eth-1, the *Neurospora crassa* Locus Encoding S-Adenosylmethionine Synthetase: Molecular Cloning, Sequence Analysis and *in Vivo* Overexpression

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Manuscript received November 7, 1995
Accepted for publication November 30, 1995

ABSTRACT

Intense biochemical and genetic research on the *eth-1*^{r} mutant of *Neurospora crassa* suggested that this locus might encode S-adenosylmethionine synthetase (S-Adomet synthetase). We have used protoplast transformation and phenotypic rescue of a thermosensitive phenotype associated with the *eth-1*^{r} mutation to clone the locus. Nucleotide sequence analysis demonstrated that it encodes S-Adomet synthetase. Homology analyses of prokaryotic, fungal and higher eukaryotic S-Adomet synthetase polypeptide sequences show a remarkable evolutionary conservation of the enzyme. *N. crassa* strains carrying S-Adomet synthetase coding sequences fused to a strong heterologous promoter were constructed to assess the phenotypic consequences of *in vivo* S-Adomet synthetase overexpression. Studies of growth rates and microscopic examination of vegetative development revealed that normal growth and morphogenesis take place in *N. crassa* even at abnormally high levels of cellular S-Adomet. The degree of cytosine methylation of a naturally methylated genomic region was dependent on the cellular levels of S-Adomet. We conclude that variation in S-Adomet levels in *N. crassa* cells, which in addition to the status of genomic DNA methylation could modify the flux of other S-Adomet-dependent metabolic pathways, does not affect growth rate or morphogenesis.

S-ADENOSYLMETHIONINE (S-Adomet) is the major cellular donor of methyl groups on a variety of biochemical pathways, being second to ATP as the most abundant cofactor in metabolic reactions (CANTONI 1977; TABOR and TABOR 1984). S-Adomet is produced from L-methionine and ATP by the enzyme S-adenosyl-L-methionine synthetase (S-Adomet synthetase; EC 2.5.1.6) (CANTONI 1953). The study of S-Adomet metabolism in eukaryotic cells has generated a growing interest in the recent years in the potential involvement of alternative cellular S-Adomet levels in normal and pathological processes associated with DNA methylation (JONES and BUCKLEY 1990; COUNTS and GOODMAN 1994; SIMILE *et al.* 1994; LAIRD *et al.* 1995). It has been proposed that abnormal cellular levels of S-Adomet and/or abnormal DNA methyltransferase activity could drive the occurrence of C → T transitions having dramatic consequences on rates of DNA mutation as well as on epigenetic phenomena (SELKER 1990; KRICKER *et al.* 1992; LAIRD and JAENISCH 1994; LAIRD *et al.* 1995). This hypothesis has been supported by studies performed on a prokaryotic DNA methyltransferase (SHEN *et al.* 1992) and on transgenic mice carrying mutations in a DNA methyltransferase gene (LI *et al.* 1993; LAIRD *et al.* 1995).

S-Adomet metabolism has been largely explored in simple microbial systems by using mutant organisms resistant to ethionine. This drug exerts cellular toxicity by misincorporation into a wide spectrum of normal metabolic pathways (ALIX 1982). Ethionine resistance has been attributed to a competitive effect of methionine overproduction caused by the additive effect of both a lower rate of conversion of methionine to S-Adomet and a derepression of some enzymes involved in the methionine biosynthetic pathways that are normally repressed by S-Adomet (KAPPY and METZENBERG 1965; KERR and FLAVIN 1970; JACOBSON *et al.* 1977; THOMAS and SURDIN-KERJAN 1991). Studies of S-Adomet synthetase gene expression and regulation of methionine and S-Adomet biosynthetic pathways in *eth*^{r} (ethionine resistant) mutants of the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (LAWRENCE *et al.* 1968; GREENE *et al.* 1970; HOLLOWAY *et al.* 1970; HOBSON and SMITH 1973; HAFNER *et al.* 1977; see SAINT-GIRONS *et al.* 1988), the yeast *Saccharomyces cerevisiae* (CHEREST *et al.* 1969, 1973; THOMAS and SURDIN-KERJAN 1991), the filamentous fungus *Neurospora crassa* (METZENBERG *et al.* 1964; KERR and FLAVIN 1970; BURTON and METZENBERG 1975), and the plant *Arabidopsis thaliana* (INABA *et al.* 1994), indicate that one of the key selected targets for mutations conferring resistance to ethionine is the S-Adomet synthetase locus. In particular, the *eth-1*^{r} mutant of *N. crassa* has been exhaustively analyzed at the genetic and biochemical levels (METZENBERG *et al.* 1964; KAPPY and METZENBERG 1965; METZENBERG and PARSON 1966; BURTON and METZENBERG 1975). It

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was suggested for the first time by KERR and FLAVIN (1970) that this locus may encode *N. crassa* S-Adomet synthetase. The presence of a thermosensitive S-Adomet synthetase activity in protein extracts from the mutant *eth-1*^{r} provided stronger evidence supporting this idea (JACOBSON *et al.* 1977).

N. crassa is an organism particularly suited to address some of the questions mentioned above related to S-Adomet metabolism, DNA methylation and mutation. This fungus has a low but measurable level of DNA cytosine methylation (RUSSELL *et al.* 1985; FOSS *et al.* 1993) and a strong ability to methylate cytosine on duplicated genomic sequences during vegetative or sexual growth. In particular, two phenomena of inactivation of duplicated DNA sequences, intimately related to DNA methylation, have been described. These are known as "quelling" (vegetative gene silencing of duplicated DNA sequences; PANDIT and RUSSO 1992; ROMANO and MACINO 1992) and repeat-induced point mutation (RIP) (taking place during the sexual cycle) (SELKER *et al.* 1987; SELKER 1990). In these studies, it has not been conclusively established whether DNA methylation is a cause or a consequence of gene inactivation. Although the isolation and preliminary characterization of putative *N. crassa* DNA methyltransferase mutants have recently been performed (FOSS *et al.* 1993), the relationship between DNA methylation and the phenomena of quelling and RIP, as well as their dependence on endogenous S-Adomet levels, remains to be analyzed. As an alternative approach to study the consequences of abnormal S-Adomet metabolism on phenomena associated to DNA methylation, we have first cloned the S-Adomet synthetase gene of the fungus *N. crassa* and initiated the analysis of the phenotypic consequences of *in vivo* S-Adomet overproduction.

MATERIALS AND METHODS

Strains and culture conditions: *N. crassa* strains *eth-1*^{r} (FGSC No. 1212/1220), *nic-1* (FGSC No. 763) and wild-type 74-OR23-1A (FGSC No. 987) were obtained from the Fungal Genetics Stock Center. Strain N276 (SELKER and GARRETT 1988) carries a duplication of the NADP-specific glutamate dehydrogenase (*am*) gene and was kindly provided by Dr. E. U. SELKER. Standard *N. crassa* methodologies were used according to DAVIS and DE SERRES (1970). L-methionine was added to solid and liquid media at 2.5 mM, when indicated.

