AGCTTATCCACATGACGTTTC-3′ for R2; and 5′-TGGAATTCATATGGATGATAATG-3′ and 5′-ATAAGCTTATCCACATGACGTTTC-3′ for R3. The PCR products were digested with Eco RI and Hind III, and then inserted in pGSTag.
22. K. J. Smith *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2846 (1993).
23. M. Takeichi, *Science* **251**, 1451 (1991); B. Geiger and O. Ayalon, *Annu. Rev. Cell Biol.* **8**, 307 (1992).
24. A. Nagafuchi and M. Takeichi, *Cell Regul.* 1, 37 (1989); S. Hirano *et al.*, *Cell* **70**, 293 (1992); M. Ozawa, M. Ringwald, R. Kemler, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4246 (1990).
25. S. Hirano *et al.*, *J. Cell Biol.* **105**, 2501 (1987); W. J. Nelson *et al.*, *ibid.* **110**, 349 (1990).
26. S. Tsukita *et al.*, *J. Cell Biol.* **113**, 867 (1991); N. Matsuyoshi *et al.*, *ibid.* **118**, 703 (1992); J. Behrens *et al.*, *ibid.* **120**, 757 (1993); T. Volberg *et al.*, *EMBO J.* **11**, 1733 (1992).
27. J. A. Trofatter *et al.*, *Cell* **72**, 791 (1993); G. A. Rouleau *et al.*, *Nature* **363**, 515 (1993).
28. J. Behrens *et al.*, *J. Cell Biol.* **108**, 2435 (1989); U. H. Frixen *et al.*, *ibid.* **113**, 173 (1991); K. Vleminckx *et al.*, *Cell* **66**, 107 (1991); Y. Shimoyama *et al.*, *Cancer Res.* **52**, 5770 (1992); R. A. Morton *et al.*, *ibid.* **53**, 3585 (1993).
29. E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
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Abnormal Chromosome Behavior in *Neurospora* Mutants Defective in DNA Methylation

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The function and regulation of DNA methylation in eukaryotes remain unclear. Genes affecting methylation were identified in the fungus *Neurospora crassa*. A mutation in one gene, *dim-2*, resulted in the loss of all detectable DNA methylation. Abnormal segregation of the methylation defects in crosses led to the discovery that the methylation mutants frequently generate strains with extra chromosomes or chromosomal parts. Starvation for *S*-adenosylmethionine, the presumed methyl group donor for DNA methylation, also produced aneuploidy. These results suggest that DNA methylation plays a role in the normal control of chromosome behavior.

In the DNA of many organisms including mammals, plants, and various fungi, some cytosines are modified by methylation at position 5. DNA methylation in eukaryotes has been correlated with reduced gene activity, and evidence has accumulated that methylation can inhibit gene expression (*1*). In mice, an approximately 70% reduction in DNA methylation, resulting from a mutation in the DNA methyltransferase gene, leads to death during embryogenesis (*2*). Although DNA methylation is required in mammals, it is not yet clear whether its basic function has been identified. The consequences of undermethylation in eukaryotes may be more apparent in organisms that contain methylation but do not require it.

In *Neurospora crassa*, about 1.5% of the cytosines are methylated (*3*), but the bulk of the genome appears devoid of methylation. Only three methylated regions have been characterized in any detail: the tandemly arranged ribosomal RNA genes [ribosomal DNA (rDNA)] on linkage group V (*4*), the zeta-eta (ζ-η) region on linkage group I (*5–7*), and the psi-63 (ψ_{63}) region on linkage group IV (*8*, *9*). The latter two regions are relics of RIP (repeat-induced point mutation), a process associated with the sexual phase of the life cycle of *Neurospora*, in which duplicated sequences are riddled with G:C to A:T mutations (*10*). Treatment of *Neurospora* with 5-azacytidine causes substantial reductions in methylation without drastically reducing growth, which suggests that DNA methylation is not essential in this organism (*5*). This observation and the relative paucity of methylation in *N. crassa* prompted us to hunt for *Neurospora* mutants defective in DNA methylation.

To make the mutant hunt as broad as possible, we screened for methylation defects directly by Southern (DNA) hybridization. Reduced methylation in the ψ_{63} region may be recognized by increased digestion of a Bam HI site that is normally blocked by methylation (Fig. 1, A and B) (*11*). A standard laboratory strain, N242, was mutagenized under conditions that induced mutations in a control gene, *mtr*, at a frequency of 1 to 3 out of 1000 survivors (*12*). Approximately 1250 survivors of the mutagenesis were screened, and 25 strains were identified in which methylation at the ψ_{63} Bam HI site had been reduced or eliminated. Thirteen of the putative mutants consistently showed the defects and could be propagated through crosses. Two strains are illustrated (Fig. 1B). Complementation tests performed on pairs of mutants grown as forced heterokaryons revealed that our collection includes mutations in at least three genes important for DNA methylation. An example of complementation between two mutants, *dim-2* and *dim-3* (defective in DNA methylation), is shown in Fig. 1C. The *dim-1* mutant was isolated in a separate mutant screen (*13*). None of the three mutations could always be completely complemented (Fig. 1C) even in control heterokaryons between mutant and wild-type strains, which suggests that the defects are partially dominant (*14*).

