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Mutations affecting the biosynthesis of S-adenosylmethionine cause reduction of DNA methylation in *Neurospora crassa*

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ABSTRACT

A temperature-sensitive methionine auxotroph of *Neurospora crassa* was found in a collection of conditional mutants and shown to be deficient in DNA methylation when grown under semipermissive conditions. The defective gene was identified as *met-3*, which encodes cystathionine-γ-synthase. We explored the possibility that the methylation defect results from deficiency of S-adenosylmethionine (SAM), the presumptive methyl group donor. Methionine starvation of mutants from each of nine complementation groups in the methionine (*met*) pathway (*met-1*, *met-2*, *met-3*, *met-5*, *met-6*, *met-8*, *met-9*, *met-10* and *for*) resulted in decreased DNA methylation while amino acid starvation, *per se*, did not. In most of the strains, including wild-type, intracellular SAM peaked during rapid growth (12–18 h after inoculation), whereas DNA methylation continued to increase. In *met* mutants starved for methionine, SAM levels were most reduced (3–11-fold) during rapid growth while the greatest reduction in DNA methylation levels occurred later. Addition of 3 mM methionine to cultures of *met* or cysteine-requiring (*cys*) mutants resulted in 5–28-fold increases in SAM, compared with wild-type, at a time when DNA methylation was reduced ~40%, suggesting that the decreased methylation during rapid growth in Neurospora is not due to limiting SAM. DNA methylation continued to increase in a *cys-3* mutant that had stopped growing due to methionine starvation, suggesting that methylation is not obligatorily coupled to DNA replication in Neurospora.

INTRODUCTION

Methylation at the 5 position of cytosine residues has been found in DNA of many eukaryotes, including mammals, plants and fungi. While many questions remain concerning the function of DNA methylation in eukaryotes, numerous correlations between the presence of DNA methylation and a lack of gene activity have been noted (see 1–3), and there is evidence that DNA methylation can, directly or indirectly, interfere with transcription [see (4–6)]. It is known that DNA methylation is essential for embryogenesis in mice, as reduction of DNA methylation by ~70% is lethal (7). Mutants of Arabidopsis with similar reductions in DNA methylation are viable, although they do exhibit altered growth characteristics (8,9). In *Neurospora crassa*, a mutant has been isolated that appears to lack detectable DNA methylation, implying that DNA methylation is not essential in this organism (10).

About 1.5% of the cytosines are methylated in vegetative tissue of wild-type *N.crassa*, except during early times after conidial germination, when the 5-methylcytosine content drops ~40% (11; H. Foss, C. Roberts and E. Selker, unpublished data). DNA methylation in Neurospora is concentrated in distinct chromosomal patches where nearly all cytosines appear methylated (12,13). Three sites of heavy methylation have been reported: the zeta–eta (ζ–η) region of linkage group I (12–15), the psi63 (ψ_{63}) region on linkage group IV (16; B. Margolin, E. Selker, J. Stevens, P. Garrett-Engele, C. Garrett-Engele, D. Fritz and R. Metzenberg, unpublished data), and the ribosomal DNA (rDNA) on linkage group V (17,18).

We reported that two Neurospora mutants harboring mutations in the biosynthetic pathway leading to S-adenosylmethionine (SAM) displayed a conditional Dim (defective in methylation) phenotype (10). The *eth-1* mutant, which harbors a temperature-sensitive SAM synthetase (19), showed reduced DNA methylation at semi-permissive growth temperatures. Also, a *met-7* mutant, which is defective in methionine biosynthesis due to a lack of cystathionine γ-synthase (20), showed decreased levels of methylation when grown in medium supplemented with low concentrations of methionine. Since methionine is the direct precursor to SAM, and SAM is the methyl group donor for 5-cytosine DNA methylation in bacteria and mammals (1,21), we proposed that *eth-1* and *met-7* showed reduced DNA methylation under restrictive conditions due to reductions of intracellular SAM concentrations. Here we report the results of investigating this possibility by testing other Neurospora mutants for reduction of DNA methylation when starved for methionine, and by examining the relationship between intracellular SAM concentration and DNA methylation.

MATERIALS AND METHODS

Strains and reagents

The strains used in this study are listed in Table 1. Methods for culturing *N.crassa* and performing genetic analyses were standard (22). [α^{32}P]dCTP, [^{3}H]dopamine, catechol O-methyltransferase

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and S-adenosylmethionine were from New England Nuclear (Boston, MA). All other reagents were from Sigma Chemical Co. (St Louis, MO) unless otherwise noted.

Southern analysis

Preparation and restriction of genomic DNA were carried out as previously described (10). For the experiments described in Figures 1, 3, 4 and 5, all cultures were inoculated with a small amount of conidia and incubated without shaking for 3–5 days in minimal liquid growth medium (with the indicated supplements). Methods for Southern analysis, including transfer of DNA to nylon membranes (Zetabind, Cuno Inc., Meriden, CT), hybridization with radioactive probes, and stripping of blots, were also performed as before (23). Quantitation of Southern blots was performed on a Radioanalytic Imaging System (AMBIS Inc., San Diego, CA). To control for incomplete restriction digestion, all southern blots described in this work were stripped and reprobed with the unmethylated *am* gene of *N.crassa*. For analysis of methylation at the rDNA spacer region, blots were probed with a 3.4 kb *Eco*RI fragment spanning the spacer region between the repeated rRNA coding regions.

Table 1. Strains used in this study

Strain	Relevant genotype	Source
N150 (74A-OR23-IV)	(wild-type)	FGSC[a] #2489
N322	*met-3*^{ts}	David R. Stadler
N608	*met-3*^{ts} *his-3 al-3*	This study
N610	*met-3 inv inl mei-2*	Robert L. Metzenberg
N558	*eth-1*	FGSC #1220
N213	*met-1*	Edward G. Barry
N190	*met-2*	FGSC #3184
N552	*met-3*	FGSC #502
N554	*met-5*	FGSC #3862
N555	*met-6*	FGSC #4249
N557	*met-8*	FGSC #98
N601	*met-9*	FGSC #552
N600	*met-10*	FGSC #2937
N602	*for*	FGSC #9
N691	*met-7*	This study
N411	*his-3*	FGSC #6524
N692	*cys-5*	FGSC #1274
N603	*hom*	FGSC #946
N553	*cys-10*	FGSC #4054
N267	*cys-3*	FGSC #4028
N693	*cys-11*	FGSC #1276
N431	*dim-1*	This study
N535	*dim-2*	ref. 10
N590	*dim-3*	ref. 10
N694	*dim* #850	This study
N695	*dim* #924	This study
N696	*dim* #140	This study

[a]FGSC = Fungal Genetic Stock Center.