DNA manipulations and protoplast transformation: Total *N. crassa* DNA was purified and subjected to Southern blot analysis as described (HAEDO *et al.* 1992). Probes were oligolabelled according to the method of FEINBERG and VOGELSTEIN (1983). Densitometric analyses of Southern blots were performed by scanning autoradiographic films, obtained at different exposure times, on a dual-wavelength Chromato scanner model CS-930 (Shimadzu). Cosmid and λZAP DNA was purified using standard protocols (SAMBROOK *et al.* 1989). Protoplasts were prepared and transformed following the protocol of VOLLMER and YANOFSKY (1986). Cotransformations of restriction enzyme digested cosmid DNA, or gel isolated restriction fragments, were carried out using 1 μg of pBT6 DNA (ORBACH *et al.* 1986; MAUTINO *et al.* 1993). Protoplasts

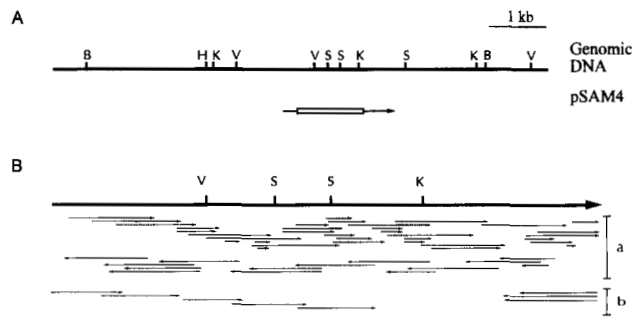

FIGURE 1.—Restriction map of the region around the *N. crassa eth-1* locus. (A) The position of the cDNA clone pSAM4 (arrow) is indicated relative to the genomic restriction map (top line). The coding region is indicated as an empty box. (B) Restriction map of the cDNA clone pSAM4 (top arrow), and sequencing strategy. Two groups of sequence stretches (thin arrows) were obtained. Sequences in group a were obtained from the pSAM4-Exonuclease III deletion clones, while sequences in group b were obtained from the cDNA clones pSAM1 to pSAM6. Relevant restriction sites shown are B, *Bam*HI; V, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; and S, *Sal*I.

transformation with restriction fragments isolated from low melting point agarose was done as previously described (LIU and DUNLAP 1988). Benomyl at 0.75 μg/ml was included in the bottom agar to select for transformant cells. Novozym 234 and Benomyl (95%) were gifts from Novo BioLabs (Denmark; via CRISPEX SRL, Argentina), and DuPont, respectively. α-^{32}PdATP (3000 Ci/mmol) was from NEN-DuPont.

cDNA cloning and DNA sequencing: A λZAP mycelial cDNA library (ORBACH *et al.* 1990), was plated on *E. coli* BB4 and ~50,000 plaques were screened with an 11-kb *Hin*dIII genomic fragment obtained from the cosmid clone X22H2 (*N. crassa* pMOcosX library) (ORBACH 1994). Eight lambda clones were obtained from the screening and their inserts were excised *in vivo* as described (SHORT *et al.* 1988). Clone pSAM4 was subcloned in both orientations in the *Eco*RI site of the vector pBKS(−). Unidirectional deletions of these clones were made with Exonuclease III (HENIKOFF 1987) after digestion with the enzymes *Hin*dIII and *Apa*I which do not cut into the insert fragment. Deletion clones were sequenced by the dideoxy chain termination method (SANGER *et al.* 1977) using the reverse sequencing primer and SEQUENASE (USB). Sequence was determined completely from both strands as indicated in Figure 1B. Genbank accession numbers are U21546, U21547.

Sequence comparisons: Database searchings and sequence comparisons were done using the CDGENE program (Hitachi, Japan). Multiple sequence alignments were performed with the program PILEUP and PRETTYBOX of the GCG sequence analysis package (DEVEREUX *et al.* 1984). Percentages of aminoacid sequence homology between different S-Adomet synthetases were calculated with the program DISTANCES. Parameters used for comparison were 0.6 for the threshold of comparison, and the "length of the shorter sequence without gaps" as the denominator.

Primers and PCR assays: To search for the presence of introns, oligonucleotides *d1* and *d3* were used to amplify by PCR a fragment from cDNA and from genomic DNA. Primers *d1* and *d3* are degenerate oligonucleotides that were used for cloning the *Ascobolus immersus* S-Adomet synthetase gene (M. R. MAUTINO, C. GOYON and A. L. ROSA, unpublished results), whose sequences are 5′ CGGGATCCGTIGGWGAIGGTCAYCCI 3′ and 5′ CCCTCGAGAACTTSAIA GYCTTIGGCTT 3′. Target sequences to which these primers hybridize are

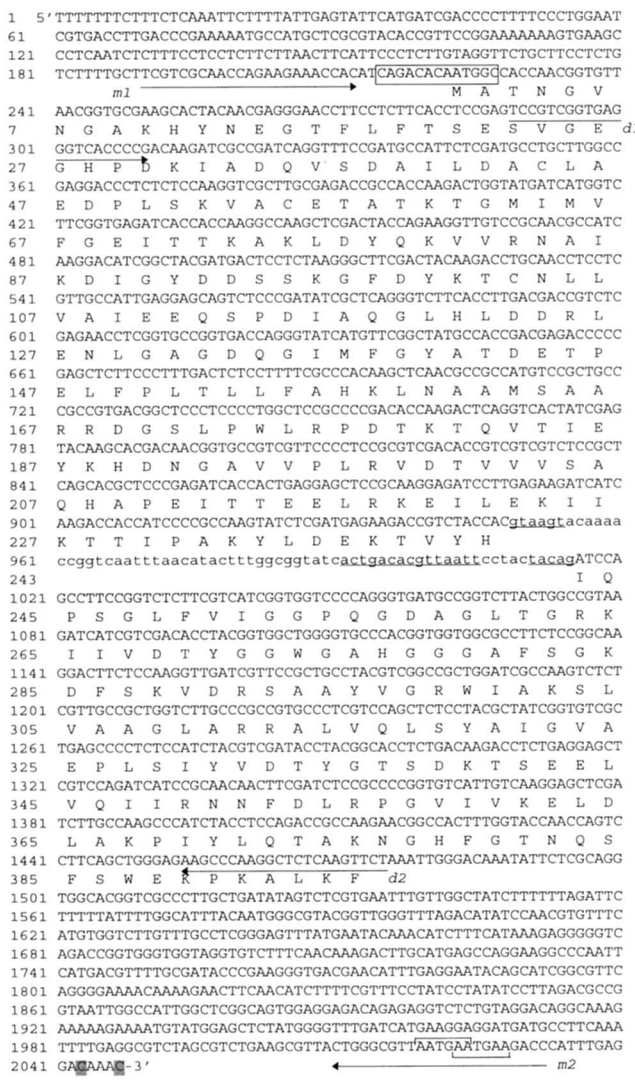

FIGURE 2.—Nucleotide and deduced amino acid sequences of the *N. crassa* S-Adomet synthetase gene. The nucleotide sequence is numbered from the first nt present in the pSAM4 cDNA. The consensus enviroment around the translation initiation codon is indicated by a box. The sequence of the intron is shown in lower case; sequences for the donor, branching and acceptor splicing sites are underlined. Two consensus polyadenylation signals are indicated with partially overlapped horizontal brackets; the two nucleotides found to precede the poly-(A) tail in different cDNA clones are shaded. Positions of primers *m1, m2, d1* and *d2* are indicated by arrows.

indicated in Figure 2. The PCR reaction was performed with *Taq* polymerase (Perkin Elmer Cetus Corp.) using 100 ng of genomic DNA or 3.5 pg of plasmid pSAM4 as templates. Reactions were carried out for 35 cycles at 94° for 45 sec, 60° for 30 sec, and 72° for 1 min 20 sec in a 50-μl reaction volume.