No eukaryotic mutants have been described in which DNA methylation is completely abolished. The mouse mutation generated by disruption of the DNA methyltransferase gene (*2*) causes overall reduction, but not elimination, of methylation, whereas a mutation in cultured CHO cells appears to affect only a few sites (*15*). Mutants isolated in *Arabidopsis* show reduced methylation at many, but not all, chromosomal regions (*16*). To further characterize our mutants, we assayed their effects on a second normally methylated region, ζ-η. All of the mutants showed a methylation deficiency in this region. The *dim-3* mutant was typical in showing reduced methylation at both of the diagnostic Bam HI sites in the ζ-η region (Fig. 1, A and D). Another mutant, *dim-2*, appeared devoid of methylation at ψ_{63} and ζ-η (Fig. 1, A, B, and D) and was chosen for further analysis. It showed no methylation at the rDNA region (Fig. 1E) nor at six other chromosomal regions that are methylated in wild-type strains (*17*). The absence of methylation detectable by Southern analysis in a total of nine normally methylated regions suggested that *dim-2* abolishes methylation throughout the genome and distinguishes *dim-2* from other known mutations affecting methylation in eukaryotes.

To test whether the *dim-2* mutation abolishes methylation throughout the genome, we measured the total 5-methylcytosine (5mC) content in DNA from *dim-2* and wild-type strains by high-performance liquid chromatography (HPLC) (Fig. 2). Approximately 1.5% of the cytosines were methylated in the wild-type strain, which is consistent with previous determinations (*3*). No 5mC was detected in DNA from the *dim-2* strain (Fig. 2). Analysis of deoxynucleoside samples from *dim-2* DNA mixed with decreasing amounts of pure 5-methyldeoxycytidine indicated that 5mC amounts corresponding to ≥5% of those found in wild-type *Neurospora* would have been detected. The apparent lack of DNA methylation in *dim-2* strains suggests that DNA methylation is not essential for

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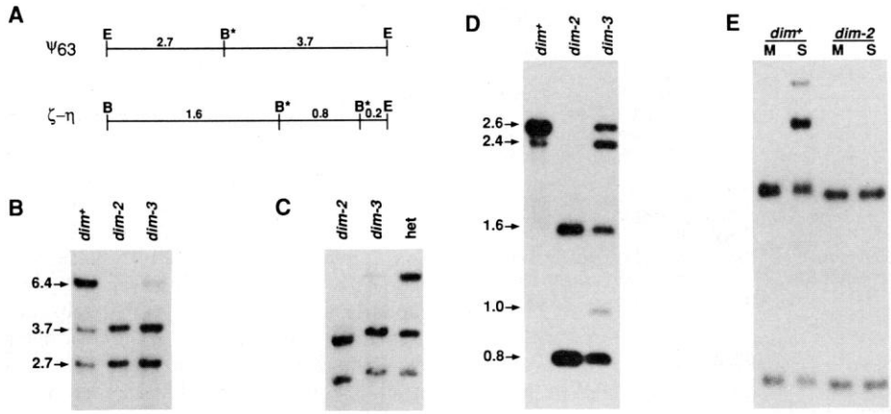

Fig. 1. Methylation deficiencies induced by mutations in *Neurospora*. (**A**) Maps of the ψ_{63} and ζ-η regions, showing Eco RI (E) and Bam HI (B) sites (*5–9*). Sites marked by asterisks display methylation in methylation-proficient strains. (**B**) Assay for methylation of the Bam HI site of ψ_{63} in wild-type (N150), *dim-2* (N535), or *dim-3* (N590) strains. DNA samples were digested with 5mC-sensitive enzymes Eco RI and Bam HI and probed with the plasmid pJS63, which contains the 6.4-kb Eco RI fragment of ψ_{63} (*8, 11*). (**C**) Complementation of the methylation defects caused by *dim-2* (N538; left) and *dim-3* (N590; center) in a heterokaryon (het.) of *dim-2* and *dim-3* (N538/N590; right) as assayed at the Bam HI site of ψ_{63}. (**D**) Assay for methylation at two Bam HI sites of ζ-η in wild-type (N150), *dim-2* (N535), or *dim-3* (N590) strains. DNA samples were digested with Eco RI and Bam HI and hybridized with the 0.8-kb Bam HI fragment of the ζ-η region. This probe detects both the 0.8- and 1.6-kb Bam HI fragments because the ζ-η region is a diverged tandem duplication (*5*). (**E**) Assay for methylation in the rDNA spacer region of wild-type (N150) or *dim-2* (N535) strains. DNA samples isolated from each strain were digested with Mbo I (M), which cuts GATC and GAT^{m}C, or with Sau 3A1 (S), which cuts GATC but not GAT^{m}C. The digests were probed with a 3.4-kb fragment containing most of the rDNA spacer region (*11*).

