Cytosine Methylation Associated With Repeat-Induced Point Mutation Causes Epigenetic Gene Silencing in *Neurospora crassa*

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ABSTRACT

Repeated DNA sequences are frequently mutated during the sexual cycle in *Neurospora crassa* by a process named repeat-induced point mutation (RIP). RIP is often associated with methylation of cytosine residues in and around the mutated sequences. Here we demonstrate that this methylation can silence a gene located in nearby, unique sequences. A large proportion of strains that had undergone RIP of a linked duplication flanking a single-copy transgene, *hph* (hygromycin B phosphotransferase), showed partial silencing of *hph*. These strains were all heavily methylated throughout the single-copy *hph* sequences and the flanking sequences. Silencing was alleviated by preventing methylation, either by 5-azacytidine (5AC) treatment or by introduction of a mutation (*eth-1*) known to reduce intracellular levels of S-adenosylmethionine. Silenced strains exhibited spontaneous reactivation of *hph* at frequencies of 10^{-4} to 0.5. Reactivated strains, as well as cells that were treated with 5AC, gave rise to cultures that were hypomethylated and partially hygromycin resistant, indicating that some of the original methylation was propagated by a maintenance mechanism. Gene expression levels were found to be variable within a population of clonally related cells, and this variation was correlated with epigenetically propagated differences in methylation patterns.

PROPER regulation of gene expression during development and differentiation is achieved by a variety of mechanisms. Some regulatory processes that cause reductions in gene expression are stable over the course of multiple cell divisions, yet are reversible. These processes do not alter the DNA sequence of the silenced gene and hence are referred to as epigenetic modifications. Mechanisms of epigenetic gene silencing that involve modifications of chromatin structure have been described in several systems. These include position effect variegation in *Drosophila melanogaster* (reviewed in WEILER and WAKIMOTO 1995) and position effects and suppression of silent mating type loci in both *Saccharomyces cerevisiae* (reviewed in LAURENSON and RINE 1992) and *Schizosaccharomyces pombe* (ALLSHIRE *et al.* 1994; GREWAL and KLAR 1996). DNA methylation at cytosine residues is associated with other epigenetic gene-silencing mechanisms, such as *X* chromosome inactivation (reviewed in RASTAN 1994) and genomic imprinting (reviewed in BARLOW 1995) in mammals. Some epigenetic silencing mechanisms may not rely on chromatin structure modifications or DNA methylation. Unknown processes that may involve post-transcriptional mechanisms have been shown to silence transgenes in various plant systems (reviewed in MATZKE and MATZKE 1995).

Recently, studies using mouse mutants with greatly reduced DNA methyltransferase levels have established a requirement for normal levels of cytosine methylation for proper development (LI *et al.* 1992), genomic imprinting (LI *et al.* 1993), and *X* inactivation (BEARD *et al.* 1995). Methylation mutants in *Arabidopsis thaliana* have been reported to have morphological abnormalities (KAKUTANI *et al.* 1995; FINNEGAN *et al.* 1996; RONEMUS *et al.* 1996). Thus it is clear that cytosine methylation can play important roles in the regulation of gene expression in these organisms. The mechanism(s) by which cytosine methylation influences gene expression are not yet fully understood, however. One barrier to gaining a more detailed understanding of how cytosine methylation inhibits gene expression is the complexity of the systems (higher plants and mammals) used to study cytosine methylation.

In the filamentous fungus *Neurospora crassa*, ~1.5% of the cytosines are methylated (RUSSELL *et al.* 1987; FOSS *et al.* 1993), and it appears that most methylated cytosines are clustered in dense patches corresponding to regions of the genome that have undergone repeat-induced point mutation (RIP; SELKER 1990; E. SELKER, B. MARGOLIN, N. TAUTAS, S. CROSS and A. BIRD, unpublished data). RIP is a process whereby duplicated sequences are littered with G:C to A:T mutations during the sexual cycle (reviewed in SELKER 1990). A sequence that has been altered by RIP is frequently heavily methylated at cytosines, even when present as a single copy in the genome (SELKER and GARRETT 1988; SINGER *et al.* 1995b). Experiments in which cytosine methylation was prevented clearly demonstrated that the mutations caused by RIP are often, but not always, sufficient to cause methylation *de novo* in vegetative cells (SELKER *et*

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al. 1987a; SINGER *et al.* 1995b). The methylation associated with RIP can inhibit transcription of genes located within the sequences that have undergone RIP (M. ROUNTREE and E. SELKER, unpublished data) and can spread well outside the boundaries of the mutated sequences (SELKER *et al.* 1993; V. MIAO and E. SELKER, unpublished data).

Epigenetic silencing involving transgenes has been observed in *N. crassa* (PANDIT and RUSSO 1992; ROMANO and MACINO 1992). Although silencing was sometimes correlated with methylation of cytosines in transgenic sequences, prevention of methylation with the nucleotide analogue 5-azacytidine (5AC) did not always prevent silencing (PANDIT and RUSSO 1992; ROMANO and MACINO 1992). Furthermore, a silenced, native homologue of the transgenic sequences did not exhibit methylation (ROMANO and MACINO 1992; COGONI *et al.* 1996), and the complete absence of cytosine methylation in a mutant strain did not affect silencing (COGONI *et al.* 1996). Thus most or all of the transgene silencing observed in Neurospora occurs by a mechanism that is independent of methylation. This mechansim may involve post- or co-transcriptional alterations in messenger RNA (mRNA) stability (COGONI *et al.* 1996).

In a previous study we observed inactivation during the sexual cycle of a single copy of *hph* (encoding hygromycin B phosphotransferase) inserted between the elements of a linked duplication of *am* (encoding the NADP-specific glutamate dehydrogenase). We showed that some of these inactivation events were due to RIP-type mutations that occurred outside the boundaries of the duplicated sequences (IRELAN *et al.* 1994). Other inactivated strains were observed to revert to the active (hygromycin-resistant) state at significant frequencies. Since cytosine methylation is often found in and around sequences that have undergone RIP and can inhibit gene expression in a reversible manner, we hypothesized that *hph* was epigenetically silenced in these strains due to the cytosine methylation associated with RIP. Here we report the results of experiments designed to test this hypothesis and to characterize this mechanism of gene silencing.

MATERIALS AND METHODS

Manipulation of *N. crassa*: Standard techniques for culturing Neurospora were carried out essentially as described by DAVIS and DE SERRES (1970), but using the crossing medium of RUSSO *et al.* (1985). For hygromycin resistance tests, conidia were suspended in sterile water, samples were counted in a hemacytometer, diluted appropriately, and either spotted in 5-μl aliquots or spread on plates containing Vogel's medium, 2% sorbose, and the appropriate supplements. Microconidia were produced by the procedure of PANDIT and MAHESHWARI (1993). Neurospora strains are described in Table 1.