For the construction of the fusion plasmids pSGT, a DNA fragment from clone pSAM4 was amplified by PCR using primers *m1* and *m2* (Figure 2). The sequences of these primers are 5′ GGGAATTCGCAACCAGAAGAAACCAC 3′ and 5′ CGGAATTCATTCATTAACGCCC AG3′, respectively. PCR reaction was carried out for 33 cycles at 94° for 40 sec, 57° for 30 sec, and 71° for 2 min 30 sec, and a final extension step at 72° for 5 min, in a 50-μl reaction volume.

Construction of the expression plasmids pSGT42 and pSGT43: In a first step, the expression vector pPT2 was constructed from plasmid pCSN44 (STABEN *et al.* 1989) as follows: a 600-bp *Bam*HI fragment containing the transcription terminator sequences of the *Aspergillus nidulans trpC* gene was cloned in the *Bam*HI site of pBSK(+) to give plasmid pT1. A 230-bp *Sal*I-*Cla*I fragment from pCSN44, containing promoter sequences of *trpC*, was then ligated into *Sal*I-*Cla*I digested pT1 plasmid to give plasmid pPT2. To construct plasmid pSGT, a DNA fragment of the *N. crassa* S-Adomet synthetase gene was amplified from pSAM4 DNA by using primers *m1* and *m2*. This fragment was cloned in the *Eco*RV site of pBSK(+) to give a set of clones named pSAP. To avoid working with a single pSAP clone that could contain a potentially nonfunctional gene due to mutations introduced by the *Taq* polymerase during the amplification process, we continued working with a pool of pSAP clones. Inserts were removed from this pool by digestion with *Hin*dIII plus *Eco*RI, and ligated into pPT2 digested with the same restriction enzymes. This gave a set of independent recombinant clones named pSGT. Sixteen clones were chosen at random and were cotransformed independently with pBT6 into *eth-1*^{r} protoplasts. Clones pSGT42 and pSGT43 (Figure 4A) complemented *eth-1*^{r} thermosensitivity at the expected frequency and were chosen as functional expression plasmids.

S-Adomet synthetase and S-Adomet determinations: S-Adomet synthetase assays were done essentially as described (CHOU and LOMBARDINI 1972). Mycelium (1.5 g) was ground in liquid nitrogen, and extracted with 2 vol of buffer (10 mM KCl, 30 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol). After microcentrifugation at 13,000 *g* for 10 min at 4°, the protein concentration of the supernatant was determined by the method of BRADFORD (1976). Between 30 and 300 μg of total protein were incubated in 0.15 ml of a reaction mixture containing 100 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM KCl, 20 mM ATP, and 5 mM [^{35}S]-L-methionine (5 μCi). Control reactions contained all reagents except ATP. After incubation for 30 min at 30°, reactions were terminated by adding 3 ml of ice-cold water. Dowex 50W-X8 cation exchange resin (350 μl) (100–200 μm mesh; NH_4^+ form; Sigma) was added to the mixture. Unreacted methionine and ATP were washed out with water and adsorbed S-Adomet was eluted with 5 ml of 4 M NH_4OH. Scintillation liquid (Optiphase Hisafe 3) was added and the sample was counted by scintillation spectrometry. [^{35}S]-L-methionine (1000 Ci/mmol) was from NEN DuPont.

S-Adomet levels were determined essentially as described (SHAPIRO and EHNINGER 1966). Briefly, mycelia from 100 ml cultures grown in minimal medium, supplemented or not with 2.5 mM L-methionine, were extracted with 2 vol of 1.5 M $HClO_4$ for 1 hr with intermittent shaking. The extracts were neutralized with 3 M $KHCO_3$ and centrifuged at 10,000 rpm for 20 min. The supernatant was incubated with 300 μl of Dowex 50W-X8 (equilibrated in 0.1 M NaCl), for 30 min with agitation. The resin was washed with 0.1 M NaCl until the A_{260} was <0.05. S-Adomet was eluted from the resin by washing three times with 5 ml of 6 N H_2SO_4, and its concentration was determined measuring A_{256} (ϵ = 15,400).

RESULTS

Recessiveness of the thermosensitive phenotype associated to the *eth-1*^{r} allele: The main phenotypic features associated with the *eth-1*^{r} mutation are: resistance to ethionine and thermosensitivity (METZENBERG *et al.* 1964), overproduction of methionine (KAPPY and METZENBERG 1965), reversal of the mutant phenotypes in high osmolarity media (METZENBERG 1968), derepres-

sion of the enzymes aryl sulfatase (METZENBERG and PARSON 1966) and cystathionine γ-synthase (KERR and FLAVIN 1970), and low S-Adomet synthetase activity (KERR and FLAVIN 1970) and easy inactivation by heat or dialysis (JACOBSON *et al.* 1977). These phenotypic characteristics disappear at the same time in natural revertants of the mutant (METZENBERG 1968), indicating that *eth-1*^{r} involves a single mutation. Previous results obtained in partially duplicated strains (*eth-1*$^+$/*eth-1*^{r}) suggested that the thermosensitivity associated to *eth-1*^{r} was recessive (PERKINS 1972). To confirm this, we constructed primary forced heterokaryons between *nic-1 A* and *eth-1*^{r} *A* strains and cultured them at 39°. All heterokaryons grew identically to the wild-type strain at this restrictive temperature, indicating that the thermosensitive phenotype is recessive. Accordingly, we decided to clone the *eth-1* gene by protoplast transformation and rescue of the thermosensitivity associated to the *eth-1*^{r} mutation.

Isolation of a genomic fragment complementing *eth-1*^{r} thermosensitivity: In previous work (MAUTINO *et al.* 1993), we estimated that the locus *eth-1* was linked (30 ± 14 kb; mean ± SD) to an Oregon-Lindegren *Eco*RI RFLP by determining the frequency of meiotic crossover events in the genetic interval *eth-1–un-2*. Taking this into account, we transformed *eth-1*^{r} protoplasts with cosmid clones obtained by chromosome walking (MAUTINO *et al.* 1993), positioned at the expected distance from the *Eco*RI RFLP. A single cosmid clone, X22H2, complemented the thermosensitive phenotype associated with the *eth-1*^{r} mutation. To obtain a smaller complementing fragment, cotransformation experiments of *eth-1*^{r} protoplasts were made with plasmid pBT6 and cosmid X22H2, digested with *Bgl*II, *Eco*RI, *Eco*RV or *Hin*dIII. No thermoresistant transformants were obtained with X22H2 digested with *Eco*RV, suggesting that *eth-1*$^+$ has an internal *Eco*RV site. Restriction fragments obtained after *Hin*dIII digestion were isolated and separately used to cotransform *eth-1*^{r} protoplasts. An 11-kb *Hin*dIII restriction fragment was identified as the complementing one and was subsequently cloned and used as a probe to screen a mycelial cDNA library.