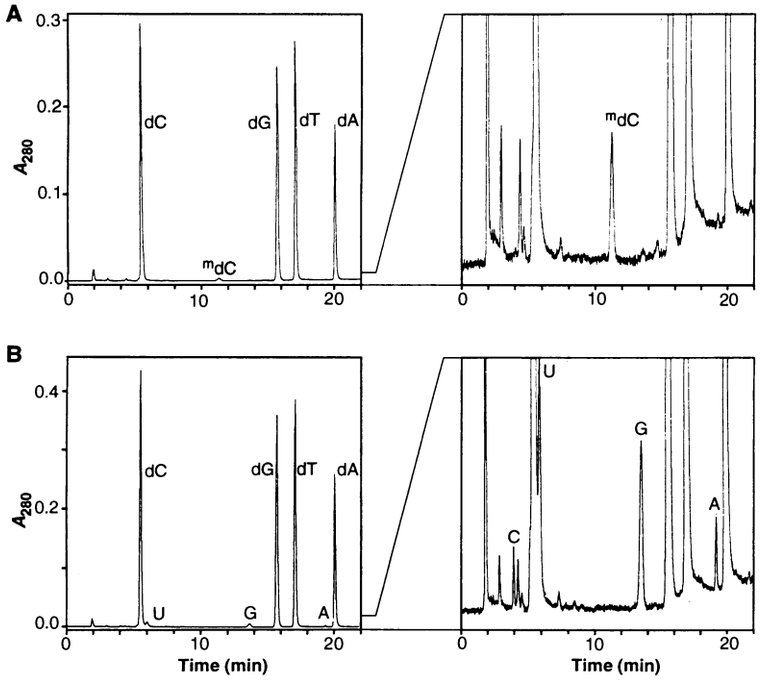

Fig. 2. High-performance liquid chromatography analysis of DNA in wild-type or *dim-2* strains. Deoxynucleosides were prepared from DNA purified in CsCl gradients and digested with P1 nuclease and bacterial alkaline phosphatase, as described by Cock *et al.* (*37*). The chromatograms on the left show results for the *dim*$^+$ (N150) (**A**) and *dim-2* (N535) (**B**) strains; 50-fold expansions of the *y* axis are shown on the right. The *dim-2* profile reveals contaminating ribonucleosides that are a result of incomplete digestion with ribonuclease during the preparation of the genomic DNA. Peak identities were determined by the ratio of absorbance at 280 and 260 nm (A_{280}/A_{260}) and by comparisons to the retention times of standards. Abbreviations: dC, deoxycytidine; ^{m}dC, 5-methyldeoxycytidine; dG, deoxyguanosine; dT, deoxythymidine; dA, deoxyadenosine; C, cytidine; U, uridine; G, guanosine; and A, adenosine.

growth of *Neurospora*. It is unknown if the loss of DNA methylation will be lethal to mammalian cells in culture.

Initially, the *dim* mutants appeared to share no phenotype beyond variable reduction in vigor and fertility and subtle changes in growth morphology. A common phenotype was revealed only when the putative mutations were tested for heritability in crosses that also served to remove possible extraneous mutations and introduce markers. Although methylation levels in progeny from crosses between *dim* and *dim*$^+$ strains typically resembled those of either parent, some progeny strains showed intermediate levels of methylation (Fig. 3). Phenotypic lag could not account for the intermediate methylation phenotype (Dim^{int}) because little change in methylation was found in a *dim-2* derivative passed through five serial transfers (*18*). Analysis of the ζ-η region revealed DNA molecules with methylation at only one or the other of the normally methylated Bam HI sites (Fig. 3). Thus, the intermediate methylation did not reflect a mixture of nuclei, some with wild type and others with mutant levels of methylation. The frequencies of the Dim^{int} phenotypes varied among progeny from different mutant isolates and even among crosses involving the same mutant. For crosses involving *dim-2* or *dim-3*, the frequency of progeny with intermediate methylation levels was typically 2 to 20%.

We postulated that intermediate methylation levels in Dim^{int} strains might result from simultaneous expression of *dim*$^+$ and *dim*$^-$ alleles. Defects in DNA methylation might lead to production of spores with alleles from both parents. *Neurospora* is normally haploid, and aneuploids normally arise only rarely (~1%) (*19*). Unstable aneuploidy, occurring spontaneously or as a result of some meiotic mutations (*20–22*), may be easily recognized in strains heterozygous for certain morphology markers. For example, aneuploids heterozygous for an albino (*al*) gene appear mosaic if the breakdown products, which are initially heterokaryotic, assort into homokaryotic sectors bearing orange or white conidia (vegetative spores); stable *al*$^+$/*al*$^-$ disomics or heterokaryons bear orange conidia only. Aneuploidy, whether stable or unstable, would also be detectable in the DNA of strains heterozygous for a restriction fragment length polymorphism (RFLP).