HPLC analysis

5-methyldeoxycytidine levels in genomic DNA of a Neurospora *met-7* mutant were determined by HPLC analysis as described previously (10). Strain N691 was inoculated to 5×10^6 conidia/ml in 400 ml of minimal medium containing either 3.3 or 0.03 mM methionine, and shaken at 33°C for 24 or 36 h respectively. After harvesting the tissue, genomic DNA was prepared as noted above, and then further purified on ethidium bromide–CsCl gradients (24). The relative amounts of 5-methyldeoxycytidine in *met-7* grown in high or low levels of methionine were determined by cutting out the peaks from five photocopies of each chromatogram, and weighing the peaks, and calculating the average values.

Preparation of tissue extracts for SAM assays

100 ml (for wild-type or fully supplemented strains) or 400 ml cultures (for strains grown on low levels of an essential supplement or at semi-permissive growth temperatures) were inoculated with 5×10^6 conidia/ml and shaken (250 r.p.m.) at the appropriate temperatures. Wet tissue samples were harvested by filtration, rinsed with water, blotted with paper towels, and weighed. The percentage of dry weight in the wet tissue was determined by lyophilizing ~0.4 g tissue samples and then weighing the dried tissue. The dried tissue was then used as a source of genomic DNA. The remaining wet tissue was ground using a mortar and pestle in 10 ml of 2% perchloric acid with 3 g of Ottawa sand (Fisher Scientific, Fair Lawn, NJ) for one min. The ground mixture was transferred to a 30 ml Corex tube and centrifuged for 10 min at 10 000 *g*, and the supernatant was frozen at –80°C in aliquots.

SAM assay

Intracellular SAM concentrations were measured using a radioenzymatic assay developed by Yu (25,26). Aliquots of the extracts were thawed slowly on ice, and then adjusted to pH 7–9 (monitored by pH paper) by adding ~0.4 ml of 0.5 M NaOH to 0.7 ml of extract. Samples (50–100 µl) of each extract were assayed in reactions containing 250 mM Tris–HCl, pH 8.6, 15 mM DTT, 6.25 mM $MgCl_2$, 0.63 mM (0.5 µCi) [^{3}H]dopamine and 0.5 U catechol O-methyltransferase. The reactions were incubated for 30 min at 37°C, and stopped by adding 0.6 ml 0.5 M borate, pH 10.0. The reaction product, 3-[^{3}H]methoxytyramine, was extracted with 0.75 ml 3:2 toluene:isoamyl alcohol. Aliquots of the organic phase of each sample were transferred to scintillation vials containing 5 ml Ecolum (ICN Biomedicals, Inc., Irvine, CA), and radioactivity in each of the samples was counted for 2 min using a Beckman LS6800 liquid scintillation counter. For each experiment, a standard reaction curve was generated using 0, 1, 3.3, 10, 33, 100 and 330 pmol of pure SAM. Typically, the number of ^{3}H counts increased approximately linearly up to 33 pmol of SAM. Each sample for the standard curve was measured in duplicate, and the resulting values were averaged. Likewise, each experimental sample was assayed in duplicate. The SAM concentration in each extract was interpolated

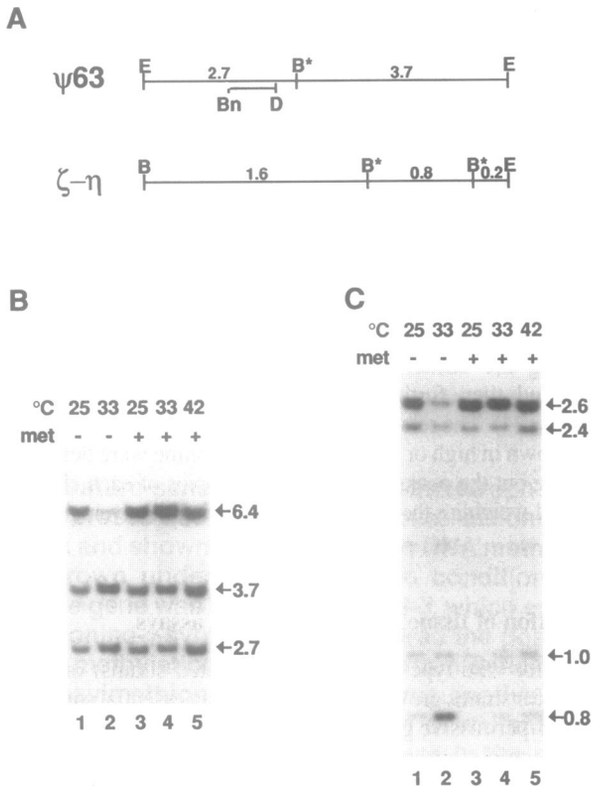

Figure 1. Reduced DNA methylation in *dim*^{ts} grown at elevated temperature. (**A**) Restriction maps of the ψ_{63} and ζ–η loci of *N.crassa*. E = *Eco*RI; B = *Bam*HI; Bn = *Ban*II; D = *Dra*II. The asterisks denote sites that are blocked by cytosine methylation. The *Ban*II–*Dra*II fragment of ψ_{63} was used as a probe in the experiment described in Figure 6B. (**B**) Southern analysis of the ψ_{63} locus in a *dim*^{ts} strain. The growth conditions used for each culture are shown above the lanes of the figure. Genomic DNA was prepared from *dim*^{ts} grown at 25, 33 or 42°C in the presence (+) or absence (–) of 3.3 mM methionine (met). The DNAs were cleaved with *Bam*HI and *Eco*RI and probed with the plasmid pJS63, which contains the 6.4 kb *Eco*RI fragment including ψ_{63} (16). The sizes of the fragments are indicated in kb. (**C**) Southern analysis of ζ–η locus. The blot from (B) was stripped and reprobed with the 0.8 kb *Bam*HI fragment of the ζ–η region (12).

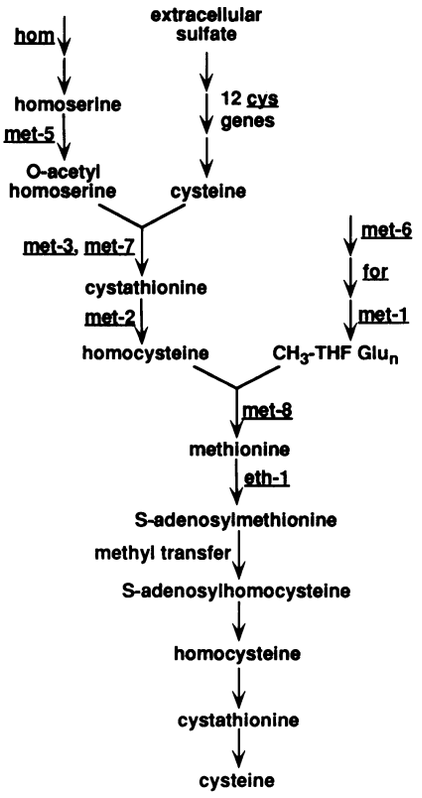

Figure 2. Biosynthetic pathways leading to and from SAM in *N.crassa* (adapted from ref. 20). The mutations that block specific steps of the pathway are indicated. Only the genes germane to this study are indicated. Included in the 12 *cys* genes are *cys-3*, which is a transcriptional regulator of many *cys* genes; *cys-5*, which is required for 3′-phosphoadenosine-5′-phosphosulfate reductase activity; and *cys-10*, which is required for sulfite reductase activity. CH_3-THF Glu_n denotes methyl-tetrahydrofolate-polyglutamate. The mutations that block specific steps of the pathway are indicated. The *met-9* and *met-10* mutants have not been assigned to specific steps in the methionine biosynthetic pathway.

from the standard curve. Control experiments showed that one round of freezing and thawing the extracts had a negligible effect on SAM activity.