Isolation of cDNAs encoding S-Adomet synthetase: Eight independent (ZAP recombinant clones were isolated after three successive rounds of screening of a *N. crassa* mycelial cDNA library (ORBACH *et al.* 1990). Inserts were excised *in vivo* and the resulting plasmids were compared by restriction mapping, Southern blot and partial sequence analysis from their insert ends. Cross-hybridization experiments as well as partial sequence analyses allowed us to define two cDNA groups (pSAM1-6 and pUNK7-8), probably corresponding to two different genes located on the 11-kb *Hin*dIII fragment. Sequence homology analysis of the pSAM1-6 group showed that they were highly similar to S-Adomet synthetase genes from other species. Accordingly, this group was chosen for further analysis. pSAM1-6 clones have the same sequences at their 3' ends, differing in size at their 5' ends. A restriction map of the corresponding genomic region (Figure 1A) showed that these cDNAs were apparently colinear with genomic DNA, except pSAM5, which had a noncolinear fragment at its 5' end, perhaps as a result of a cloning artifact during library construction (data not shown). Therefore, the sequence of the clone with the largest insert, pSAM4, was determined on both strands (Figure 1B). The nucleotide sequence shows a 1188-bp open reading frame, encoding a polypeptide of 395 amino acids with a calculated M_r of 42,785 (Figure 2). The ATG translation initiation codon is in the context sequence CAGACACA**ATG**GC, which is very close to the $C_{57}NNNC_{77}A_{81}(A_{44}/C_{43})T_3A_{99}T_{100}G_{99}G_{51}C_{53}$ consensus defined for *N. crassa* (BRUCHEZ *et al.* 1993a; EDELMANN and STABEN 1994). Two partially overlapping putative polyadenylation signals (5' AATGAA 3') were found in the 3' portion of the S-Adomet synthetase gene (Figure 2). This sequence is also present in the 3' ends of the genes *atp-2, ilv-2* and *nit-3* of *N. crassa* at 17–21 nt from the poly-(A) tail addition site (BRUCHEZ *et al.* 1993b). The finding of two alternative positions of the poly-(A) tail in our S-Adomet synthetase cDNAs, differing in 4 nt, in agreement with the positions of the two polyadenylation signal sequences (see Figure 2), strongly supports the physiological relevance and functionality of this signal in *N. crassa*. The G+C content in the S-Adomet synthetase ORF (59.5%) is significantly elevated when compared with the 5'UTR (43.2%) and 3'UTR (44.9%), in agreement with *N. crassa* preference for codons ending in C or G. Codon usage is strongly biased towards codons ending in pyrimidines or guanine. Indeed only 36 out of 61 possible sense codons are used by the S-Adomet synthetase gene. This kind of bias is typical of constitutively expressed genes of *N. crassa* (GERMANN *et al.* 1988; EDELMANN and STABEN 1994).

Molecular characterization of the S-Adomet synthetase structural gene: To detect the possible presence of introns in the *N. crassa* S-Adomet synthetase gene, the entire ORF region was amplified by PCR with the set of primers *d1* and *d2*, from genomic DNA and from pSAM4, and compared by detailed restriction mapping. A *Sal*I restriction fragment having different sizes when amplified from genomic DNA (280 nt) or from pSAM4 (210 nt) was cloned and sequenced, showing the presence of an intron of 67 bp (Figure 2). The sequences of the donor, branching and acceptor sites (Figure 2) match perfectly the consensus derived for Neurospora (GTAAGT, RCTRACMnnnnnnYY and WACAG, respectively) (EDELMANN and STABEN 1994).

Amino acid sequence analysis of S-Adomet synthetase polypeptides: The availability of S-Adomet synthetase gene sequences of two filamentous fungi, as well as of yeast, plants, insect, mammals and bacteria allowed us to examine the overall sequence homologies among S-

TABLE 1

Percentage of amino acid sequence homology between different S-Adomet synthetase polypeptides

		1	2	3	4	5	6	7	8	9	10	11	12	13
E. coli metK	1	100	94	63	64	64	64	64	64	64	64	65	64	62
E. coli metX	2		100	67	67	67	67	68	68	68	68	69	68	66
A thaliana sam-1	3			100	98	93	73	72	73	75	75	74	75	68
A. thaliana sam-2	4				100	93	73	72	73	75	76	75	76	69
D. caryophyllus	5					100	74	72	73	74	74	73	74	68
S. cerevisiae sam-1	6						100	94	84	84	81	81	80	74
S. cerevisiae sam-2	7							100	84	84	81	81	79	74
A. immersus	8								100	90	80	80	79	75
N. crassa	9									100	79	78	80	74
Human liver	10										100	98	92	84
Rat liver	11											100	92	84
Rat kidney	12												100	83
D. melanogaster	13													100

Adomet synthetases from evolutionarily distant organisms. A comprehensive representation of the overall sequence homology found between all pairs of S-Adomet synthetases is presented in Table 1. The minimun degree of homology between the least related pair is 62%, indicating an exceptionally high degree of conservation throughout evolution. *N. crassa* S-Adomet synthetase is more similar to the corresponding enzyme of the filamentous fungus *A. immersus,* having 94% of homology, and has the second highest homology to both S-Adomet synthetases of the budding yeast *S. cerevisiae,* having 84% of amino acid sequence homology. S-Adomet synthetases from fungi have more homology with the corresponding enzymes of mammals (80% on average) and insects (74%) than with plants (73%) or bacteria (66%). Figure 3 shows that there are remarkable amino acid sequence similarities over the whole length of the S-Adomet synthetase molecule. In particular, very well conserved domains are located in the amino-terminal portion (position 30–56 in the figure), and in the region between positions 262–320. The domain that has been proposed to be involved in ATP binding, Gly^{145}-Ala-Gly-Asp-Gln-Gly^{150} and Lys^{174} (HORIKAWA *et al.* 1989; KAMPS *et al.* 1984), is perfectly conserved through all the species except in *E. coli* and *A. immersus,* where Lys^{174} is replaced by Arg and Gln, respectively. The cysteine residues Cys^{90} of *E. coli* (MARKHAM and SATISHCHANDRAN 1988) and Cys^{150} (PAJARES *et al.* 1991) of rat liver have been proposed to be relevant for S-Adomet synthetase function. It can be seen that Cys^{90} (here in position 116) is perfectly conserved, whereas Cys^{150} (here in position 165) is not. A typical signature sequence for S-Adomet synthetases, without a known function, is an almost perfectly conserved glycine-rich nonapeptide Gly^{294}-Gly-Gly-Ala-Phe-Ser-Gly-Lys-Asp^{302}, which is not detected in any other kind of protein sequences. A protein kinase C (PKC) phosphorylation consensus sequence [Ser-(Lys/Gln)-Lys-(Ser/Thr358)-Glu-Arg], present on the most hydrophilic region of the rat liver and human S-Adomet synthetases, has been demonstrated to be physiologically relevant for regulation of the dimer-monomer equilibrium of the enzyme (PAJARES *et al.* 1994). This sequence is partially conserved in the S-Adomet synthetase sequence of *N. crassa* (Ser-Asp-Lys-Thr-Ser), but is absent in the S-Adomet synthetase genes of a higher plant, in *A. immersus* and in *S. cerevisiae.*

***In vivo* overexpression of the S-Adomet synthetase gene:** To investigate the phenotypic consequences of overexpression of the S-Adomet synthetase gene in *N. crassa,* we constructed the plasmids pSGT, which carry S-Adomet synthetase-cDNA sequences under the control of the *A. nidulans trpC* gene promoter (Figure 4A; see MATERIALS AND METHODS). Functionality of the *A. nidulans trpC* promoter in *N. crassa* has been well documented (STABEN *et al.* 1989). To construct the pSGT plasmids, we took care to preserve both the native environment of the translation initiation codon and the 3′ untranslated region of the S-Adomet synthetase gene, including its polyadenylation signal. The fusion does not include the long 5′UTR of the gene, thus avoiding possible effects of posttranscriptional regulation. The functionality of the fusion construct was confirmed by transformation of *eth-1*^{r} protoplasts and phenotypic complementation of the thermosensitive phenotype associated to this mutation (see MATERIALS AND METHODS).