If the strains with intermediate methylation reflect heterozygosity for *dim*, then even a small sample of *dim*-derived ascospores (sexual spores) might yield evidence of aneuploidy. Thus, we examined progeny from a cross (N114 × N510) (Fig. 4) heterozygous for *dim-2*, *al-2*, *mt*, and the RFLP marker *mt-flank* (*11*). Strains N114 and N510 were also potentially polymor-

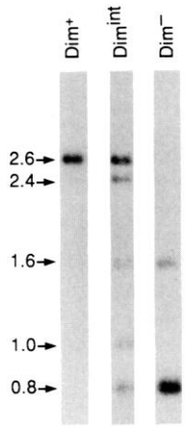

Fig. 3. Intermediate methylation in progeny from a *dim-2* × *dim*$^+$ cross. DNA samples from Dim$^+$ (N285), Dim$^-$ (N614), or Dim^{int} (N595) strains were assayed for methylation at the Bam HI sites of the ζ-η region as described (Fig. 1D).

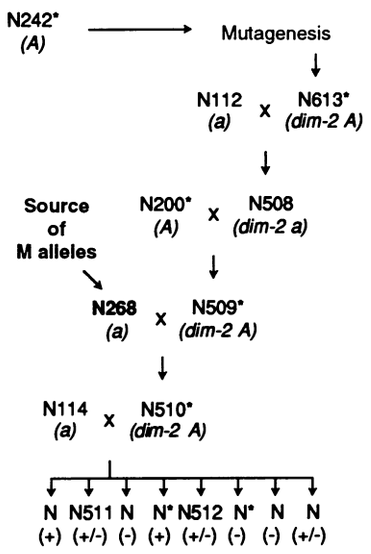

Fig. 4. Lineage of *dim-2* and Dim^{int} strains described here. Mauriceville (M) alleles, introduced by means of strain N268, provided RFLP markers used in the detection of aneuploidy. Mating types are indicated as *A* or *a*; methylation levels are indicated as wild-type (+), mutant (−), or intermediate (+/−); Al$^-$ strains are marked with an asterisk.

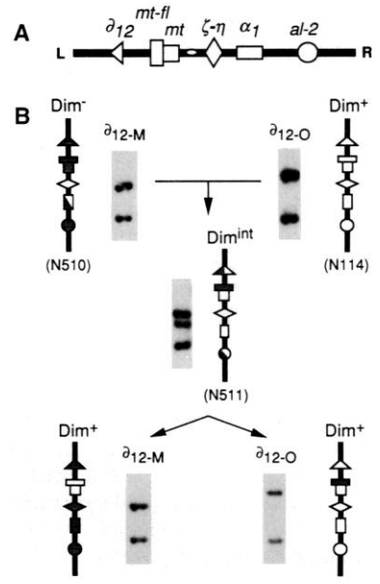

Fig. 5. Transmission of genetic markers on linkage group I (LG I) through a cross heterozygous for *dim-2*. (**A**) Partial map of left (L) and right (R) arms of LG I with a key to the symbols for loci analyzed. (**B**) Pedigree for LG I of mosaic strain N511, including its parents and its vegetative derivatives. RFLP phenotypes for δ_{12} are illustrated. Open symbols indicate O alleles of δ_{12}, ζ-η, and α_1, *a* parental alleles of *mt* and *mt-flank* (*mt-fl*), and the wild-type allele of *al-2*; filled symbols indicate M alleles of δ_{12}, ζ-η, and α_1, *A* parental alleles of *mt* and *mt-fl*, and mutant (albino) allele of *al-2*; half-filled symbols indicate mosaic phenotypes (*11*). At the α_1 locus of strain N510, the M allele was evident in DNA isolated from a secondary culture, but not in the primary culture. Top tier: LG I of Dim$^-$ parent N510 and Dim$^+$ parent N114; center tier: LG I of Dim^{int} strain N511; bottom tier: LG I representative of ten Al$^-$ clones (left) and of three Al$^+$ and two Al$^+$/Al$^-$ mosaic clones (right).

phic at other loci, including δ_{12}, ζ-η, and α_1, because the *dim-2* parent was derived in part from an exotic *Neurospora* strain, Mauriceville (M), used for RFLP mapping (8) (Fig. 4). Among 23 progeny scored for methylation immediately upon germination, five were Dim^{int}. Three of these, along with two Dim$^+$ and three Dim$^-$ strains representing different combinations of Al-2 and Dim-2 phenotypes, were inspected for mosaicism at *al-2*. One of the Dim^{int} strains (N511) appeared mosaic and produced orange, white, or mosaic vegetative derivatives (Fig. 5). One other strain (N512), also Dim^{int}, produced orange and white vegetative derivatives although the original culture was not noticeably mosaic. These two Dim^{int} strains were tested for segregation of Dim$^+$ and Dim$^-$ phenotypes among the conidial isolates. Analysis of 13 vegetative derivatives from strain N511 revealed no segregation of *dim-2* and *dim-2*$^+$ alleles. Strain N512, however, yielded one Dim$^-$ clone plus ten clones that were Dim$^+$ or nearly so (*23*). These observations support the hypothesis that the Dim^{int} phenotype reflects heterozygosity for *dim* and that generation or tolerance of aneuploidy in outcrosses is a *dim* phenotype (*24*).