5-Azacytidine treatments: Strains were grown on agar slants containing 24 μM 5AC and, just before formation of conidia (2–3 days after inoculation), they were given a second dose of the drug by adding aqueous 5AC to yield a final concentration of 96 μM. Twenty-four micromolar 5AC was used for germination of conidia in liquid medium for RNA extractions (see below). In neither case was 5AC included in the plates upon which the 5AC-treated conidia were tested, because pilot experiments indicated that this had little effect on *hph* reactivation relative to the effect of the 5AC pretreatment.

Southern analysis: DNA was purified as previously described (IRELAN *et al.* 1993), except that glass beads (240–300 μm; Sigma) were used for grinding germinated conidia in the cases where both RNA and DNA was isolated. DNA was fractionated by electrophoresis in 1.5% agarose gels, followed by depurination in 0.25 N HCl, capillary transfer in 0.4 N NaOH to Zetabind nylon membrane (Cuno), and UV crosslinking (Fisher UV Crosslinker). DNA labeling and hybridization were as described by SELKER and STEVENS (1987). Each blot was stripped and reprobed with a fragment corresponding to an unmethylated region of the genome (either *Bml* or *mtr*), to verify complete restriction enzyme digestion and comparable loading for all lanes.

Northern analysis: Approximately 10^7 conidia were germinated in 50 ml of liquid Vogel's medium for 6 hr with shaking at 34° and then split into three fractions. One was use to prepare total RNA using a procedure adapted from MCKINNEY *et al.* (1993) by M. ROUNTREE (personal communication). The culture of germinated conidia was added to a 30-ml Corex centrifuge tube filled to approximately one-third volume with crushed ice and centrifuged at 5000 rpm in a Sorvall SS34 rotor at 4°. The resulting pellet was resuspended in 1 ml of ice-cold 10 mM Tris (pH 7.6), 1 mM EDTA (TE), transferred to a microcentrifuge tube, and centrifuged for 1 min at 15,000 rpm at 4° in an Eppendorf 5402 microcentrifuge. The resulting pellet was resuspended with ~300 μl of acid-washed 240–300-μm glass beads suspended in H_2O, 350 μl of phenol:chloroform:isoamyl alcohol (25:24:1), and 350 μl of 300 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 0.2% SDS (NETS), vortexed for 10 min at top speed in a multitube vortexer (VWR), and then centrifuged for 5 min as above. The resulting aqueous phase was removed and nucleic acids were ethanol precipitated as described by MCKINNEY *et al.* (1993). Samples (15 μg) of each total RNA preparation were denatured and fractionated in 1.2% agarose gels containing MOPS buffer as described (MCKINNEY *et al.* 1993), with the exception that formaldehyde was omitted from the gels. RNA was blotted to a Zetabind nylon membrane in 0.75 M NaCl, 75 mM sodium citrate, pH 7.0 (5× SSC) and crosslinked by UV irradiation. The ribosomal RNA (rRNA) was visualized by soaking the membrane in 0.03% methylene blue, 0.3 M NaOAc pH 5.2 for 2–3 min followed by several washes in H_2O. Membranes were incubated for 1 hr at 60° in 250 mM sodium phosphate, pH 7.4; 7% SDS; 1 mM EDTA; and 5% dextran sulfate before addition of the probe DNA, which was prepared by the random hexamer method using the large fragment of *Escherichia coli* DNA polymerase I under conditions suggested by the manufacturer (USB). Hybridizations were carried out at 60° overnight, followed by three or four washes at 60° in 0.1× SSC, 0.5% SDS at 60°.

RESULTS

Reversible silencing associated with RIP: In a previous study, several independent transgenic Neurospora strains, each harboring one copy of an inverted or direct duplication of the *am* gene, separated by a single copy of *hph* (Figure 1), were crossed and the resulting progeny were tested for *am* and *hph* activity. Loss of *am* function was taken as an indication of RIP and, among strains that had suffered RIP, occasional loss of *hph*

TABLE 1

Neurospora strains

Name	Genotype	Source
N24	*am_{132} inl A*	SELKER *et al.* (1987b)
N204	*his-2 nuc-1 am_{132} inl a*	R. METZENBERG
N528	*am_{132} $(am^+/hph^+/am^+)^{ec51\ pJI1}$ his-2 nuc-1 inl a*	IRELAN *et al.* (1994)
N558	*eth-1 a*	FGSC no. 1220
N571	*am_{132} $(am^+/hph^+/am^+)^{ec53\ pJI1}$ his-2 nuc-1 inl a*	IRELAN *et al.* (1994)
N583	*am_{132} $(am^+/hph^+/am^+)^{ec28\ pJI2}$ his-2 nuc-1 inl a*	IRELAN *et al.* (1994)
N584	*am_{132} $(am^+/hph^+/am^+)^{ec42\ pJI2}$ his-2 nuc-1 inl a*	IRELAN *et al.* (1994)
N591	*am_{132} $[(am/hph/am)^{ec51\ pJI1}]^{RIP54}$ his-2 inl a*	This work
N637	*am_{132} $(am^+/hph^+/am^+)^{ec28\ pJI2}$ inl A*	IRELAN *et al.* (1994)
N638	*am_{132} $(am^+/hph^+/am^+)^{ec42\ pJI2}$ inl A*	IRELAN *et al.* (1994)
N639	*am_{132} $(am^+/hph^+/am^+)^{ec53\ pJI2}$ inl A*	IRELAN *et al.* (1994)
N644	*am_{132} $[(am/hph/am)^{ec42\ pJI2}]^{RIP77}$ inl A*	This work
N1232	*am_{132} $[(am/hph/am)^{ec42\ pJI2}]^{RIP2}$ inl*	This work
N1233	*am_{132} $[(am/hph/am)^{ec42\ pJI2}]^{RIP11}$ his-2 inl*	This work
N1234-1238	*$[(am/hph/am)^{ec42\ pJI2}]^{RIP77'}$*	This work
N1239-1243	*$[(am/hph/am)^{ec42\ pJI2}]^{RIP77'}$ eth-1*	This work

The nature and status of the transgenic DNA is indicated as follows: within the parentheses, the arrangement of genes within the transforming DNA is indicated. Outside the parentheses, the superscripted information designates transformant type (ectopic) and isolate number and the name of the plasmid that was the source of the transgenes. Outside the brackets, the superscripted information designates allele number (when due to RIP) and the number of generations since the initial RIP event is designated by the number of prime (*e.g.*, 77′) symbols. FGSC, Fungal Genetics Stock Center.

function was observed. Some of these hygromycin-sensitive strains had deleted the *hph* sequences, and some others contained mutations due to RIP in the *hph* coding sequences (IRELAN *et al.* 1994). A minority of the progeny that had undergone RIP grew weakly on hygromycin plates and/or reverted to hygromycin resistance (Hyg^R) at moderate frequencies, resulting in an occasional colony on hygromycin plates. We proposed that, in these strains, the cytosine methylation that often accompanies RIP was responsible for reduced *hph* expression.