Two identical constructs (pSGT42 and pSGT43) were cotransformed, in independent experiments, with plasmid pBT6 into protoplasts of the N276 strain. A total of 24 Bml^r transformants were chosen and their S-Adomet content was determined (Figure 4B). Two selected transformant strains, N4216 (pSGT42) and N4316 (pSGT43), show 20- to 33-fold increased S-Adomet levels in comparison with the untransformed N276 strain growing in a medium supplemented with 2.5 mM L-methionine (Figure 4C). S-Adomet levels in the wild-type strain were eightfold higher in this medium in comparison with minimal medium, suggesting that pro-

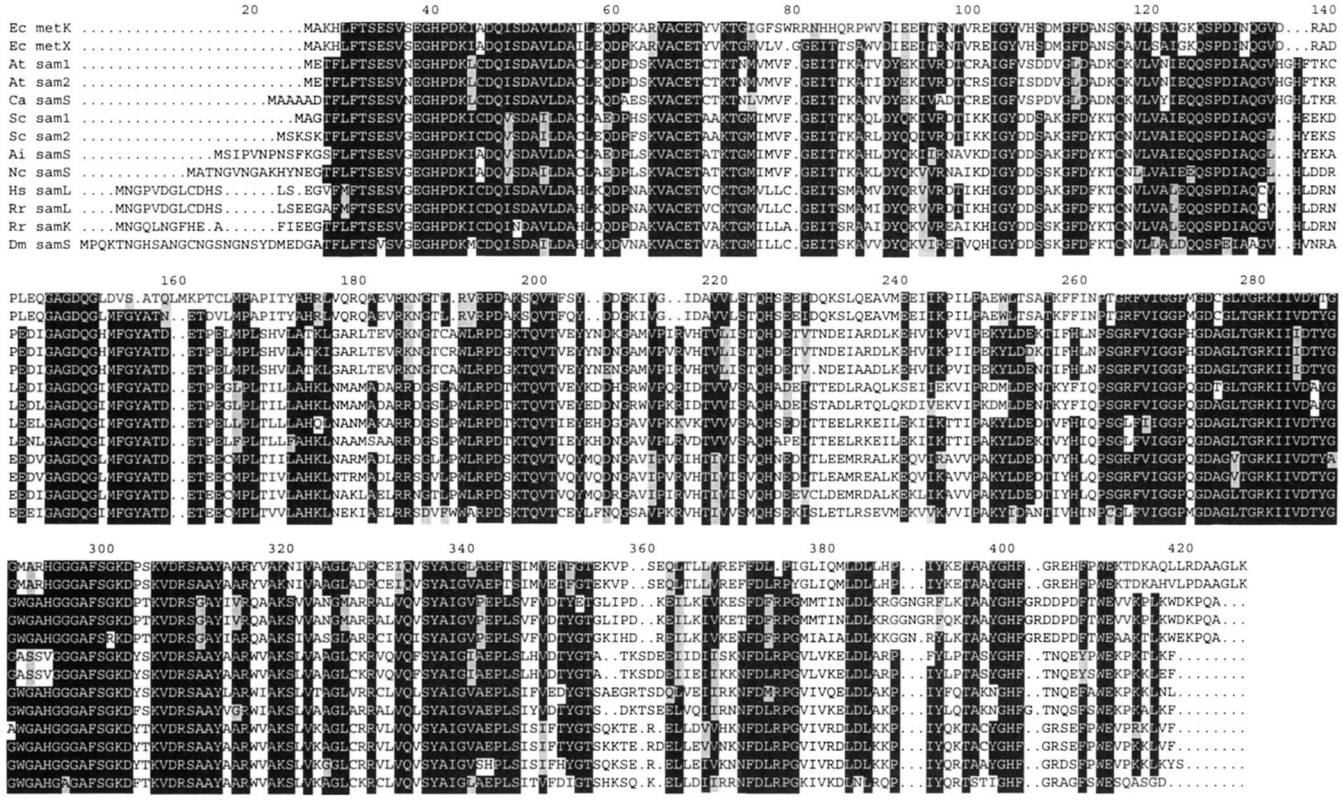

FIGURE 3.—Multiple amino acid comparison of S-Adomet synthetase sequences from bacteria, fungi and higher eukaryotic organisms. The S-Adomet synthetase amino acid sequences of *E. coli* (Ec) *metK* (MARKHAM *et al.* 1984) and *metX* (Genbank No. M98266); *A. thaliana* (At) *sam1* (PELEMAN *et al.* 1989a) and *sam2* (PELEMAN *et al.* 1989b); *D. caryophyllus* (Ca) (LARSEN and WOODSON 1991); *S. cerevisiae* (Sc) *sam1* (THOMAS and SURDIN-KERJAN 1987) and *sam2* (THOMAS *et al.* 1988); *A. immersus* (Ai) (Genbank No. U21548); *N. crassa* (Nc) (this work); human liver (Hs) (ALVAREZ *et al.* 1993); rat liver (Rr) *samL* (HORIKAWA *et al.* 1989); rat kidney (Rr) *samK* (HORIKAWA *et al.* 1990) and *D. melanogaster* (Dm) (LARSSON and RASMUSON-LESTANDER 1994) are compared. Amino acid residues that are identical in at least eight of the 13 analysed sequences are indicated with black boxes. Conservative amino acid changes are shown in shaded boxes. The alignment was performed with the PILEUP program from the GCG package (DEVEREUX *et al.* 1984).

duction of S-Adomet is limited by the endogenous availability of L-methionine. Considering these results, we have achieved a 260-fold increase of the S-Adomet level in the N4216 strain in comparison to that of N276 growing under standard conditions. Confirming previous results (BURTON and METZENBERG 1975), no significant increase in S-Adomet level was measured in the *eth-1*^{r} when growing in medium supplemented with L-methionine (Figure 4C). S-Adomet levels in the transformant strains, growing in media without externally added L-methionine, were approximately twofold higher than that of N276 in the same medium (Figure 4C) supporting the contention that production of S-Adomet is limited by the endogenous availability of L-methionine. Determination of S-Adomet synthetase activity present in total protein extracts of these strains is shown in Figure 4D. A threefold increase in the enzyme activity in N4216 accounts for the 260-fold increase in its S-Adomet level in media containing L-methionine. Southern blot analyses show that N4216 has a single ectopic copy, while N4316 has at least eight copies of pSGT integrated in the genome (not shown). Thus, no correlation was found between the number of transgene copies integrated in the genome and the S-Adomet synthetase activity or S-Adomet levels.