To look directly for aneuploidy, we examined the DNA of N511 and N512 and their vegetative derivatives for heterozygosity at five loci linked to *al-2* (Fig. 5A). The mosaic strain N511 clearly contained both the standard, Oak Ridge (O), and the M alleles at δ_{12}. Moreover, the O and M alleles had segregated among the vegetative derivatives (Fig. 5). Although at *mt* only the *a* allele was apparent, at three of the other loci (*mt-flank*, ζ-η, and α_1), both alleles had segregated among the vegetative derivatives, which confirms that the N511 ascospore had been aneuploid. The cosegregation of the Al$^-$ phenotype with the M alleles of α_1 and ζ-η in all 10 Al$^-$ vegetative clones examined (Fig. 5) provided independent evidence that for *al-2* also the mosaicism was caused by the presence of two alleles. Strain N512 was heterozygous for one of the RFLP markers, *mt-flank*, and it, too, showed segregation among its vegetative clones.

Mosaicism or heterozygosity in cultures undoubtedly represents an underestimate of aneuploidy in ascospores. Our assay recognized only aneuploids that were originally heterozygous and retained both alleles in the culture. Heterozygosity may be lost by mitotic recombination, and aneuploidy itself may be lost through segregation of homologs. In strain N511, both homozygosis and loss of aneuploidy appears to have occurred. The presence of M alleles of ζ-η and α_1 in some clones, but not in tissue grown from a large sector of the original culture, suggested that recombination had resulted in homozygosis for these loci (Fig. 5B) (*25*). Mitotic gene conversion followed by selection could also account for the absence of the *A* allele at *mt*, particularly because heterozygosity at *mt* and other loci that control vegetative incompatibility is known to inhibit growth (*26*). Loss of aneuploidy, too, appears to have occurred in strain N511 as manifested by the cosegregation of markers at five linked loci to yield two classes of vegetative clones (Fig. 5). That these loci span approximately two-thirds of *Neurospora's* longest chromosome indicates that aneuploidy can involve at least a large portion of a chromosome.

The observation that mutations in several genes required for DNA methylation can cause the Dim^{int} phenotype suggested that DNA methylation is required for normal chromosome behavior. It remained possible, however, that all of the mutants had a defect that affected both methylation and chromosome behavior. We therefore wished to assay chromosome behavior in *dim*$^+$ strains under conditions that reduce methylation. The drug 5-azacytidine can be used to reduce DNA methylation in *Neurospora* (*5*, *27*), but it has pleiotropic effects and exact concentrations are impossible to control during the course of crosses. It was important therefore to explore other means of reducing methylation in *dim*$^+$ strains. We used the *eth-1* mutation to investigate whether methylation levels would drop in response to reductions in cellular concentrations of S-adenosylmethionine (SAM), the methyl group donor for cytosine methylation in bacteria and mammals and presumably in *Neurospora* as well. The *eth-1* mutation results in the production of a

SAM synthetase that is labile at high temperatures (28). Strains carrying *eth-1* grow well at low temperatures but fail to grow at 37°C, presumably because of starvation for SAM. We observed decreased DNA methylation in an *eth-1* strain grown at elevated temperatures (Fig. 6). This result implicates SAM as the methyl group donor for cytosine methylation in *Neurospora* and suggested a means for inducing methylation deficiencies in *dim*$^+$ strains.

The *eth-1* mutation could not be used directly to test the effects of reduced methylation on chromosome behavior because *Neurospora* is infertile at 30°C and above. We therefore tested whether mutations in the biosynthetic pathway leading to methionine would also reduce DNA methylation and found that methionine deprivation resulted in reduced methylation in several methionine auxotrophs tested (29). This is illustrated at ψ_{63} with a *met-7* strain (Fig. 6). The conditional methylation defects, like the methionine auxotrophies, were recessive in a heterokaryon. Methionine starvation did not affect methylation in other auxotrophs (29). For example, a *cys-10* mutant, which lacks sulfite reductase and can be complemented by methionine, cysteine, cystathionine, or homocysteine, displayed normal methylation when starved for methionine. A histidine auxotroph, *his-3*, also showed normal methylation when starved for histidine. Thus, interference with SAM biosynthesis, rather than with amino acid synthesis in general, appears to cause methylation deficiencies. Reduced methylation in response to limited methionine has also been reported for cultured mammalian cells (30). In *dim-1*, *dim-2*, or *dim-3* strains, which are prototrophs, DNA methylation was unaffected by excess methionine. We conclude that several gene products besides those required to synthesize SAM are needed for DNA methylation in *Neurospora*.

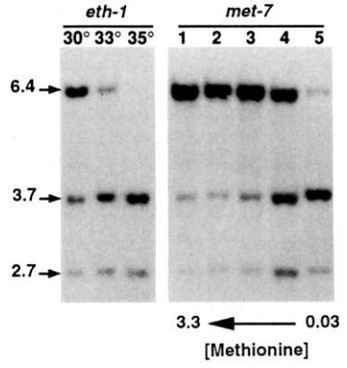

Fig. 6. Conditional methylation in mutants deficient in SAM production. Methylation was assayed at the Bam HI site in the ψ_{63} region (Fig. 1B) of an *eth-1* strain (N558, FGSC 1220) grown at 30°, 33°, or 35°C and in a *met-7* strain (N556, FGSC 3915; defective in cystathionine-γ-synthase) grown at several limiting concentrations of methionine (lane 1, 3.3 mM; lane 2, 1 mM; lane 3, 0.33 mM; lane 4, 0.1 mM; and lane 5, 0.03 mM).