To test the hypothesis that the partially hygromycin-resistant strains were the result of cytosine methylation, these strains were assayed for Hyg^R after treatment with the methylation inhibitor 5AC. Four partially silenced

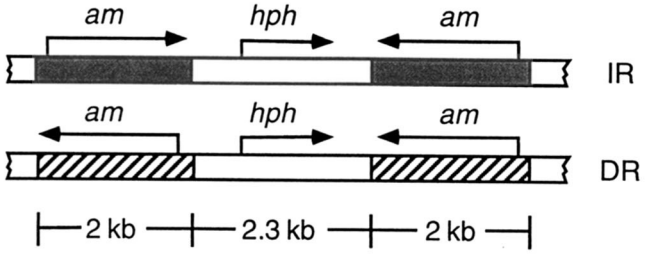

FIGURE 1.—Duplication constructs used in this study. Hatched boxes indicate duplicated sequences. The location and direction of transcripts are indicated by arrows above the boxes. The inverted duplication construct (IR) corresponds to plasmid pJI1 (present in strain N528 and derivatives) and the direct duplication construct (DR) corresponds to plasmid pJI2 (present in strain N638 and derivatives; IRELAN *et al.* 1994).

progeny strains derived from N528, which contains the inverted duplication construct (Figure 1), were tested by spotting suspensions of asexual spores (conidia) that had been grown in the presence of 5AC onto plates containing 200 μg/ml hygromycin. Each strain showed increased growth on hygromycin after 5AC treatment relative to untreated conidia; results from a representative strain N591 are shown in Figure 2. These results were consistent with the hypothesis that methylation was responsible for the silencing.

To assess the frequency of reversible silencing of *hph*, we tested progeny from three crosses that were homozygous for either the direct or the inverted, linked duplication that had not been inactivated by RIP. All progeny from these crosses that were scored as hygromycin sensitive (Hyg^S) in the previous study were treated with 5AC and tested as described above. Strains exhibiting 5AC-reversible silencing of *hph* were recovered from each cross, at frequencies of 0.7–10% (Table 2). These 5AC-reversible strains were then assessed for the frequency of spontaneous *hph* reactivation (without 5AC treatment) by plating for individual colonies on plates containing 200 μg/ml hygromycin. All 19 of the 5AC-reversible strains showed significant frequencies of spontaneous Hyg^R conidia, covering a continuous range from 10^{-5} to 10^{-2} (data not shown). A representative from cross DR5 that exhibited a low frequency of spontaneous Hyg^R conidia, strain N644, is shown in Figure 2.

All of the parental strains used in these crosses grew well on concentrations of hygromycin up to nine times higher than the level normally used (200 μg/ml; see

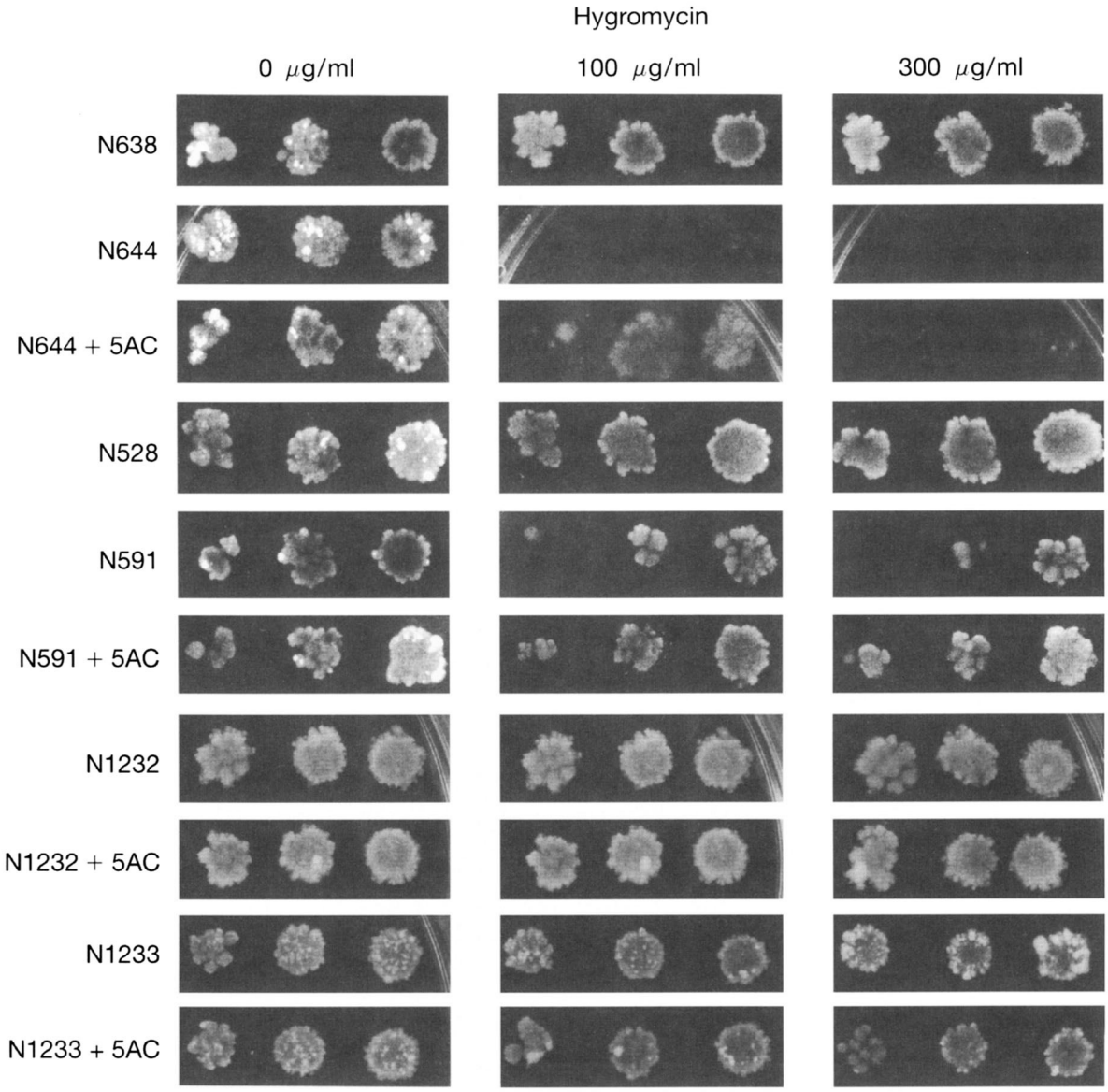

FIGURE 2.—5AC-reversible silencing of *hph*. Each panel depicts, from left to right, growth resulting from spotting 50, 500, or 5000 conidia of the indicated strain on the indicated concentration of hygromycin. Silenced strains are arranged from top to bottom in order of increasing hygromycin resistance. Strain N638 is an *hph*$^+$ parent of the silenced, direct duplication strains N644, N1232, and N1233. Strain N528 is the *hph*$^+$ parent of the silenced, inverted duplication strain N591. Strains N1232 and N1233 show no evidence of silencing at these concentrations of hygromycin.

below). Therefore, it seemed possible that strains with substantial reductions in *hph* expression might have been missed by our previous screen for resistance to standard levels of hygromycin. To explore this possibility, 10 progeny from cross DR5 that were previously scored as Hyg^R were retested by spotting conidial suspensions on a range of hygromycin concentrations (300, 600, and 1800 μg/ml). Eight of these strains showed at least a subtle increase in growth on hygromycin after 5AC treatment, suggesting that methylation associated with RIP was indeed affecting *hph* expression in the majority of strains previously scored as Hyg^R. Representative strains, N1232 and 1233, are shown in Figure 2.