To analyze the influence of varying levels of cellular S-Adomet on the status of genomic DNA methylation, we studied diagnostic *Bam*HI (Figure 5A), and *Sau*3AI (not shown), restriction sites in the tandem duplication ζ-η (zeta-eta) (SELKER and STEVENS 1985; SELKER *et al.* 1987). *Bam*HI sites in this region are ~90% blocked by methylation under standard growth conditions (SELKER *et al.* 1987). Strains N276, *eth-1*^{r} and transformants N4216 and N4316, were grown in media supplemented or not with 2.5 mM L-methionine. Strains were grown at 34°, a condition which has been shown to diminish cytosine methylation of the ψ_{63} region in the mutant *eth-1*^{r} (FOSS *et al.* 1993). Figure 5, B and C, shows that strain N276, and strains overexpressing S-Adomet synthetase have a similar degree of methylation in the ζ-η region when growing in minimal medium, which correlates with their similar endogenous levels of S-Adomet in this medium. However, with the exception of the *eth-1*^{r} mutant, which is unable to increase its endogenous levels of S-Adomet in response to added L-methionine (Figure 4C), the degree of methylation of the ζ-η region

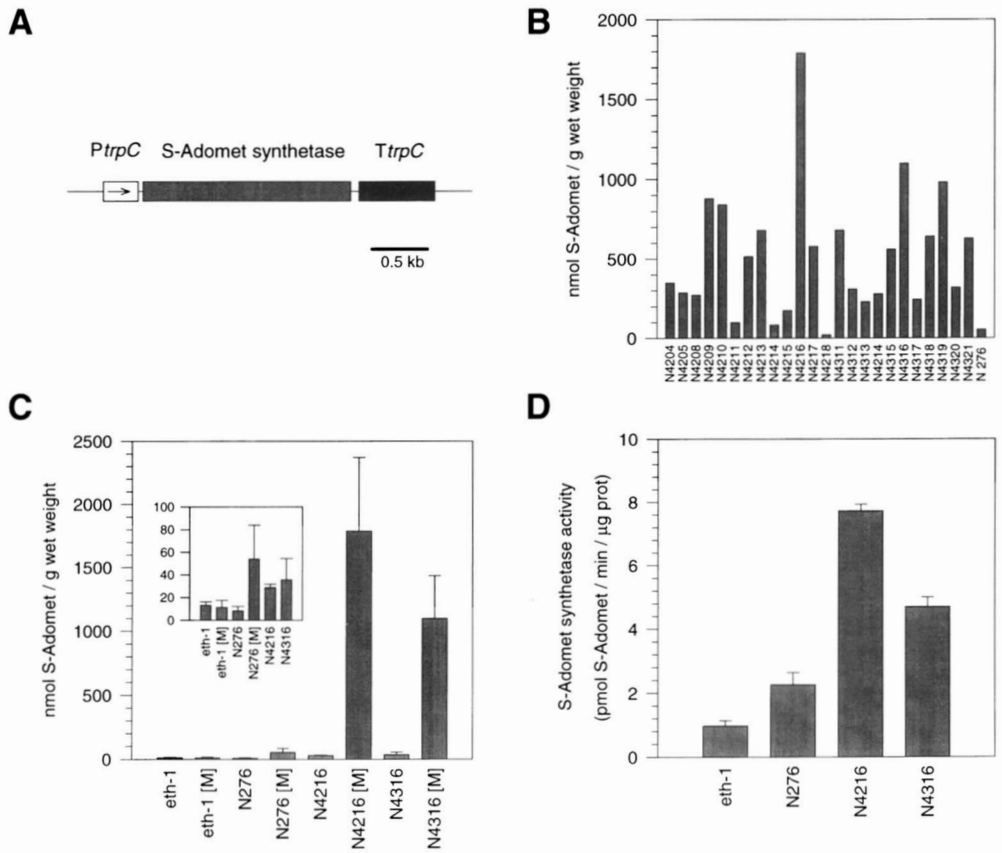

FIGURE 4.—*In vivo* overexpression of the S-Adomet synthetase gene in *N. crassa*. (A) Structure of the fusion plasmids pSGT. Promoter (P*trpC*) and terminator (T*trpC*) sequences of the *A. nidulans trpC* gene were used to drive the expression of a *N. crassa* S-Adomet synthetase cDNA, deleted for 5′UTR sequences. (B) Cellular S-Adomet levels in the wild-type strain (N276), and 24 transformant strains obtained with plasmids pSGT42 (N4204 to N4218) and pSGT43 (N4311 to N4321), grown in medium supplemented with 2.5 mM L-methionine. (C) Comparison of cellular S-Adomet levels in *eth-1*^{r}, N276, N4216 and N4316 strains growing at 25° in media suplemented (indicated as [M]) or not with 2.5 mM L-methionine. Values for those strains having low levels of S-Adomet are shown, in an enlarged scale, in the inset. (D) Determination of S-Adomet synthetase activity in *eth-1*^{r}, N276, N4216 and N4316 strains. At least three independent determinations were performed, in C and D, for each strain. Error bars represent ±SD.

was higher when strains were grown in media supplemented with L-methionine (Figures 5B and 5C). Thus, although only a low fraction of unmethylated sites in the ζ-η region ($\leq$10%) is available to test increases in DNA methylation, the sensitivity of the test was high enough to observe a dependence of DNA methylation on cellular S-Adomet levels. Full DNA methylation was not obtained even at the abnormally high levels of S-Adomet present in N4216 and N4316. Similar results were obtained when DNAs digested with *Sau*3AI were analysed (not shown). Complete digestion of the DNAs in these blots was confirmed by stripping and reprobing the blots with DNA sequences of the *am* gene (not shown). The analysis of genomic DNA methylation was extended further to two kinds of duplicated sequences: an *am* duplication, carried by the strain N276, which was not obtained directly by transformation but segregated from a cross (SELKER and GARRETT 1988), and the endogenous and ectopic copies of the S-Adomet synthetase gene present in the transformants N4216 and N4316. No methylation was associated with either the duplicated *am* sequences or the S-Adomet synthetase DNA sequences present in N4216 and N4316 (not shown). Thus, highly abnormal levels of cellular S-Adomet do not modify the unmethylated status of either the *am* duplication or the duplicated S-Adomet synthetase sequences in the transformed strains.