RESULTS

Identification and characterization of a temperature-sensitive mutant deficient in DNA methylation

As a first step to explore the possibility that essential genes might be involved in DNA methylation in *N.crassa*, a collection of 100 temperature-sensitive strains (27; D. Stadler, unpublished data) were screened by Southern analysis for decreased levels of DNA methylation at semi-permissive growth temperatures. DNA was isolated from strains grown in minimal liquid medium at standard and elevated growth temperatures. The DNA samples were analyzed for the methylation status of a *Bam*HI site in the methylated region, ψ_{63} (Fig. 1A). In *dim*$^{+}$ cells, a majority of the molecules are methylated at the *Bam*HI site, and thus uncut by *Bam*HI, giving a 6.4 kb fragment when digested with *Eco*RI and *Bam*HI (10). DNA from tissue with reduced methylation shows greater cleavage, with a corresponding increase in the 2.7 and 3.7 kb fragments in a *Bam*HI and *Eco*RI digest. A single mutant, tentatively called *dim*^{ts} (strain N322), was identified that showed substantial reduction in DNA methylation when grown at the semi-permissive temperature of 33°C, and normal methylation at 25°C (Fig. 1B, lanes 1 and 2).

The *dim*^{ts} mutant also showed reduced DNA methylation at other normally methylated loci of Neurospora, as illustrated by Southern analysis of the ζ–η region (Fig. 1C, lanes 1 and 2). Less methylation was seen at the two *Bam*HI sites at ζ–η when the strain was grown at 33°C as opposed to 25°C, as indicated by an increased amount of the 0.8 kb *Bam*HI fragment (Fig. 1C, lanes 1 and 2). As with the *dim-3* mutant of Neurospora (10), the DNA methylation defect of *dim*^{ts} appeared more pronounced at ψ_{63} than at ζ–η.

The methylation defect of *dim*^{ts} is alleviated by methionine and *dim*^{ts} is an allele of *met-3*

We found that the *dim*^{ts} strain did not show a growth defect on complete medium. Crosses to marked strains demonstrated that *dim*^{ts} is linked to the *al-3* and *inl* genes on linkage group V. Because *met-3* also maps to this region, we tested whether *dim*^{ts}

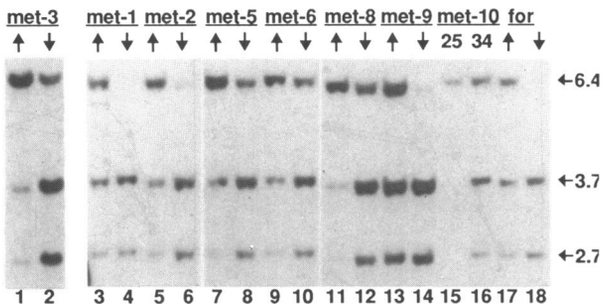

Figure 3. DNA methylation at ψ_{63} in *met* and *for* mutants. The indicated strains (see Table 1 for strain information; *met-3* = N552) were grown in a high level of methionine (upward-pointing arrow; 3.3 mM methionine) or low level of methionine (downward-pointing arrow; 0.03 mM methionine, except for *met-1*, which was grown in 0.15 mM methionine). The temperature-sensitive *met-10* strain was grown in minimal medium without methionine at either 25 or 34°C. Genomic DNA preparation and Southern analysis of ψ_{63} was performed as described in the legend of Figure 1B.

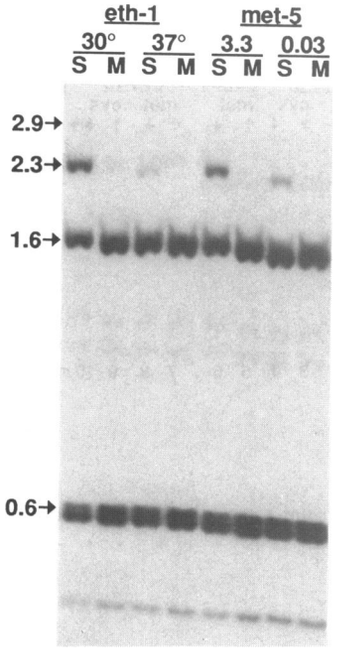

Figure 4. Assay for methylation at the rDNA spacer region of *eth-1* and *met-1* mutants. Genomic DNA was isolated from *eth-1* grown at 30 and 37°C and *met-1* grown in 3.3 or 0.03 mM methionine. The DNA was digested with *Sau*3AI (S), which cuts GATC but not GAT^{m}C, or *Mbo*I (M), which cuts both GATC and GAT^{m}C (52), and was probed with a 3.4 kb *Eco*RI fragment of the rDNA that includes the spacer region. Methylation of the spacer region results in the 2.9 and 2.3 kb fragments indicated (17).

was defective for methionine biosynthesis at restrictive temperatures. Both the growth and DNA methylation defects were relieved by the addition of methionine to growth media (Fig. 1B, lanes 3–5; Fig. 1C, lanes 3–5). Allelism of the *dim*^{ts} and *met-3* mutations was demonstrated directly in two ways. First, a heterokaryon forced between a *dim*^{ts} (N608) and a *met-3* (N610) strain was unable to grow at 37°C in the absence of methionine (data not shown). Secondly, a cross between a *dim*^{ts} (N322) and a *met-3* (N552) strain yielded only seven progeny able to grow at 37°C without methionine out of 10 000 ascospores analyzed, indicating very tight linkage. Thus, the *dim*^{ts} mutation is a temperature-sensitive allele of *met-3*, and will henceforth be referred to as *met-3*^{ts}.