To quantify the degree of silencing of *hph*, we assessed the frequency of hygromycin-resistant conidia produced with or without 5AC in the four representative silenced strains shown in Figure 2. Conidia were spread on plates containing concentrations of hygromycin that had revealed silencing in the spot test assays described above. Mean plating efficiencies, expressed as the ratio of colonies formed on hygromycin medium to colonies formed on permissive medium, varied from 1.2×10^{-4} to 0.52 for untreated conidia (Table 3). In contrast, all but one of the 5AC-treated cultures were indistinguishable from cultures of the *hph*$^+$ control strains (with plating efficiencies near 1.0). The exception, strain N644, had a plating efficiency after 5AC

TABLE 2

Frequency of epigenetic silencing associated with RIP of linked duplications

Cross	Hyg^R	Hyg^S		Revertible/ total (%)
		Nonrevertible	Revertible	
DR4 (N637 × N583)	118	20	1	0.7
DR5 (N638 × N584)	109	6	13	10.2
IR3 (N639 × N571)	73	6	5	5.9

DR and IR indicate direct and inverted repeat crosses, respectively. The frequency of revertible strains was calculated with respect to the total of the progeny tested here, which were all previously shown to have undergone RIP (as determined by loss of *am* function) and to have retained *hph* sequences (as determined by Southern analyses; IRELAN *et al.* 1994).

treatment of 0.31. This strain showed signs of mutation, presumably due to RIP, within the *hph* coding sequences (see below), and thus may contain a partially defective *hph* gene. The effect of 5AC on *hph* expression, given as the ratio of the plating efficiency of the 5AC-treated conidia to the untreated conidia, varied from two- to 2500-fold (Table 3), indicating that the degree of 5AC-reversible silencing of *hph* can vary widely from strain to strain.

Epigenetically silenced (*hph*) strains are heavily methylated at *hph*: To verify that the *hph* sequences were methylated in these strains, and that this methylation was reduced or prevented by 5AC treatment, we analyzed each of the strains shown in Figure 2 by Southern hybridization. We used the isoschizomers *DpnII* and *Sau3AI*, which recognize 14 sites in the 2.3 kilobases (kb) of single-copy sequences containing *hph* (Figure 3B) and four additional sites in each copy of the 2 kb of flanking, duplicated sequences containing *am* (see Figure 8B). *DpnII* cuts GATC sites regardless of cytosine methylation status, while *Sau3AI* cuts only sites that are not methylated at the cytosine (NELSON *et al.* 1993). All of the silenced strains tested showed heavy methylation of the GATC sites within the *hph* sequences (lanes 4, 9, 12, and 15 of Figure 3A). The majority of the fragments observed in *Sau*3AI digests of silenced strains were approximately the size of the entire duplicated region (6–8 kb; Figure 1). A similar pattern was obtained upon reprobing this blot with *am* sequences, confirming that the entire region is heavily methylated in each silenced strain (data not shown). In each case the methylation was almost completely prevented by 5AC treatment (lanes 5, 10, 13, and 16 of Figure 3A). One strain, N644, revealed loss of a restriction site within the *hph* coding sequences (site separating fragments C and D in Figure 3B), consistent with the observation of *hph* mutations due to RIP among siblings of N644 (IRELAN *et al.* 1994).

The six hph^+ parents of the crosses shown in Table 2 were all tested for methylation in the transgenes by Southern analysis; none showed evidence of methylation in *hph* or *am* sequences (lanes 2 and 7 of Figure 3A; data not shown). Three of these strains, represented by N638 (lane 7 of Figure 3A), had duplications that passed through the sexual cycle but had not undergone RIP (as assessed by *am* function; IRELAN *et al.* 1994). Thus the methylation observed in the silenced progeny was almost certainly the result of RIP triggered by the duplicated sequences and was not due to methylation of the *hph* sequences upon transformation, which has been observed in some work with *hph* (PANDIT and

TABLE 3

Frequency of *hph* reactivation in silenced strains

Strain	Hygromycin concentration	Plating efficiency		Ratio[a]
		−5AC	+5AC	
N528	200	0.84 ± 0.23	0.97 ± 0.11	1.2
N591	200	$9.2 \pm 0.64 \times 10^{-2}$	1.0 ± 0.31	11.0
N638	200	1.0 ± 0.06	0.93 ± 0.30	0.93
N644	200	$1.2 \pm 1.4 \times 10^{-4}$	0.31 ± 0.092	2.5×10^3
N584	1800	0.81	0.65	0.80
N638	1800	0.83	0.82	0.99
N1232	1800	0.52 ± 0.057	1.02 ± 0.028	2.0
N1233	600	0.22 ± 0.049	0.99 ± 0.021	4.5

Hygromcyin concentrations are expressed in μg/ml. Plating efficiencies are expressed as the mean ± SD of the ratio of colonies formed on hygromycin plates to colonies formed on permissive plates. Strain N528 is the hph^+ parent of silenced strain N591. Strains N584 and N638 are the hph^+ parents of silenced strains N644, N1232, and N1233.

[a] +5AC/−5AC.