The ability to control DNA methylation levels in methionine auxotrophs enabled us to investigate whether methylation affects chromosome behavior in *dim*$^+$ strains. Scoring Al$^+$/Al$^-$ mosaicism as a measure of heterozygosity for *al-2*, we analyzed crosses between *al-2 met-7* and *al-2*$^+$ *met-7* strains on crossing media supplemented with various amounts of methionine (Table 1). Crosses heterozygous for *met-7* or homozygous for *cys-10* served to control for factors unrelated to DNA methylation. Crosses between two *met-7* parents performed with very low concentrations of methionine (0.03 or 0.07 mM) yielded a large fraction of mosaic progeny (~29%). A similar cross performed on 1 mM methionine, a concentration that did not appear to affect methylation in *met-7* tissue grown vegetatively (Fig. 6), yielded a smaller fraction of mosaic progeny (11%). In these crosses, as in many that involve *dim-2* (13), a considerable fraction of the progeny produced many perithecia (fruiting bodies) or pseudoperithecia, which suggests the presence of both mating type alleles. The incidence of heterozygosity could not be tested at methionine concentrations greater than 1 mM because nitrogen deprivation is a requirement for fertility in *Neurospora*. When one parent was *met*$^+$, however, no cultures with mosaic phenotypes were recovered. Moreover, a cross homozygous for *cys-10* yielded no evidence of mosaicism on 100 μM methionine (Table 1). Thus, aberrant chromosome behavior appears to be a direct consequence of methylation deficiency.

Although crosses were used for detecting the aberrant chromosome behavior, the generation of aneuploidy need not have been due to a meiotic defect. Aneuploidy could have resulted from nondisjunction or overreplication of the chromosomes (31) during meiosis or mitosis. Nuclei entering meiosis with extra chromosomes or chromosomal parts acquired during vegetative growth could account for the dominance of aberrant chromosome behavior in crosses heterozygous for *dim*. Preliminary cytological examination of meioses in *dim-2* crosses has not revealed obvious defects in chromosome pairing comparable to those seen in meiotic mutants that produce aneuploids (22, 32). Thus, the timing and nature of the defect causing aberrant chromosome behavior in *Neurospora* strains deficient in DNA methylation are unknown.

It is early to speculate about the role of DNA methylation in chromosome behavior. 5-Azacytidine, an inhibitor of DNA methylation, can prevent decondensation of chromosomes at centromeres, telomeres, and other regions rich in satellite DNA (33, 34). Whether *Neurospora* chromosomes, like those in higher eukaryotes, contain methylated sequences at the centromeres is not yet known. 5-Azacytidine has also been reported to alter DNA replication patterns and disturb the cell cycle (34, 35). Finally, 5-azacytidine has been reported to cause nondisjunction of B chromosomes in rye (36). The availability of a collection of methylation mutants in a model eukaryote will allow us to examine directly the possible ties between DNA methylation and important functions of the chromosome, including replication, segregation, and gene expression.

Table 1. Induction of heterozygosity in progeny of *met-7* strains crossed under conditions that affect DNA methylation. Crossing media were supplemented with methionine as indicated. Phenotypes for *al-2* were scored by inspection; cultures with ambiguous phenotypes were further analyzed for segregation of Al$^+$ and Al$^-$ phenotypes among vegetative derivatives. Cultures containing many perithecia or pseudoperithecia were scored as mosaic for mating type (Mt A/a). The number of mosaic cultures over the total number of progeny examined is shown in parentheses.

Cross*	Methionine concentration (mM)	Percent Al$^+$/Al$^-$ mosaics	Percent Mt A/a mosaics
met-7 al$^+$ × *met-7 al-2*	0.03	29 (24/83)	42 (35/83)
met-7 al$^+$ × *met-7 al-2*	0.07	30 (22/74)	53 (39/74)
met-7 al$^+$ × *met-7 al-2*	1	11 (6/54)	20 (11/54)
met$^+$ *al-2* × *met-7 al*$^+$	0	0 (0/105)	0 (0/105)
cys-10 al$^+$ × *cys-10 al-2*	0.1	0 (0/117)	0 (0/117)

*Strain designations: *met-7 al*$^+$ (*a*) = N556, FGSC 3915; *met-7 al-2* (*A*) = N604; *met*$^+$ *al-2* (*A*) = N242; *cys-10 al*$^+$ (*a*) = N553, FGSC 4054; and *cys-10 al-2* (*A*) = N599.

