these processing reactions would have little advantage. Possibly the processing of the human proenzyme and others like it may require a specially evolved stimulatory mechanism to compensate for their unusually slow rate of processing ($t_{0.5}$ ~80 min; Xiong et al. 1997). Only a systematic survey of putrescine stimulation of *S*-AdoMetDC proenzyme processing can answer this question.

The crystal structure of the mature human AdoMetDC enzyme has been reported recently (Ekstrom et al. 1999). The αβ monomer forms a novel α/β-sandwich fold comprising two eight-stranded antiparallel β sheets facing one another and flanked by α and 3_{10} helices on the exterior of the β sheets. Comparison of the sequences of the human and *N. crassa* enzymes suggests that the additional amino acids in the *N. crassa* AdoMetDC might lie on the periphery of the monomer.

The insertion of the sequence Pro219–Glu250 of the *N. crassa* enzyme would occur in a sequence between β strands 8 and 9 of the human enzyme that connects the two facing β sheets of the monomer. The Lys282–Thr327 segment of *N. crassa* AdoMetDC would lie within the peripheral α-helix 8 of the human AdoMetDC monomer. Neither insertion, nor the large N-terminal extension, would necessarily impair the architecture of the residues surrounding the active site.

The catalytic activity of AdoMetDC from many eukaryotes is activated by putrescine. Three of the four conserved acidic amino acids involved in the putrescine stimulation of AdoMetDC proenzyme processing are also required for putrescine activation of the catalytic activity of the mature human enzyme (Stanley et al. 1994; Xiong et al. 1997). [The effects of mutation of the fourth residue (human Glu11) on putrescine activation could not be tested because it is required for catalytic activity; Stanley and Pegg 1991.] In the fungi *S. cerevisiae* and *A. nidulans*, putrescine stimulates or is required for AdoMetDC enzyme activity (Poso et al. 1975; Stevens et al. 1976). In the present work, we have demonstrated that in *N. crassa,* putrescine is an obligatory cofactor in the AdoMetDC reaction, and, in previous work, that the K_m of the enzyme for *S*-adenosylmethionine decreases with increasing putrescine concentration (Pitkin and Davis 1990). Although putrescine appears not to be involved in processing of the proenzyme, the acidic residues conserved between the human and *N. crassa* proenzymes may nevertheless be required for the stimulation of catalytic activity.

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