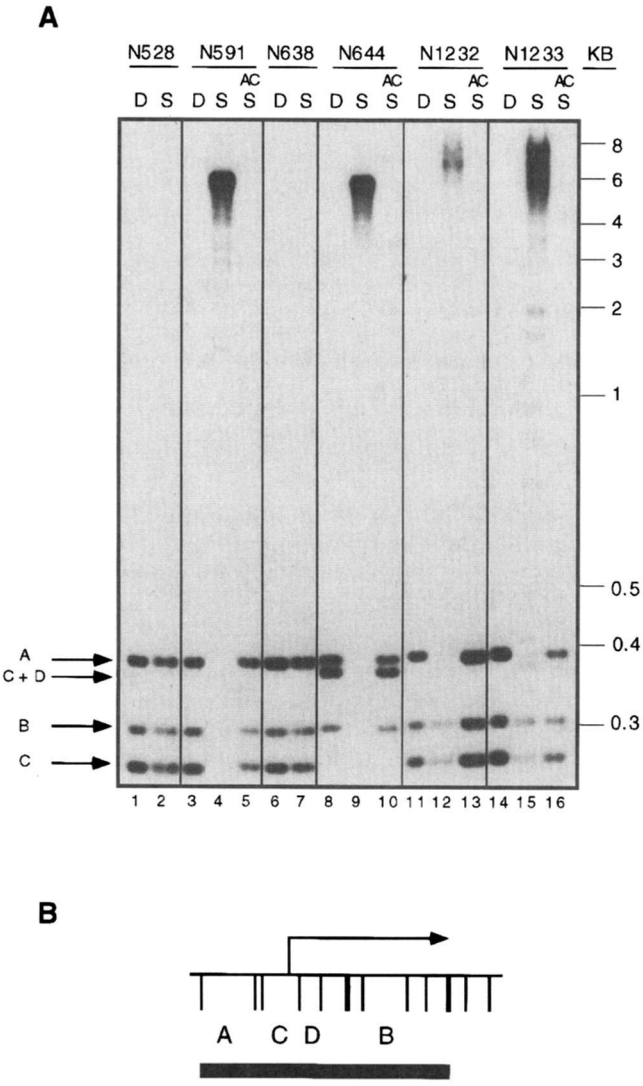

FIGURE 3.—Methylation of *hph* in reversibly silenced strains. (A) Southern analysis of genomic DNA from *hph*$^+$ parents and their reversibly silenced progeny before and after 5AC treatment. Each DNA sample was cut with *Dpn*II (D) or *Sau*3AI (S) and probed with a fragment containing *hph*. The arrows on the left indicate the identity of the fragments shown in B, and the positions of size standards (in kb) are indicated on the right. Lanes 1 and 2: *Dpn*II and *Sau*3AI digests of the inverted duplication parent strain N528. Lanes 3 and 4: *Dpn*II and *Sau*3AI digests of the silenced inverted duplication strain N591. Lane 5: *Sau*3AI digest of N591 after 5AC treatment. Lanes 6 and 7: *Dpn*II and *Sau*3AI digests of the direct duplication parent strain N638. Lanes 8 and 9: *Dpn*II and *Sau*3AI digests of the silenced direct duplication strain N644. Lane 10: *Sau*3AI digest of N644 after 5AC treatment. Lanes 11 and 12: *Dpn*II and *Sau*3AI digests of the silenced direct duplication strain N1232. Lane 13: *Sau*3AI digest of N1232 after 5AC treatment. Lanes 14 and 15: *Dpn*II and *Sau*3AI digests of the silenced direct duplication strain N1233. Lane 16: *Sau*3AI digest of N1233 after 5AC treatment. (B) *Dpn*II/*Sau*3AI restriction map of the sequences containing *hph*. The arrow above the line indicates the expected location of the *hph* transcript (CULLEN *et al.* 1987). Thin vertical bars below the line represent single GATC sites, and thick bars represent two adjacent sites. The fragments indicated by arrows in A are identified by the letters below the line. The location of the probe is indicated by the filled box.

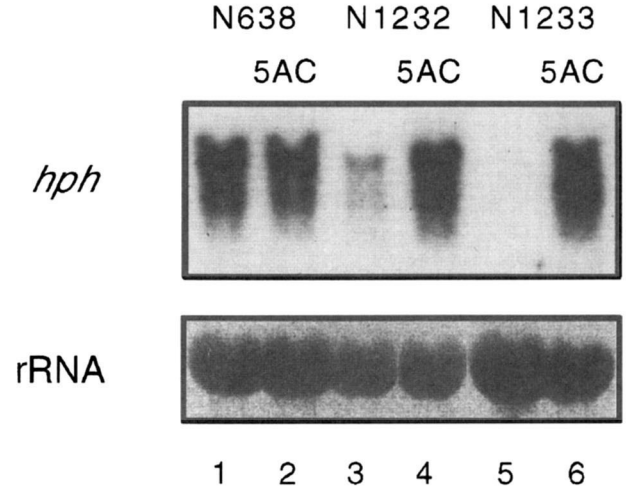

FIGURE 4.—Steady-state *hph* mRNA levels in silenced strains. A Northern blot of total RNA from an *hph*$^+$ parent (lanes 1 and 2) and its silenced progeny (lanes 3–6) before (odd lanes) and after (even lanes) 5AC treatment is shown. The top shows an autoradiogram resulting from probing total RNA with the *hph* fragment shown in Figure 3B. The bottom shows methylene blue staining of the 26S rRNA on the same gel, which served as a loading control. The *hph* construct used in these strains does not include a transcription terminator; therefore, it is possible that the two bands hybridizing to *hph* represent use of alternative termination signals in the flanking sequences.

RUSSO 1992; S. LOMMEL, B. MARGOLIN and E. SELKER, unpublished data).

Methylation prevents accumulation of mRNA: To verify that the increase in hygromycin resistance of silenced strains after 5AC treatment was due to increased expression of *hph*, we assessed the level of *hph* mRNA in silenced strains with or without 5AC treatment. Pretreated conidia were germinated in the presence or absence of 5AC and split into three fractions: one for plating assays as described above to assess silencing, one to prepare DNA for Southern analyses to assess methylation status, and one to prepare RNA for Northern analyses. Strains N1232 and N1233, which showed a subtle response to 5AC treatment in the plating assay (Table 3), showed a dramatic decrease in cytosine methylation (lanes 13 and 16 of Figure 3A) and a dramatic increase in *hph* mRNA after 5AC treatment (lanes 4 and 6 of Figure 4). Interestingly, N1232 and N1233 show little *hph* mRNA in the absence of 5AC (lanes 3 and 5 of Figure 4), even though they are quite resistant to hygromycin under these conditions (Figure 2 and Table 3). Germinated conidia from two more thoroughly silenced strains, N644 and N591, were also tested in this manner. No *hph* mRNA was detected from either strain, even using gels loaded with 50 μg of RNA (more than three times that used for Figure 4) extracted from 5AC-treated conidia that showed efficient plating on hygromycin (M. ROUNTREE, J. IRELAN and E. SELKER, unpublished data). Hygromycin B phosphotransferase enzyme assays confirmed that, even after 5AC treatment, these

TABLE 4

Frequency of *hph* reactivation in an *eth-1* background

Strain	Plating efficiency on hygromycin		
	25°	25° + 5AC	35°
Parents			
N558	$<3.0 \times 10^{-6}$	$<1.9 \times 10^{-6}$	$<4.4 \times 10^{-6}$
N644[a]	$1.2 \pm 1.4 \times 10^{-4}$	$3.1 \pm 0.092 \times 10^{-1}$	5.9×10^{-4}
eth-1$^+$ progeny			
N1234	4.1×10^{-5}	5.4×10^{-3}	1.7×10^{-5}
N1235	3.1×10^{-5}	1.4×10^{-1}	2.6×10^{-5}
N1236	4.3×10^{-5}	2.4×10^{-4}	2.2×10^{-5}
N1237	3.4×10^{-5}	4.0×10^{-2}	5.6×10^{-5}
N1238	3.6×10^{-5}	1.0×10^{-2}	2.0×10^{-5}
Mean ± SD	$3.7 \pm 0.5 \times 10^{-5}$	$4.4 \pm 5.5 \times 10^{-2}$	$2.8 \pm 1.6 \times 10^{-5}$
eth-1$^-$ progeny			
N1239	6.0×10^{-5}	2.2×10^{-2}	2.0×10^{-3}
N1240	3.5×10^{-5}	1.1×10^{-1}	6.1×10^{-2}
N1241	2.3×10^{-5}	5.1×10^{-2}	1.9×10^{-2}
N1242	2.1×10^{-5}	1.1×10^{-1}	1.7×10^{-2}
N1243	2.5×10^{-5}	2.5×10^{-2}	1.4×10^{-2}
Mean ± SD	$3.3 \pm 1.6 \times 10^{-5}$	$6.4 \pm 4.4 \times 10^{-2}$	$2.3 \pm 2.3 \times 10^{-2}$

[a] Platings of N644 at permissive temperature are from Table 1 and were conducted at 32°. Values are means ± SD.

heavily silenced strains show very little *hph* activity (M. FREITAG, J. IRELAN and M. SACHS, unpublished data). These results, together with the finding that strains N1232 and N1233 showed little *hph* mRNA but significant resistance to hygromycin in the absence of 5AC, indicate that only very low levels of hygromycin B phosphotransferase are required to confer a hygromycin-resistant phenotype.