Conditional reduction of DNA methylation in other mutants of Neurospora

We previously reported that the *eth-1* mutant of Neurospora, which is temperature-sensitive for growth due to a mutation in SAM synthetase (19; Fig. 2), is deficient in DNA methylation at semi-permissive growth temperatures (10). Because SAM is the methyl-group donor for cytosine methylation in bacteria and animal cells, this suggested that reduced SAM levels in Neurospora result in decreased DNA methylation. Since methionine is the direct biosynthetic precursor to SAM, and *met-3*^{ts} and *met-7* (10) mutants are deficient in methylation when starved for methionine, we wished to test if starvation of any *met* mutant reduces DNA methylation. A survey of representative mutants from all of the other *met* genes of *N.crassa*, including *met-1*, *met-2*, *met-3*, *met-5*, *met-6*, *met-8*, *met-9* and *met-10* (20) showed that our expectation was correct. Less DNA methylation was seen at the *Bam*HI site of ψ_{63} when these mutants were grown in 0.03 mM methionine (*met-2*, *met-3*, *met-5*, *met-6*, *met-8* and *met-9*) or 0.15 mM methionine (*met-1*) than when grown on 3.3 mM methionine (Fig. 3, lanes 1–14). The temperature-sensitive *met-10* mutant, which has a methionine requirement at 34°C, but not at 25°C, showed reduced DNA methylation at 34°C when grown without methionine (Fig. 3, lanes 15 and 16). We also tested the *for* mutant, which acts between *met-6* and *met-1* in the biosynthetic pathway of methyl-tetrahydrofolate (Fig. 2), and whose defect is relieved by either formate or a mixture of methionine and adenine (20). DNA methylation was reduced when this mutant was grown in low levels of methionine (Fig. 3, lanes 17 and 18). Curiously, different *met* mutants reproducibly displayed variable levels of methylation at ψ_{63} when supplemented with methionine (e.g. Fig. 3, lanes 11 and 13). We do not understand the basis of this variability.

In addition, several *met* mutants were tested for reduction of DNA methylation at other methylated loci. All the mutants tested showed reduced methylation at the ζ–η locus (*met-3*, *met-5*, *met-8*, *met-9*, *for* and *eth-1*; data not shown) and rDNA region (e.g., *eth-1*, *met-5*; Fig. 4) when grown in methionine-poor medium. For analysis of methylation at the rDNA, genomic DNAs were digested with *Sau*3AI or *Mbo*I and probed with the 3.4 kb *Eco*RI fragment that includes the spacer region between the rDNA repeats (see ref. 17). Methylation of *Sau*3AI sites results in the appearance of the 2.3 and 2.9 kb fragments. Southern analysis of the rDNA of the wild-type strain, N150, gave results identical to the *eth-1* strain grown at 30°C and the *met-5* strain supplemented with methionine (data not shown). Furthermore, supplementation of N150 with excess methionine appeared to cause a very small increase in methylation at the ζ–η region, but did not result in a noticeable increase in methylation at ψ_{63} (H. Foss and E. Selker, unpublished).

Southern analysis of DNA from the *met* mutants revealed substantial reductions in DNA methylation at the three loci examined, but did not provide information on overall DNA methylation in the genome. To quantify the overall decrease in DNA methylation induced by methionine starvation of a *met* mutant, HPLC analysis was performed on deoxynucleosides from genomic DNA of *met-7* cells grown either in 3.3 or 0.03 mM

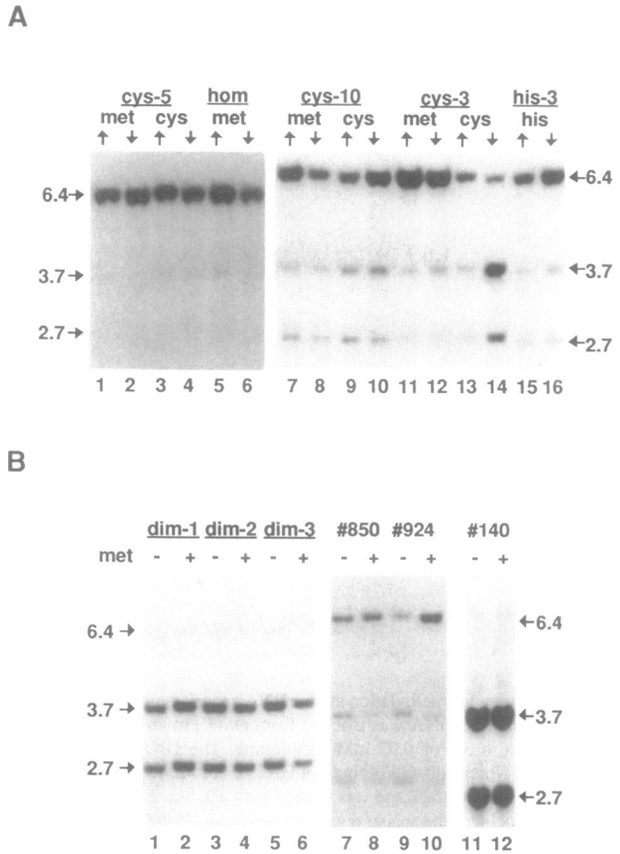

Figure 5. Assay for methylation at the *Bam*HI site of ψ_{63} in *cys*, *hom*, *his-3* and *dim* mutants. (**A**) *cys-5*, *hom*, *cys-10* and *cys-3* strains were grown in either 3.3 or 0.03 mM methionine. *cys-5*, *cys-10* and *cys-3* mutants were also grown in either 1 or 0.1 mM cysteine. The *his-3* strain was grown in 3.2 or 0.1 mM histidine. Upward-pointing and downward-pointing arrows refer to the high and low concentrations of supplements in the growth media respectively. (**B**) *dim-1*, *dim-2*, *dim-3* and the less characterized dim mutants designated as #850, #924 and #140 were grown in the presence or absence of 3.3 mM methionine. Genomic DNA was isolated and analyzed as described in the Figure 1B legend.