REFERENCES AND NOTES

1. A. P. Bird, *Nature* **321**, 209 (1986); E. U. Selker, *Trends Biochem. Sci.* **15**, 103 (1990); A. P. Bird, *Cell* **70**, 5 (1992).
2. E. Li, T. H. Bestor, R. Jaenisch, *Cell* **69**, 915 (1992).
3. P. J. Russell, K. D. Rodland, E. M. Rachlin, J. A. McCloskey, *J. Bacteriol.* **169**, 2902 (1987).
4. D. D. Perkins, R. L. Metzenberg, N. B. Raju, E. U. Selker, E. G. Barry, *Genetics* **114**, 791 (1986).
5. E. U. Selker and J. N. Stevens, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8114 (1985).
6. E. U. Selker, B. C. Jensen, G. A. Richardson, *Science* **238**, 48 (1987).

7. E. U. Selker and J. N. Stevens, *Mol. Cell. Biol.* **7**, 1032 (1987); W. S. Grayburn and E. U. Selker, *ibid.* **9**, 4416 (1989).
8. R. L. Metzenberg, J. N. Stevens, E. U. Selker, E. Morzycka-Wroblewska, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2067 (1985).
9. E. U. Selker, J. Stevens, P. Garrett-Engele, C. Garrett-Engele, D. Fritz, B. Margolin, R. Metzenberg, unpublished material.
10. E. U. Selker, *Annu. Rev. Genet.* **24**, 579 (1990).
11. Methods for culturing *Neurospora* and performing genetic analyses were standard [R. H. Davis and F. J. De Serres, *Methods Enzymol.* **17A**, 47 (1970)]. For Southern analyses, DNA samples were isolated as described [H. Foss and E. Selker, *Fungal Genet. Newsl.* **36**, 82 (1989)]. Restriction enzymes were used in buffers as suggested by the manufacturers. Genomic DNA was digested with 5 U of enzyme per 0.5 μg of sample. Restriction digests were fractionated by electrophoresis in agarose gels, transferred to Zetabind (Cuno Inc., Meriden, CT) nylon membranes by the method of Southern [E. Southern, *J. Mol. Biol.* **98**, 503 (1975)], and hybridized with radioactive probes as described (*6*). The plasmid pJS63 (*8*) was used to probe the ψ_{63} region in genomic DNA digested with Eco RI and Bam HI. Other probes used in this study include the 0.8-kb Bam HI fragment of pES174, to probe the ζ-η region (*6*); the 3.4-kb Eco RI fragment of the plasmid pKH4 (K. Haack and E. U. Selker, unpublished results), to probe the rDNA spacer region; the plasmids pJS12 and pJS1, to probe the δ_{12} and α_1 regions, respectively (*8*); and the 0.8-kb and ~11-kb Bam HI fragments of plasmid pCSN15, to probe the mating type *a*-specific sequence and its flanking sequence (*mt-flank*), respectively [C. Staben and C. Yanofsky, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4917 (1990)]. Of the RFLP markers, only ζ-η is normally methylated, and it is distinguishable from its unmethylated M allele whether or not it displays methylation. Nylon membranes were routinely reprobed after stripping with 0.4 M NaOH (42°C for 30 min) and neutralization in 0.1× saline sodium citrate [15 mM sodium chloride, 1.5 mM sodium citrate·$2H_2O$ (pH 7)], 0.5% SDS, and 0.2 M tris (pH 7.5). All conclusions are based on digests that were controlled for completeness of digestion by hybridization with labeled fragments of genes that are not methylated.
12. Vegetative spores from strain N242 [equivalent to the Oak Ridge wild-type strain (N150, FGSC 2489) except for a mutation in *al-2*] were shaken in a buffered solution of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine [MNNG: 80 mM MNNG in 0.12 M KH_2PO_4 and 0.06 M Na_2HPO_4 (pH 7)] for 3.5 or 4 hours at room temperature in the dark. The spores were washed in 0.1% $Na_2S_2O_3$ and assayed for survival (20 to 35% at this dose) and for resistance to *p*-fluorophenylalanine, a measure of mutation in the *mtr* gene, to test the effectiveness of the mutagenesis.
13. H. M. Foss and E. U. Selker, unpublished material.
14. It is possible that the intermediate levels of methylation are due to uneven distribution of nuclei in the heterokaryon. If the *dim*$^+$ product is barely sufficient for normal methylation in wild-type *Neurospora* strains, a regional preponderance of *dim*$^-$ nuclei may cause the heterokaryon to be hypomethylated.
15. F. Gounari, G. R. Banks, K. Khazaie, P. A. Jeggo, R. Holliday, *Genes Dev.* 1, 899 (1987).
16. A. Vongs, T. Kakutani, R. A. Martienssen, E. J. Richards, *Science* **260**, 1926 (1993).
17. E. U. Selker, unpublished material.
18. Methylation in Dim^{int} progeny from a *dim-2* strain was assayed before and after vegetative propagation by means of vegetative spores (conidia) that were transferred in bulk and allowed to grow on fresh medium. A conservative estimate of the number of mass doublings per transfer is five.
19. T. H. Pittenger, *Genetics* **39**, 326 (1954); D. A. Smith, *ibid.* **76**, 1 (1974); S. F. H. Threlkeld and J. M. Stoltz, *Genet. Res.* **16**, 29 (1970).
20. D. D. Perkins, A. Radford, D. Newmeyer, M. Björkman, *Microbiol. Rev.* **46**, 426 (1982).
21. D. A. Smith, *Genetics* **80**, 125 (1975).
22. A. L. Schroeder and N. B. Raju, *Mol. Gen. Genet.* **231**, 41 (1991).
23. Subsequent experiments confirmed that the Dim$^+$ and Dim$^-$ phenotypes can segregate among vegetative clones from Dim^{int} strains (M. Rountree and H. Foss, unpublished results). All 13 clones from strain N511 that were assayed for methylation, including three Al$^+$, eight Al$^-$, and two mosaic cultures, were Dim$^+$ or nearly so, which suggests that *dim-2* and *al-2* are not closely linked.
24. We use the word aneuploid (and the associated modifiers, heterozygous or homozygous) to describe cultures that were derived from individual ascospores (sexual spores) containing extra chromosomes or chromosome parts, even though these cultures may no longer be aneuploid at the time of analysis.
25. Similarly, strain N510 appears to have transmitted the M alleles of ζ-η and α_1 from its Dim$^+$Al$^+$ *a* parent (N268) to strain N511 (Fig. 4) but lacked evidence of the M allele of ζ-η in its own DNA and revealed the transmitted M allele of α_1 only in a DNA sample from a secondary culture (Fig. 5). This indicates that aneuploidy can be found in both Dim$^-$ and Dim^{int} strains. Studies in progress to determine the incidence and extent of apparent aneuploidy have confirmed heterozygosity among progeny from *dim-2* strains in a variety of crosses. Heterozygosity in Dim$^+$, Dim$^-$, or Dim^{int} strains was observed at more than 10 loci representing five of the seven *Neurospora* chromosomes (H. Foss, M. Rountree, E. U. Selker, unpublished results). Among 38 progeny strains examined from one cross, one Dim^{int} strain was heterozygous for markers on three of five linkage groups scored (linkage groups I, II, and V) (*13*). These results suggest that the behavior of all chromosomes can be affected by *dim-2*.
26. D. Newmeyer and C. W. Taylor, *Genetics* **56**, 771 (1967); D. D. Perkins, *ibid.* **80**, 87 (1975).
27. J. H. Bull and J. C. Wootton, *Nature* **310**, 701 (1984).
28. E. S. Jacobson, G. S. Chen, R. L. Metzenberg, *J. Bacteriol.* **132**, 747 (1977).
29. C. J. Roberts and E. U. Selker, unpublished results.
30. L. A. Culp and P. H. Black, *Biochim. Biophys. Acta* **247**, 220 (1971).
31. D. J. Bond, *Mutat. Res.* **181**, 257 (1987).
32. A. Hagemann and E. U. Selker, unpublished material.
33. E. Viegas-Pequignot and B. Dutrillaux, *Hum. Genet.* **34**, 247 (1976).
34. T. A. Hori, *Mutat. Res.* **121**, 47 (1983).
35. D. A. Shafer and J. H. Priest, *Am. J. Hum. Genet.* **30**, 534 (1984); M. Poot, J. Koehler, P. S. Rabinovitch, H. Hoehn, J. H. Priest, *Hum. Genet.* **84**, 258 (1990).
36. N. Neves *et al.*, *Genome* **35**, 650 (1992).
37. J. M. Cock *et al.*, *J. Phycol.* **26**, 361 (1990).