N4216 and N4316 strains were chosen for further phenotypic analysis. Growth rates of these strains were assessed in race tubes (RYAN *et al.* 1943) on minimal media supplemented or not with L-methionine, and compared with those of *eth-1*^{r} and N276. In these experiments, strains were grown in media containing different concentrations of glucose (0.1 and 1.5% w/v) or glucose plus ethanol (0.1 and 1.0% w/v, respectively). Although different growth rates were observed for each strain in these various media, there were no significant differences between wild type and strains overexpressing S-Adomet synthetase (not shown). To see if the altered S-Adomet levels present in the strains N4216 and N4316 lead to some abnormal vegetative phenotype, we studied several morphological characteristics

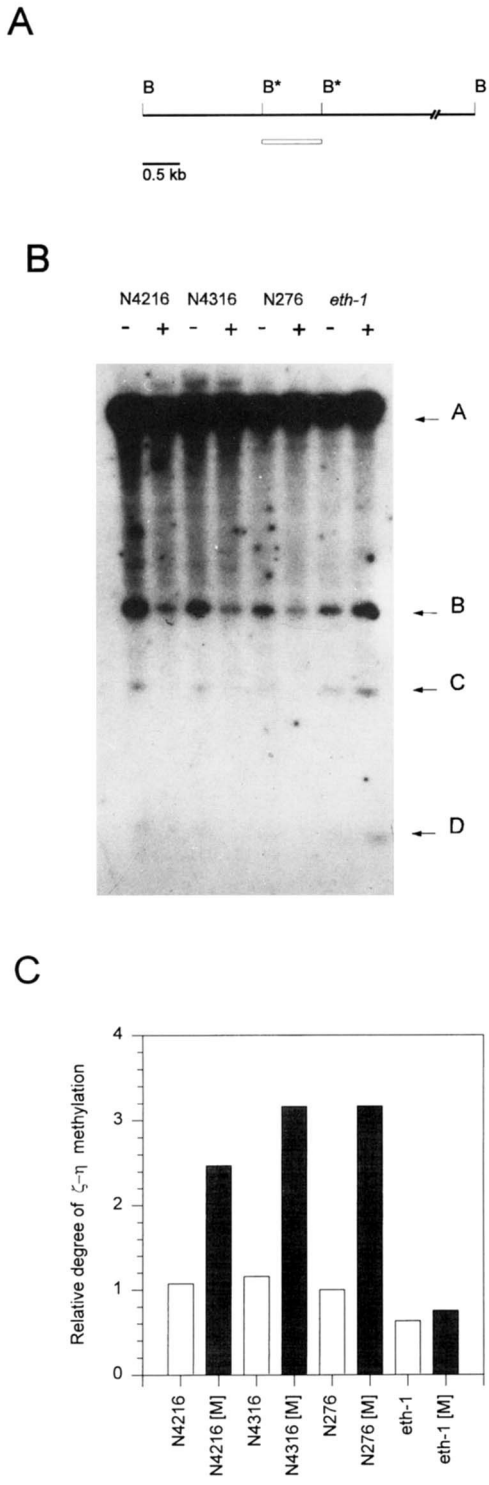

FIGURE 5.—DNA methylation at the ζ-η region. (A) Map of the ζ-η region (SELKER and STEVENS 1985) showing *Bam*HI restriction sites. Asterisks indicate restriction sites that display DNA methylation in B. The 0.8-kb fragment used as a probe is indicated by a box. (B) Total genomic DNA of the strains *eth-1*^{r}, N276, N4216 and N4316, purified from mycelia growing at 34° in minimal medium supplemented (+) or not (−) with 2.5 mM L-methionine, was digested with *Bam*HI and subjected to Southern blot analysis. A 0.8-kb *Bam*HI restriction fragment containing part of the ζ-η region (SELKER and STEVENS 1985) was gel purified, oligolabeled and used as a probe. Bands of 0.8 kb (D), 1.6 kb (C), 2.4 kb (B) and >10.0 kb (A) were considered (see SELKER and STEVENS 1985). It is assumed that bands A and B correspond to DNA molecules having the sequence GGATmCC methylated, thus rendering a *Bam*HI restriction site resistant to cleavage by this enzyme. (C) Densitometric analysis of the autoradiographic film shown in B. Given the intensity of the signal of band A, films with different exposure times were scanned to obtain the values corresponding to the signal ratio B/A for each condition The B/A ratios obtained were divided by that of N276 DNA, isolated from mycelia grown in minimal medium, to obtain values that reflect the relative degree of methylation of the different DNAs in comparison with the standard growth condition. Bars represent the relative degree of DNA methylation for the indicated strains grown in medium supplemented (grey) or not (white) with 2.5 mM L-methionine.

of cells grown in solid and liquid media containing or not L-methionine. In this analysis, we tested: conidial size, shape and germination rate; branching frequency; distance between septa; and presence and abundance of vacuoles. Although the germination rate of all the strains was lower in medium containing L-methionine, no additional differences were found in the transformant strains when compared with the wild type and *eth-1*^{r} strains growing in the tested media.

DISCUSSION

Recent work in our laboratory established a genetic and physical map on a *N. crassa* 200-kb region that includes the locus *eth-1* (MAUTINO *et al.* 1993). We have now cloned and sequenced *eth-1* and demonstrated that it encodes S-Adomet synthetase, as first suggested by KERR and FLAVIN (1970). Almost all organisms studied so far have two different genes encoding highly similar S-Adomet synthetases. The reason for this is not clear, but it can be suspected that this pattern is of central importance due to its presence in a wide range of organisms from bacteria to man. Considering that growth failure of the *eth-1*^{r} mutant at 39° represents a lethal inactivation of S-Adomet synthetase, it means that, if there is a second gene coding for an additional S-Adomet synthetase in *N. crassa*, it is not being expressed in our standard growth conditions. Differential expression of the alternative S-Adomet synthetase genes of *S. cerevisiae* and *E. coli*, in response to the media used for growth, has been reported (THOMAS and SURDIN-KERJAN 1991; SATISHCHANDRAN *et al.* 1993). Pairs of S-Adomet synthetase genes of a same organism share >90% amino acid sequence homology (see Table 1), and their genes showed cross-hybridization in Southern blots performed at relative high stringency. In *N. crassa*, we have not detected a putative second S-Adomet synthetase gene by Southern blot hybridization. Therefore, if a second gene exists, it must have a higher degree of nucleotide sequence divergence than that between pairs of S-Adomet synthetase genes of other species. This is a point of relevance when considering the phenomenon of RIP: a putative functional second *N. crassa*

S-Adomet synthetase gene should have enough divergence to allow it to become resistant to RIP (CAMBARERI *et al.* 1991) or, alternatively, it should be resistant by another mechanism. If there is a single S-Adomet synthetase gene in *N. crassa,* it should be of great interest to analyze why this fungus does not maintain the pattern of two S-Adomet synthetase genes.

Even though we have not performed primer extension analysis to determine the transcription initiation site of the *N. crassa* S-Adomet synthetase mRNA, the longest S-Adomet synthetase cDNA isolated has a 5′ untranslated region (5′UTR) of 222 bp. A long 5′UTR is a common feature among other S-Adomet synthetase mRNAs: 210 nt in the S-Adomet synthetase from rat liver (ALVAREZ *et al.* 1991), 224 nt in S-Adomet synthetase from murine liver (SAKATA *et al.* 1993), and 231 nt in the S-Adomet synthetase from Drosophila (LARSSON and RASMUSON-LESTANDER 1994). This is also true of the mRNAs of S-Adomet decarboxylase (RUAN *et al.* 1994) and ornithine decarboxylase (GRENS and SCHEFFLER 1990; MANZELLA and BLACKSHEAR 1990; WILLIAMS *et al.* 1992), enzymes which link the metabolism of S-Adomet to polyamine synthesis. It has been proposed that small 5′UTR-ORFs and/or secondary structures of the mRNAs of S-Adomet decarboxylase (RUAN *et al.* 1994) and ornithine decarboxylase (ITO *et al.* 1990) could be involved in posttranscriptional regulation by polyamines. In addition, it has been suggested that long 5′UTRs in S-Adomet synthetase mRNAs may also be involved in feedback translational regulation by polyamines (LARSSON and RASMUSON-LESTANDER 1994). Two small ORFs were found in the 5′UTR of the longest *N. crassa* S-Adomet synthetase cDNA. However, as in the case of *N. crassa* ornithine decarboxylase (WILLIAMS *et al.* 1992), the environment of their putative ATG start codons do not match the consensus for *N. crassa* (EDELMANN and STABEN 1994). In addition, no stable secondary structures could be found in the 5′UTR of the S-Adomet synthetase mRNAs mentioned above. Thus, it is unlikely that these structural determinants could represent functional regulatory elements involved in translational regulation of S-Adomet synthetase mRNAs. A very long 3′ untranslated region (574 nt) was found in the *N. crassa* S-Adomet synthetase cDNA. This includes two consensus polyadenylation sequences, which appear to be functional, as cDNAs having the corresponding alternative positions in their poly-(A) tails were isolated. To our knowledge, this represents the first strong evidence supporting a functional role of a consensus sequence for polyadenylation in *N. crassa.*