Silencing is relieved by reducing levels of S-adenosylmethionine: 5-Azacytidine has effects on metabolism in addition to the prevention of cytosine methylation. In Neurospora, 5AC results in reduced cell viability (CAMBAREERI *et al.* 1996; J. IRELAN and E. SELKER, unpublished data) and increased mutation frequency (M. ROUNTREE and E. SELKER, unpublished data), and, in animal cells, 5AC treatment has been shown to result in alterations in heterochromatin (HAAF and SCHMID 1989). Thus it was possible that the effect of 5AC treatment on silencing involved a mechanism independent of the effect on cytosine methylation (*e.g.*, by altering chromatin structure). Therefore, we wished to test the role of cytosine methylation in *hph* silencing by preventing methylation using an independent method. We used a mutant defective in the last step in biosynthesis of S-adenosylmethionine (SAM), the presumed source of the methyl group in the DNA methyltransferase reaction. Strain N558, which contains a temperature-sensitive allele of *eth-1* (encoding SAM synthetase; JACOBSON *et al.* 1977), showed reduced levels of both SAM and cytosine methylation when grown at a semipermissive temperature (ROBERTS and SELKER 1995).

To test the effect of reductions in intracellular SAM on silencing and methylation of *hph*, strain N558 was crossed to strain N644, which contains a heavily silenced allele of *hph*. Previous crosses of strain N644 had produced a large fraction of progeny with altered hygromycin-resistance phenotypes, presumably due to the effects of further rounds of RIP on *hph* expression. To reduce this problem, we collected the very earliest spores produced from this cross, as duplicated sequences from early spores are less likely to have undergone RIP (SINGER *et al.* 1995a). Progeny strains were tested for *eth-1* by scoring growth at the nonpermissive temperature (38°) and were tested for the presence of the silenced allele of *hph* by comparing growth on hygromycin at the *eth-1*-permissive temperature (25°) before and after 5AC treatment. Five *eth-1* and five *eth-1*$^+$ control strains, all containing a silenced allele of *hph*, were then tested for hygromycin resistance after growth and conidiation at a semipermissive temperature (35°). All five *eth-1* strains showed large increases in the frequency of Hyg^R conidia after growth at 35°, while the five *eth-1*$^+$ controls showed little change (Table 4). A representative strain of each class was retested for silencing by spot assays (Figure 5) and for cytosine methylation levels by Southern analysis. The *eth-1* strain N1240 showed a dramatic reduction in *hph* methylation after growth at 35°, while the *eth-1*$^+$ control N1235 differed little from wild type (Figure 6). The small increase in hygromycin resistance (Figure 5) and reduction in methylation in the *eth-1*$^+$ strains after growth at 35° (compare band B of N644 in Figure 6 and Figure 3) is

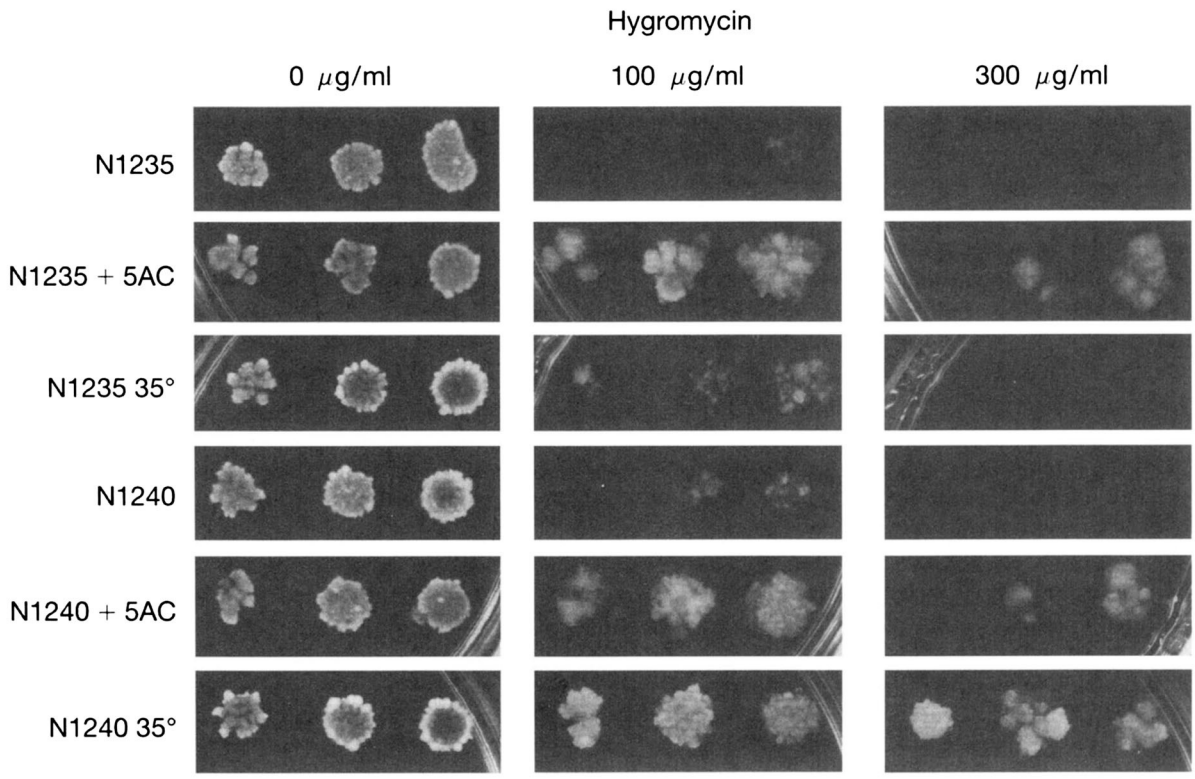

FIGURE 5.—Release of silencing in an *eth-1* strain. Each panel depicts, from left to right, growth resulting from spotting 50, 500, or 5000 conidia of the strain indicated for each concentration of hygromycin. Strain N1240 is an *eth-1* strain containing the silenced *hph* allele from strain N644. Strain N1235 is an *eth-1*$^+$ sibling of N1240, also containing the silenced *hph* allele from strain N644. Prior to plating, strains were grown at a temperature permissive for *eth-1* (25°) without 5AC (rows 1 and 4), at permissive temperature with 5AC (rows 2 and 5), or at a semipermissive temperature without 5AC (35°; rows 3 and 6).

consistent with the observation that cytosine methylation is somewhat temperature sensitive in wild-type *N. crassa* (E. SELKER and E. CAMBARERI, unpublished data).