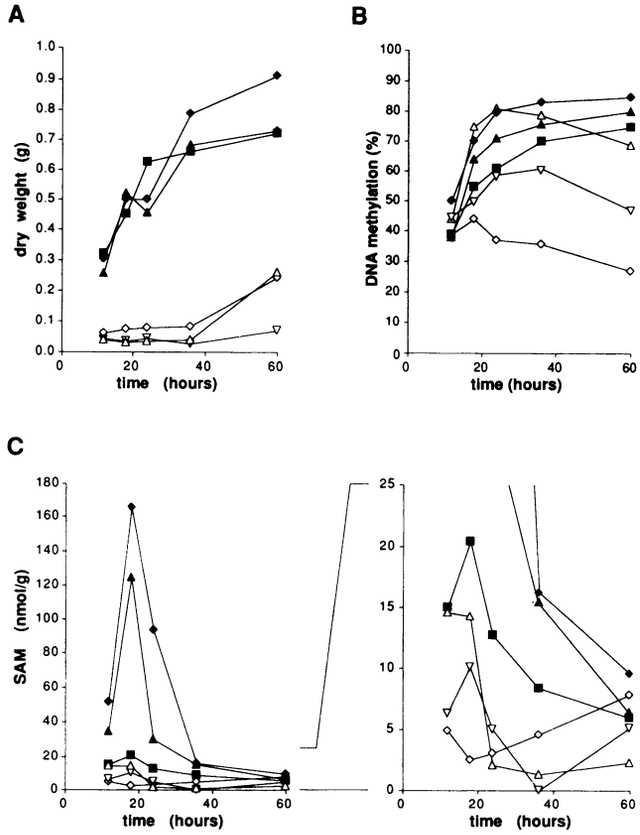

Figure 6. Time course study of DNA methylation levels and SAM concentrations. Wild-type (filled squares), *met-1* grown in 3.0 mM (filled diamonds) or 0.03 mM methionine (open diamonds), and *cys-3* grown in 3 mM methionine (filled triangles), 0.03 mM methionine (open triangles) or 0.3 mM cysteine (inverted open triangles) were grown for 12, 18, 24, 36 or 60 h. They were then assayed for growth (**A**), DNA methylation at the *Bam*HI site of the ψ_{63} region (**B**), and SAM concentration (**C**). The data presented in (B) were derived from AMBIS quantification of the blots.

methionine. Starvation for methionine decreased the fraction of 5-methyldeoxycytidine in the genomic DNA by 47% (data not shown).

Amino acid starvation, *per se*, does not reduce DNA methylation

Starvation of amino acid auxotrophs for their required amino acid, *per se*, does not decrease DNA methylation levels. For example, DNA methylation was not reduced by starving a *his-3* mutant for histidine (Fig. 5A, lanes 15 and 16). Also, methionine starvation, *per se*, does not cause reduction of DNA methylation. This was shown for the mutants *cys-5*, *hom* and *cys-10*, (Fig. 5A, lanes 1, 2 and 5–8), whereas the *cys-3* mutant showed a slight reduction in methylation (Fig. 5A, lanes 11 and 12). The *cys* mutants are complemented by addition of either cysteine or methionine to the growth medium, whereas the *hom* mutant requires both methionine and threonine (20). Because cysteine, like methionine, is a precursor to SAM, but is earlier in the biosynthetic pathway (Fig. 2), we tested if starvation of these mutants for cysteine would caused decreased DNA methylation. Figure 5A shows that *cys-5* (lanes 3 and 4) and *cys-10* (lanes 9 and 10) mutants exhibited normal DNA methylation when grown in limiting cysteine, whereas *cys-3* showed a significant decrease in methylation at the *Bam*HI site of ψ_{63} when starved for cysteine (lanes 13 and 14). As with *cys-5* and *cys-10*, starvation of *cys-11* for either methionine or cysteine did not reduce DNA methylation (data not shown). It is unclear why the different *cys* mutants behave differently with respect to DNA methylation upon starvation for cysteine.

Several mutants defective in DNA methylation (*dim*) have been isolated in our laboratory. Three complementation groups, *dim-1* (H. Foss, C. Roberts and E. Selker, unpublished), *dim-2* and *dim-3* (10) have been defined so far, and a number of other mutants remain unclassified (H. Foss, K. Claeys and E. Selker, unpublished). Although the screens used to isolate these mutants involved growth of Neurospora on minimal medium, we decided to examine the possibility that the mutants have defects in the pathway leading to SAM. Thus, methylation in all of the *dim* mutants was assayed following growth in medium containing a high level of methionine (3.3 mM). The methylation defects of *dim-1*, *dim-2* and *dim-3* were not relieved by the addition of methionine (Fig. 5B, lanes 1–6). Several other *dim* isolates from

the screen that yielded *dim-2* and *dim-3* showed increased methylation, however, when methionine was added to the growth medium (e.g., #850, #924; Fig. 5B, lanes 7–10). Since the *dim* strains #850 and #924 grow in the absence of methionine, albeit more slowly (data not shown), these mutants may be partially defective for methionine biosynthesis. We have not tested this hypothesis directly. Several other unclassified *dim* isolates from this screen were not relieved by the addition of methionine (e.g. *dim*#140; Fig. 5B, lanes 11 and 12).

Reduction of intracellular SAM is associated with starvation for methionine

To explore the possibility that the reductions in DNA methylation seen in the various mutants tested reflected decreases in intracellular SAM concentrations, we measured the SAM levels using a radioenzymatic assay (25,26). The assay used cellular extracts as the SAM source for a reaction catalyzed by the enzyme catechol O-methyltransferase, which transfers a methyl group from SAM to dopamine. The results of assaying SAM levels in a number of strains under different growth conditions are shown in Table 2. Cultures were inoculated with 5×10^6 conidia/ml, and tissue samples were harvested after 12–18 h of incubation at 33°C (except for *eth-1*). Under these conditions, fully supplemented Neurospora strains are typically in mid to late logarithmic growth after 12–18 h of growth (28). Auxotrophic strains starved for the required nutrient typically stop growing by 12 h (for example, see Fig. 6A). The concentration of SAM in a wild-type strain, N150, was 19.4 nmol SAM/g of dry tissue (4.6 nmol SAM/g of wet tissue). This level of SAM is much lower than that reported previously for Neurospora mycelial tissues using different methods for assaying SAM [62 nmol SAM/g wet tissue (29); 500 nmol SAM/g wet tissue (30)].

The SAM concentration measured in *eth-1* tissue grown at 30°C was equivalent to that found in the wild-type strain, whereas in tissue grown at 37°C the level was ~5-fold lower than that in the wild-type (Table 2), consistent with the *eth-1* mutant harboring a temperature-sensitive SAM synthetase (19). The SAM pools measured in *met* mutants grown in 3.3 mM methionine were 5–28-fold higher than that in the wild-type strain grown in the absence of added methionine (Table 2). Growth of *met* mutants in low methionine (0.03 mM) resulted in 3–11-fold reductions of the SAM pools relative to wild-type. Thus, the SAM pools of *met* mutants starved for methionine are significantly smaller than in a wild-type strain.

Starvation of the *cys-3* or *cys-10* mutants for methionine, which caused little or no reduction of DNA methylation, did not dramatically reduce the SAM concentration in cultures harvested after 12–18 h of growth (e.g. *cys-3* showed 14.3 nmol SAM/g, compared with 19.4 nmol/g for wild-type). Starvation of a *cys-3* mutant for cysteine, which did result in some reduction in DNA methylation, resulted in an ~70% reduction in SAM (6.1 nmol/g, compared with 19.4 nmol/g for wild-type). Starvation of the *his-3* strain for histidine did not affect intracellular SAM levels.