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Elements of the Yeast Pheromone Response Pathway Required for Filamentous Growth of Diploids

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Transmission of an external signal from receptors to downstream targets is often mediated by a conserved set of protein kinases that act in sequence (a kinase cascade). In haploid strains of *Saccharomyces cerevisiae*, a signal initiated by peptide pheromones is transmitted through this kinase cascade to a transcription factor STE12, which is required for the expression of many mating-specific genes. Here it was shown that in diploids some of the same kinases and STE12 are required for filamentous growth, but the pheromone receptors and guanosine triphosphate–binding protein are not required for filament formation. Thus, a similar kinase cascade is activated by different signals in haploids and diploids and mediates different developmental outcomes in the two cell types.

In haploid cells of the fungus *S. cerevisiae*, extracellular peptide pheromones control the switch from vegetative growth to the sexual cycle. Each cell type secretes a unique pheromone (**a** cells secrete **a**-factor and α cells α-factor) that binds a cell type–specific receptor on cells of opposite mating type (α-factor to STE2 in *MAT***a** cells and **a**-factor to STE3 in *MAT*α cells) and thereby induces a sequence of events—arrest of cell division in G1, formation of projections, agglutination of two cells of opposite mating type, and cell fusion—that culminate in nuclear fusion (*1*). The signal initiated by the binding of pheromone to its receptor (Fig. 1) is transmitted by a heterotrimeric guanosine triphosphate–binding protein (G protein) encoded by *GPA1*, *STE4*, and *STE18* (*2*) to an ensemble of

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