Reestablishment of silencing in reactivated strains: The experiments described above indicate that methylation associated with RIP is responsible for silencing of *hph*. Since previous work has demonstrated that mutations by RIP often create potent *de novo* methylation signals (SELKER *et al.* 1987a; SINGER *et al.* 1995b), strains that had reactivated *hph* might be expected to become rapidly remethylated and therefore resilenced. However, some sequences that have undergone RIP are methylated, yet lack a *de novo* methylation signal, suggesting that methylation patterns established during the sexual cycle are sometimes propagated by a maintenance mechanism (SINGER *et al.* 1995b). To determine whether reactivated strains were susceptible to reestablishment of methylation and silencing, strains that had reactivated *hph*, either spontaneously or as a result of 5AC treatment, were assayed for methylation and silencing through several subsequent rounds of growth. For the 5AC experiment, ~1000 conidia from strain N644 treated with 5AC were transferred to agar slants lacking 5AC and allowed to grow and conidiate, a process involving roughly 12–14 divisions. This procedure was repeated serially for a total of four rounds of growth and conidiation on permissive medium. For the spontaneous hygromycin-resistant strains, conidia were plated on 200 μg/ml hygromycin and the resulting colonies were transferred to slants lacking hygromycin and allowed to grow to conidiation. These conidia were then used to inoculate new slants in series as described for the 5AC experiment. Conidia from each round were then plated on hygromycin to assess silencing and were used to grow mycelial tissue in liquid culture to assess methylation status.

Spot assays of N644 derivatives grown permissively after treatment with 5AC or after selection on hygromycin revealed that these strains did not become completely resilenced. For each of 10 spontaneous hygromycin-resistant colonies and in the 5AC treated culture, the derived cultures grew significantly better on hygromycin than did the original strain (representatives are shown in Figure 7). Five strains derived from colonies obtained without hygromycin selection did not differ from the original culture, indicating that the colony isolation procedure did not affect silencing (Figure 7). All of the strains derived from hygromycin-resistant colonies or the 5AC-treated culture showed somewhat less growth on 300 μg/ml hygromycin than did the original 5AC-treated culture (Figure 7). In each case, the first four transferred cultures showed no dramatic changes in expression levels relative to one another (Figure 7). Additional cultures derived from these strains through

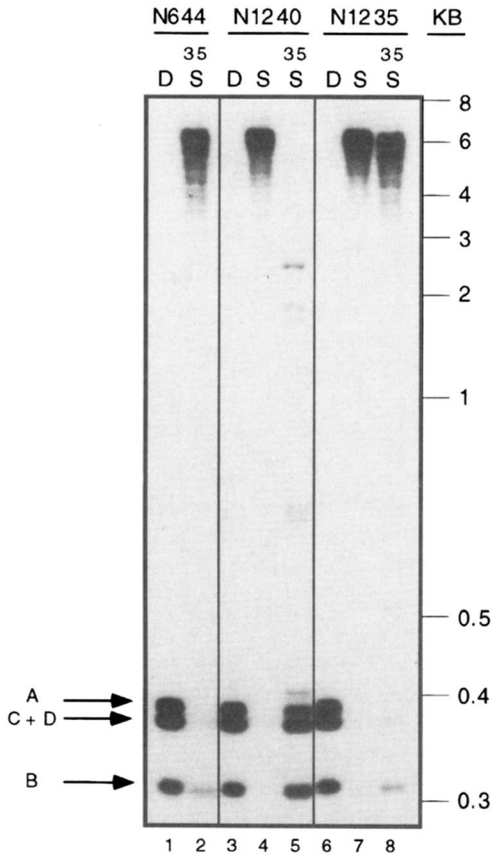

FIGURE 6.—Reduced *hph* methylation in an *eth-1* strain. DNA samples were cut with either *Dpn*II (D) or *Sau*3AI (S) and probed with *hph* as in Figure 3. The arrows, size standards, and restriction map are as in Figure 3. Lanes 1 and 2: the parent strain N644 grown at 35°. Lanes 3 and 4: the *eth-1* strain N1240 grown at 25°. Lane 5: N1240 grown at 35°. Lanes 6 and 7: the *eth-1*$^+$ control N1235 grown at 25°. Lane 8: N1235 grown at 35°.

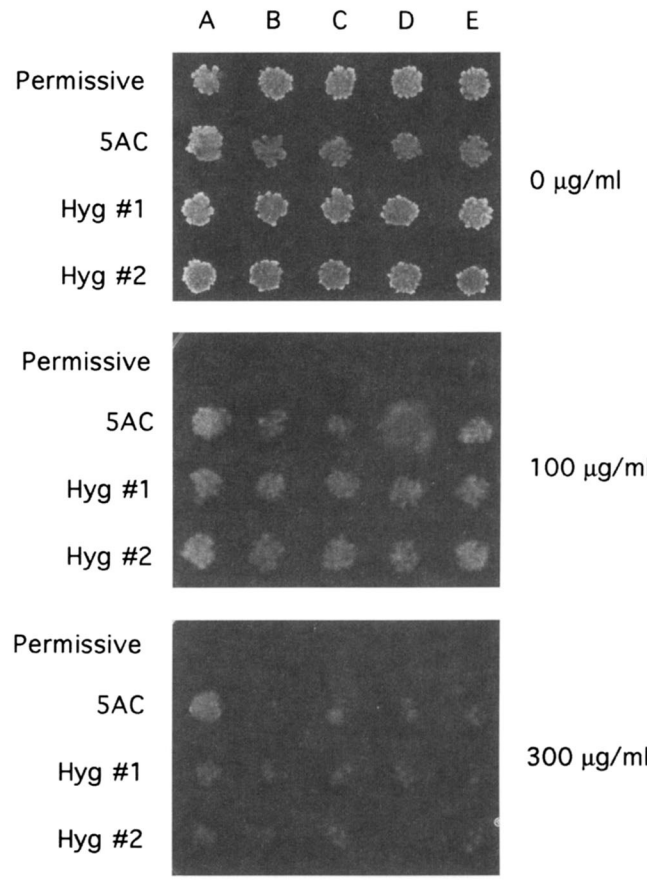

FIGURE 7.—Partial reestablishment of silencing after *hph* reactivation. Five hundred conidia from each culture were spotted on plates containing 0 (top), 100 (middle), or 300 (bottom) μg/ml hygromycin. In row 1 (Permissive), columns A–E represent five independent N644 colonies isolated from permissive medium. In row 2 (5AC), column A represents N644 conidia treated with 5AC and columns B–E represent four serial cultures derived by mass transfer of conidia from the culture shown in A. In rows 3 and 4 (Hyg #1 and #2), column A represents an independent N644 colony isolated from hygromycin medium and columns B–E represent four serial cultures derived by mass transfer of conidia from the culture shown in A.

three subsequent serial transfers showed similar expression levels (data not shown), suggesting that the intermediate level of silencing present in the first round of permissive growth was the steady-state level.