The results of Southern analyses presented above were from cultures grown to for 3–4 days. To gain a clearer understanding of the relationship of DNA methylation to growth phase and intracellular SAM concentration in Neurospora, we examined wild-type, *met-1*, and *cys-3* strains grown in high or low methionine or cysteine for 12, 18, 24, 36 and 60 h (Fig. 6). Figure 6A shows the growth of wild-type, *met-1* (grown in 3 or 0.03 mM methionine), and *cys-3* (grown in 3 mM methionine, 0.03 mM methionine or 0.3 mM cysteine) over the course of the experiment. The wild-type and fully supplemented *met-1* and *cys-3* cultures were growing rapidly at 12 and 18 h of growth, whereas the cultures containing low levels of methionine or cysteine showed essentially no growth through the 36 h time point. Each of the starved strains showed significant growth between 36 and 60 h of growth, however. This may have been due to the growth of met^+ or cys^+ revertants, considering that the cultures were heavily inoculated (2×10^9 conidia/400 ml culture), and in an independent test the *met-1* and *cys-3* strains used in this study reverted at frequencies of 8.7×10^{-6} and 2.6×10^{-6} per conidium respectively, when plated on growth medium lacking methionine (data not shown). Note that the cultures grown for the experiments described in Figures 1, 3, 4 and 5 did not accumulate revertants, probably because these cultures were inoculated with a small number of conidia (see Materials and Methods).

Table 2. Intracellular SAM concentrations of wild-type and mutant *N.crassa*

Strain	Supplement	SAM (nmol/g)
N150	none	19.4 ± 2.8[a]
eth-1, 30°	none	19.4 ± 2.0[b]
eth-1, 37°	none	3.6 ± 0.6[a]
met-1	3.3 mM met[c]	137.9, 137.7[d]
met-1	0.03 mM met	3.6, 3.6[d]
met-2	3.3 mM met	212.0, 217.0[d]
met-2	0.03 mM met	5.2, 5.2[d]
met-3	3.3 mM met	407.0, 399.6[d]
met-3	0.03 mM met	4.9, 5.1[d]
met-7	3.3 mM met	94.8, 108.4[d]
met-7	0.03 mM met	2.3, 2.5[d]
met-8	3.3 mM met	526.2, 550.8[d]
met-8	0.03 mM met	1.5, 2.1[d]
cys-3	3.3 mM met	106.6, 112.6[d]
cys-3	0.03 mM met	14.3 ± 2.4[b]
cys-3	0.3 mM cys[e]	5.8, 6.4[d]
cys-10	3.3 mM met	397.5, 370.3[d]
cys-10	0.03 mM met	21.7 ± 0.8[b]
his-3	3.2 mM his	17.8 ± 0.6[b]
his-3	0.03 mM his	20.1 ± 1.8[b]

Each strain was grown in the indicated concentration of supplement (at 33°C unless noted otherwise noted), and the tissues were harvested after 12–18 h and assayed for intracellular SAM concentration. SAM concentration is expressed per dry weight tissue.

[a]Duplicate tissue samples were harvested from two different cultures, and all four samples were assayed in duplicate. The eight values were averaged and the standard deviation is indicated.

[b]Two tissue samples were harvested from the same culture and assayed in duplicate. The four values were averaged and the standard deviation is indicated.

[c]Methionine.

[d]The two values represent duplicate assays of the same sample.

[e]Cysteine.

Genomic DNA prepared from all samples was examined for methylation at the *Bam*HI site of the ψ_{63} region (Fig. 6B). All of the strains initially showed 40–50% methylation at the *Bam*HI site, whether grown in high or low levels of supplement. Over the course of the experiment, *met-1* and *cys-3* grown in 3 mM methionine, and wild-type showed increasing methylation to give ~70–80% of the sites methylated at 60 h. Figure 6C shows that the SAM concentration of wild-type peaked at 18 h, and then gradually decreased. Addition of 3 mM methionine to *met-1* and *cys-3* strains resulted in a dramatic increase in intracellular SAM levels early in the growth phase, peaking at ~165 and 120 nmol/g, respectively, at 18 h. By 60 h the SAM concentrations in these strains fell to approximately the level of wild-type. In spite of the 2–3-fold increase in the SAM pool of *met-1* and *cys-3* over wild-type at 12 h of growth (Fig. 6C), DNA methylation was not markedly increased in these strains. This suggests that SAM is not limiting for DNA methylation during the rapid growth phase.

The methylation levels of *met-1* grown in 0.03 mM methionine, and *cys-3* grown in 0.3 mM cysteine, were reduced at the 60 h time point compared with wild-type, consistent with our previous observations (Fig. 3, lanes 3 and 4, and Fig. 5A, lanes 13 and 14, respectively). Both of these strains also displayed lower intracellular SAM concentrations through the 36 h time point, consistent with decreased SAM levels resulting in lower DNA methylation. The starved *met-1* and *cys-3* cultures showed increased SAM levels at 60 h, possibly due to the growth of met^+ and cys^+ revertants.

The *cys-3* strain grown in 0.03 mM methionine showed nearly normal levels of methylation at 36 and 60 h of growth, consistent with the previous findings (Fig. 5, lanes 11 and 12). DNA methylation increased from ~45 to ~80% at 24 h, and then fell slightly to ~70% (Fig. 6B). The intracellular SAM concentration was comparable with that of wild-type at 12 h, and fell slightly at 18 h before dropping precipitously at 24 h. Even though the mass of the culture had not increased from 12 to 24 h (Fig. 6A), the level of DNA methylation increased to ~80% by 24 h.

DISCUSSION

All known DNA (5-cytosine) methyltransferases use SAM as the methyl-group donor (1,21). While the methyltransferase from *N.crassa* has not been identified, the results reported here are consistent with SAM being the methyl-group donor in Neurospora as well. Auxotrophic strains with defects in the SAM biochemical pathway (i.e., the *met* and *for* mutants) showed reduced DNA methylation under starvation conditions, whereas starvation of a *his-3* mutant did not affect methylation. Curiously, we found that the intracellular SAM concentration at early growth stages seemed to predict the levels of DNA methylation at stationary phase (e.g. see *cys-3* and *met-1* cultures starved for methionine or cysteine; see Fig. 6). A possible partial explanation for the lack of a direct temporal relationship between DNA methylation and SAM levels is that a shortage of SAM should not affect methylation until after DNA replication creates its substrate, unmodified cytosines.