Gene constructs carrying the S-Adomet synthetase coding sequences were designed to overexpress the S-Adomet synthetase gene after introduction into *N. crassa* protoplasts. In these studies, it was observed that L-methionine is a limiting metabolite for the cellular production of S-Adomet. Growth rates of wild-type and transformed strains were studied in minimal medium and in medium supplemented with L-methionine and containing various carbon sources. Although growth rates of the analysed strains were different in the various media, no differential effect of any medium on the growth rates of wild-type and transformed strains could be seen. From these observations, it is tempting to speculate that the endogenous availability of S-Adomet is not limiting for growth. Certainly, growth rates were not increased in transgenic strains containing up to 260-fold higher endogenous levels of S-Adomet.

No obvious morphological alteration occurs in cells having abnormally high levels of S-Adomet. This could indicate that although S-Adomet is an extremely important metabolite, it may not be subjected to a tight control, and that large variations in the intracellular levels of S-Adomet are harmless to the cell. This is consistent with observations made on the bacterium *E. coli,* in which variations between 200-fold reduced (SATICHANDRAN *et al.* 1990) and 400-fold augmented endogenous levels of S-Adomet have no consequences in cellular growth (ALVAREZ *et al.* 1994). Alternatively, the putative "excess" of S-Adomet could be compartmentalized in the cell, to maintain the cytoplasmic levels in a constant concentration, as has been described in *S. cerevisiae* (FAROOQUI *et al.* 1983). However, considering the correlation found in this work between different S-Adomet levels and alternative degrees of DNA methylation, we suggest that most endogenous S-Adomet is fully available for the different S-Adomet-requiring metabolic pathways. It has recently been demonstrated that unregulated, high ornithine decarboxylase activity in *N. crassa* results in high cellular levels of putrescine whereas spermidine levels do not vary greatly (PITKIN *et al.* 1994). Externally added L-methionine (1.0 mM), which should modify the cellular S-Adomet levels, did not lead to enlargement of the spermidine pool (PITKIN *et al.* 1994). Thus, increases in S-Adomet do not appear to modify the flux of this metabolic pathway, at least at S-Adomet levels found in cells growing in the presence of 1.0 mM L-methionine. This observation is consistent with the notion that S-AdoMet decarboxylase limits spermidine synthesis when putrescine levels are high, although spermidine synthase has not been excluded as a limiting factor (R. H. DAVIS, personal communication).

The status of DNA methylation at the normally methylated ζ-η region was dependent on the cellular level of S-Adomet. This result confirms and extends recent observations (FOSS *et al.* 1993) indicating both that S-Adomet is the methyl group donor for cytosine DNA methylation in *N. crassa* and that externally added L-methionine could alter the status of genomic DNA methylation. We demonstrate that this effect depends on alternative cellular levels of S-Adomet and that the defect in DNA methylation of the *eth-1*^{r} mutant, growing at 34°, is probably due to its low endogenous level of

S-Adomet at this temperature. A maximum degree of DNA methylation was reached at cellular levels of S-Adomet lower than that present in transformed strains. This effect may be due to a limiting activity of the putative DNA (cytosine-5) methyltransferase, topological restrictions imposed by a heavily but not completely methylated substrate DNA, a low affinity of the putative DNA (cytosine-5) methyltransferase for particular nucleotide sequences, or a fraction of newly synthesized unmethylated DNA. Considering that in some analysed *N. crassa* DNA molecules 100% of cytosines could be subjected to methylation (SELKER *et al.* 1993) we consider unlikely that topological restrictions or particular nucleotide sequences may limit the degree of genomic DNA methylation. In addition, we doubt that the fraction of unmethylated DNA detected may correspond to newly synthesized molecules, as DNA was purified from mycelium in stationary phase. Thus, we favor the possibility that DNA methylation reaches a maximum due to limiting activity of the putative DNA methyltransferase.

A high proportion of strains overproducing S-Adomet was found in our initial screening of primary pSGT transformants (Figure 4B). In the analyzed strains, we did not find vegetative DNA methylation of the multiple copies of the S-Adomet synthetase gene. Thus, no evidences of vegetative silencing and/or DNA methylation of the transforming S-Adomet synthetase sequences ("quelling") (PANDIT and RUSSO 1992; ROMANO and MACINO 1992) were found. This result may indicate that quelling is not a general phenomenon. Alternatively, putative quelling of the S-Adomet synthetase gene could result in cellular lethality, thus rendering only nonaffected transformant cells. Certainly, a low frequency of quelling has been observed in the case of genes apparently essential for cellular viability (ROMANO and MACINO 1992).

N. crassa is able to grow in media containing high concentrations of the drug 5-azacytidine, which causes substantial reduction in DNA methylation (SELKER and STEVENS 1985). This result, as well as evidences from the study of *N. crassa* mutant strains defective in DNA methylation (FOSS *et al.* 1993) and results reported in this work, indicate that a wide spectrum of both cellular S-Adomet levels and DNA methylation states have no dramatic consequences on growth or morphology in *N. crassa.* This may not be the case in higher eukaryotic cells, where subtle variations in cellular S-Adomet levels and/or DNA (cytosine-5) methyltransferase activity (LI *et al.* 1993; BOERJAN *et al.* 1994; LAIRD *et al.* 1995) could modify the pattern or intensity of DNA methylation, thus altering a variety of cellular phenomena including gene regulation, gene expression or DNA mutation.

We thank H. J. F. MACCIONI for critical reading of the manuscript; E. SELKER for kindly providing the strain N276 and plasmids pJR2 and pES174; C. WILSON, from the FGSC, for the *N. crassa* λZAP and pMOcosX libraries, plasmid pCSN44 and strains; the Neurospora team at the University of Córdoba, for stimulating discussion along the course of this work. We thank to Novo BioLabs (*via* CRISPEX SRL) and S. R. FOOR (Du Pont) for gifts of Novozym 234 and benomyl, respectively. This work was supported by grants from International Centre for Genetic Engineering and Biotechnology, Trieste, Italy; Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina; Consejo de Investigaciones de Córdoba; SECYT-UNC, Córdoba, Argentina; and Fundación Antorchas.

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Communicating editor: R. H. DAVIS