The concentration of SAM in wild-type Neurospora changed during growth, increasing to ~20 nmol/g dry tissue (4.6 nmol/g wet tissue) during rapid growth of cultures, then decreasing to ~7 nmol/g dry tissue. These values are much lower than the SAM concentrations, measured in rapidly growing tissue, reported previously [62 (29) and 500 (30) nmol/g wet tissue]. We cannot readily account for the discrepancy between these measurements of SAM in Neurospora, except to state that different methods were employed. SAM concentrations have also been determined for *E.coli* (~50 nmol/g wet cells) (31), rat brain (16–27 nmol/g wet tissue) (25) and various rat tissues (1). Since SAM is inherently unstable, all of these measurements should be regarded as lower limits of the actual values. Given the similarity among the SAM levels measured for wild-type, *eth-1* grown at 30°C, *cys-10* grown in high or low methionine, or the *his-3* mutant (Table 2), it seems safe to make comparisons between the different mutant strains examined in this study.

It is difficult to relate the value of 20 nmol SAM/g dry tissue to a cytosolic or nuclear concentration of SAM, because the fractions of Neurospora mycelial tissue made up of intracellular and extracellular water is not known. A further potential complication is that, at least in yeast, a sizable fraction of the intracellular SAM is stored in vacuoles (32). It is unknown how much SAM is stored in the vacuoles of Neurospora. Values for the content of intracellular water in various cell types range from 60 to 85% (e.g., see 33–35). Typically, water comprised ~75% of the wet tissue samples harvested in the experiments presented here. Assuming that the intracellular water content of Neurospora is between 60 and 75%, and that all of the intracellular SAM is available to the methyltransferase for DNA methylation, then the intracellular SAM concentration peaked between 6–13 μM during rapid growth, and decreased to 2–4 μM at stationary phase. Our finding that 3–11-fold reductions of the SAM pool were correlated with decreased DNA methylation suggests that the intracellular SAM concentration is not vastly different from the K_m of the DNA methyltransferase. While the K_m of the Neurospora enzyme for SAM is unknown, other eukaryotic DNA methyltransferases have values between 1 and 10 μM (36–38). Our results suggest that the Neurospora enzyme has a similar K_m. Critical testing of this hypothesis will await biochemical characterization of the Neurospora DNA methyltransferase.

Russell *et al.* (11) showed that ~1.5% of the cytosine residues are methylated in germinating conidia at 0, 3 and 24 h. The 5-methylcytosine content was 40% lower at 6 and 12 h, however. Considering that the doubling time of Neurospora is ~3 h under these conditions, very little, if any, DNA methylation could have occurred between the 3 and 6 h time points. Our results from examining methylation at specific sites are consistent with those observations. All of the strains studied in our time course experiment (Fig. 6) showed 40–50% methylation at the ψ_{63} *Bam*HI site after 12 h of growth, with the methylation increasing to 70–80% at 36 h in wild-type and fully supplemented cultures (Fig. 6). Starvation of *met-1* for methionine and *cys-3* for cysteine resulted in decreased levels of SAM at the 12 and 18 h time points, and decreased levels of DNA methylation at 36 h of incubation. Interestingly, while 12 h cultures of methionine-starved *met-1* and cysteine-starved *cys-3* displayed ~3-fold less SAM than did wild-type cultures, their methylation levels appeared comparable with wild-type at this time. Also, addition of 3 mM methionine to the growth media of *met-1* and *cys-3* mutants resulted in dramatic increases in SAM after 12 h of growth, but like wild-type, these strains still showed reduced DNA methylation at this time point. Although the SAM levels at times earlier than 12 h were not examined, these results suggest that the reduction of DNA methylation seen in rapidly growing Neurospora is not due to limiting SAM. Presumably, some other factor essential for DNA methylation such as the methyltransferase, or an unknown associated factor, is limiting during the period of rapid growth.

In animal cells, at least 50% of all DNA methylation occurs within 5 min after the synthesis of the nascent DNA chain (39). The timing of DNA methylation during the Neurospora cell cycle is unknown. It is noteworthy, however, that while starvation of the *cys-3* mutant for methionine resulted in the cessation of growth by 12 h (Fig. 6A), the level of DNA methylation at the ψ_{63} *Bam*HI site increased from 45 to 80% between the 12 and 24 h time points (Fig. 6B). Thus, Neurospora DNA can become methylated in the absence of growth, suggesting that there is no obligatory coupling between DNA methylation and DNA replication in Neurospora. This is consistent with the situation in the slime mold *Physarum polycephalum*, in which a substantial fraction of DNA methylation occurs outside of S phase (40,41).

In the specialized dikaryotic cells of the premeiotic phase of Neurospora, a process called RIP (repeat-induced point mutation) riddles sequence duplications with CG to AT transition mutations (23,42,43). Based on the enzymatic mechanism of DNA (5-cytosine) methyltransferases, it was proposed that the methyltransferase of Neurospora might catalyze deamination of cytosine or methylcytosine under special conditions, e.g., low SAM concentration (42). Support for this idea has recently come from observations that bacterial DNA methyltransferases can catalyze cytosine deamination when SAM is limited (44,45). Enzymatic deamination was not detected when the SAM concentration was above ~300 nM (*Hpa*II methylase) (44) or 250 nM (*Eco*RII methylase) (45). Since the K_m values for SAM of bacterial DNA methyltransferases are considerably lower than the eukaryotic enzymes (46–48), eukaryotic MTases may catalyze cytosine deamination at higher concentrations of SAM. While it is unlikely that the Neurospora DNA methyltransferase catalyzes cytosine deamination to any significant extent in vegetative tissue under normal conditions, our findings raise the possibility that it may do so under conditions of starvation for SAM (e.g., in *met* mutants growing on limiting methionine). It would be interesting to know the SAM concentration in the tissue in which RIP occurs, but due to difficulty in getting sufficient quantities of the pure tissue, we did not attempt to determine this.

It is likely that reduction of the intracellular SAM pool in animal cells causes decreased DNA methylation. Culp and Black (49) found that starvation of mouse 3T3 cells for methionine resulted in a 30–40% decrease in total 5-methylcytosine in newly synthesized DNA. It is interesting in this regard that feeding animals a diet low in the precursors of SAM, i.e., folate and methionine, results in increased malignancies and, in some cases, hypomethylated DNA (50,51). The methyltransferase in these cells may be catalyzing deamination deoxycytosine residues under these conditions.

This work grew from a small survey of temperature-sensitive mutants of Neurospora to identify possible essential genes involved in DNA methylation. With the characterization of the *dim-2* mutant, which is devoid of detectable methylation in vegetative tissue (10), it became virtually certain that methylation is not essential for viability. It remains possible, however, that essential gene products are involved in DNA methylation in Neurospora. A better understanding of key components in this process, such as the DNA methyltransferase, and the relationship of DNA methylation to essential cellular processes, such as DNA replication and transcription, should clarify this issue.

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REFERENCES

1 Adams, R. L. P. and Burdon, R. H. (1985) Molecular Biology of DNA Methylation. In Rich, A. (ed.) *Springer Series in Molecular Biology*, Springer-Verlag, New York.
2 Bird, A. (1992) *Cell*, **70,** 5–8.
3 Bird, A. P. (1986) *Nature*, **321,** 209–213.
4 Boyes, J. and Bird, A. (1991) *Cell*, **64,** 1123–1134.
5 Eden, S. and Cedar, H. (1994) *Curr. Opin. Genet. Dev.*, **4,** 255–259.
6 Barry, C., Faugeron, G. and Rossignol, J.-L. (1993) *Proc. Natl Acad. Sci. USA*, **90,** 4557–4561.
7 Li, E., Bestor, T. H. and Jaenisch, R. (1992) *Cell*, **69,** 915–926.
8 Vongs, A., Kakutani, T., Martienssen, R. A. and Richards, E. J. (1993) *Science*, **260,** 1926–1928.
9 Kakutani, T., Jeddeloh, J. A. and Richards, E. J. (1995) *Nucleic Acid Res.*, **23,** 130–137.
10 Foss, H. M., Roberts, C. J., Claeys, K. M. and Selker, E. U. (1993) *Science*, **262,** 1737–1741.
11 Russell, P. J., Rodland, K. D., Rachlin, E. M. and McCloskey, J. A. (1987) *J. Bacteriol.*, **169,** 2902–2905.
12 Selker, E. U. and Stevens, J. N. (1985) *Proc. Natl Acad. Sci. USA*, **82,** 8114–8118.
13 Selker, E. U., Fritz, D. Y. and Singer, M. J. (1993) *Science*, **262,** 1724–1728.
14 Selker, E. U., Jensen, B. C. and Richardson, G. A. (1987) *Science*, **238,** 48–53.
15 Selker, E. U. and Stevens, J. N. (1987) *Mol. Cell. Biol.*, **7,** 1032–1038.
16 Metzenberg, R. L., Stevens, J. N., Selker, E. U. and Morzycka-Wroblewska, E. (1985) *Proc. Natl Acad. Sci. USA*, **82,** 2067–2071.
17 Perkins, D. D., Metzenberg, R. L., Raju, N. B., Selker, E. U. and Barry, E. G. (1986) *Genetics*, **114,** 791–817.
18 Russell, P. J., Rodland, K. D., Cutler, J. E., Rachlin, E. M. and McClosley, J. A. (1985) In Timberlake, W. (ed.), *Molecular Genetics of Filamentous Fungi*. Alan Liss, Inc., pp. 321–332.
19 Jacobson, E. S., Chen, G. S. and Metzenberg, R. L. (1977) *J. Bacteriol.*, **132,** 747–748.
20 Perkins, D. D., Radford, A., Newmeyer, D. and Björkman, M. (1982) *Microbiol. Rev.*, **46,** 426–570.
21 Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J. and Wilson, G. G. (1994) *Nucleic Acids Res.*, **22,** 1–10.
22 Davis, R. H. and De Serres, F. J. (1970) *Meth. Enzymol.*, **17,** 47–143.
23 Selker, E. U., Cambareri, E. B., Jensen, B. C. and Haack, K. R. (1987) *Cell*, **51,** 741–752.
24 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
25 Yu, P. H. (1978) *Anal. Biochem.*, **86,** 498–504.
26 Yu, P. H. (1983) *Methods Enzymol.*, **94,** 66–69.
27 Stadler, D. (1981) *Neurospora Newsletter*, **28,** 18–19.
28 Harold, F. M. (1960) *Biochim. Biophys. Acta*, **45,** 172–188.
29 Burton, E. G. and Metzenberg, R. L. (1975) *Arch. Biochem. Biophys.*, **168,** 219–229.
30 Kerr, D. S. and Flavin, M. (1970) *J. Biol. Chem.*, **245,** 1842–1844.

31 Greene, R. C., Hunter, J. S. V. and Coch, E. H. (1973) *J. Bacteriol.*, **115,** 57–67.
32 Schwencke, J. and De Robichon Szulmajster, H. (1976) *Eur. J. Biochem.*, **65,** 49–60.
33 Koujima, I., Hayashi, H., Tomochika, K., Okabe, A. and Kanemasa, Y. (1978) *Appl. Environ. Microbiol.*, **35,** 467–470.
34 Hoffman, D., Kumar, A. M., Spitzer, A. and Gupta, R. K. (1986) *Bioch. Biophys. Acta*, **889,** 355–360.
35 Wheatley, D. N., Inglis, M. S., Foster, M. A. and Rimington, J. E. (1987) *J. Cell Science*, **88,** 13–23.
36 Roy, P. H. and Weissbach, A. (1975) *Nucleic Acids Res.*, **2,** 1669–1684.
37 Theiss, G., Schleicher, R., Schimpff-Weiland, G. and Follmann, H. (1987) *Eur. J. Biochem.*, **167,** 89–96.
38 Giordano, M., Mattachini, M. E., Cella, R. and Pedrali-Noy, G. (1991) *Biochem. Biophys. Res. Comm.*, **177,** 711–719.
39 Razin, A., Cedar, H. and Riggs, A. D. (eds) (1984) *DNA Methylation: Biochemistry and Biological Significance*. Springer-Verlag, New York.
40 Evans, H. H. and Evans, T. E. (1970) *J. Biol. Chem.*, **245,** 6436–6441.
41 Evans, H. H., Evans, T. E. and Littman, S. (1973) *J. Mol. Biol.*, **74,** 563–572.
42 Selker, E. U. (1990) *Ann. Rev. Genet.*, **24,** 579–613.
43 Selker, E. U. and Garrett, P. W. (1988) *Proc. Natl Acad. Sci. USA*, **85,** 6870–6874.
44 Shen, J.-C., Rideout, W. M., III and Jones, P. A. (1992) *Cell*, **71,** 1073–1080.
45 Wyszynski, M., Gabbara, S. and Bhagwat, A. S. (1994) *Proc. Natl Acad. Sci. USA*, **91,** 1574–1578.
46 Rubin, R. A. and Modrich, P. (1977) *J. Biol. Chem.*, **252,** 7265–7272.
47 Günthert, U., Gentsch, S. and Freund, M. (1981) *J. Biol. Chem.*, **256,** 9346–9351.
48 Wu, J. C. and Santi, D. V. (1987) *J. Biol. Chem.*, **262,** 4778–4786.
49 Culp, L. A. and Black, P. H. (1971) *Biochim Biophys Acta*, **247,** 220–232.
50 Wainfan, E., Dizik, M., Hluboky, M. and Balis, M. E. (1989) *Cancer Res.*, **49,** 4094–4097.
51 Hoffman, R. M. (1990) *Bioessays*, **12,** 163–166.
52 Nelson, M., Raschke, E. and McClelland, M. (1993) *Nucleic Acids Res.*, **21,** 3139–3154.