Southern analysis using the strains shown in Figure 7 revealed that methylation was partially reestablished after 5AC treatment or hygromycin selection (Figure 8A), consistent with the partial level of silencing. Additional culturing of these strains through three serial passages revealed no significant increase in methylation (data not shown). Although large fractions of the DNA molecules were heavily methylated in these partially silenced strains, a significant fraction of the molecules remained unmethylated at the sites assayed, and novel bands resulting from partial methylation were observed (Figure 8A). One fragment indicative of partial methylation (indicated with an asterisk in Figure 8A) was prominent in cultures derived from Hyg^R colonies and in cultures derived from 5AC treatments. This band was observed with a probe corresponding to fragment C (Figure 8B) as well as with a probe corresponding to a *Sau*3AI fragment within *am* (white box in Figure 8B), suggesting that it resulted from cutting one of several sites within the copy of *am* upstream of *hph* and one of several sites within *hph* (Figure 8B; data not shown). A similar methylation pattern was obtained upon reprobing this blot with the duplicated sequences containing *am*, indicating that the entire region was partially methylated (data not shown). Apparently, some of the methylation observed in the original culture was not due to reiterative *de novo* methylation.

Two control experiments were done to address the possibility that the spontaneously reactivated strains were due to genetic, rather than epigenetic, alterations. First, to test the possibility that reactivants were the result of a mutation causing a global defect in cytosine methylation, the blot shown in Figure 8A was reprobed with ψ63, a region of the genome that is methylated (FOSS *et al.* 1993). No differences in methylation at ψ63

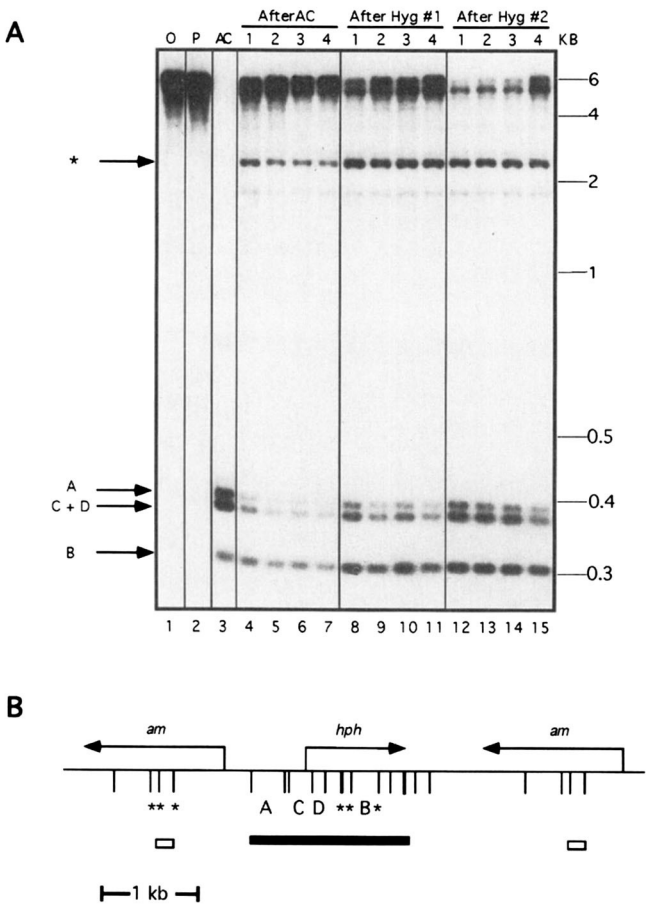

FIGURE 8.—Partial reestablishment of methylation after *hph* reactivation. (A) Southern analysis of genomic DNA from cultures derived from strain N644. Each DNA sample was cut with *Sau*3AI and probed with *hph* DNA. The arrows identify the fragments shown in B; the fragment marked with an asterisk is derived from cutting an unidentified pair of the several sites marked with asterisks in B. The positions of size standards (in kb) are indicated on the right. Lane 1: original culture of strain N644 (O). Lane 2: culture grown from a colony isolated on permissive medium (P). Lane 3: 5AC-treated culture (AC). Lanes 4–15: sets of four cultures derived from each of three treatments. For each treatment, the four cultures shown are the first (1) through the fourth (4) serial transfer on permissive medium after the indicated treatment. Lanes 4–7: culture series derived from the 5AC-treated culture. Lanes 8–11 and 12–15: culture series derived from two different colonies isolated on hygromycin medium. (B) Restriction map of the sequences surrounding *hph* in N644. The arrows above the line indicate the locations of transcripts. Thin vertical bars below the line represent single GATC sites while thick bars represent two adjacent sites. The fragments indicated by arrows in A are identified by the letters below the line. The location of the probe used in A is indicated by the filled box. The location of a second probe, used to identify the fragment marked with an asterisk in A, is indicated by the open box.

were observed between the reactivated strains and the original culture (data not shown). Second, to test for genetic modifications of *hph* sequences (*e.g.*, duplications of the DNA sequences containing *hph*), we tested whether strains that had reactivated *hph* showed any obvious DNA rearrangements involving the *hph* sequences. Southern analyses using enzymes that are not sensitive to cytosine methylation and that cut once in *hph* (*Nde*I) or once in each copy of *am* (*Eco*RV) showed identical patterns using DNA from the original strain (N644) and five of its partially reactivated derivatives (data not shown). Thus no gross rearrangements occurred in these strains.

Individual cells show intermediate levels of *hph* expression: Examples of gene silencing that involve chromatin structure alterations, such as position effect variegation in Drosophila and telomere position effect in Saccharomyces, give rise to clonal lineages containing a mixture of cells that either express or do not express markers (GOTTSCHLING *et al.* 1990; WEILER and WAKIMOTO 1995). In contrast, other chromatin-based gene silencing phenomena, such as centromere and telomere position effects in Schizosaccharomyces, give rise to intermediate gene expression states (ALLSHIRE *et al.* 1994; NIMMO *et al.* 1994). To test whether silencing of *hph* by methylation results in a mixture of fully expressing and fully silenced cells or in a continuous range of gene expression states, we assessed the frequency of conidia resistant to various levels of hygromycin. If silencing resulted in a mixture of fully expressing and fully silenced cells, the fraction of cells that would grow on any significant concentration of hygromycin would be constant and equal to the fraction that was fully expressing *hph*. If, on the other hand, silencing resulted in a range of *hph* expression levels, the fraction of cells that would grow should decrease as the concentration of hygromycin was increased.

Since Neurospora conidia are often multinucleate, and a mixture of fully expressing and fully silenced nuclei within a single conidium might give an intermediate hygromycin-resistance phenotype, we isolated uninucleate asexual spores called microconidia. Results from plating microconidia from two silenced strains, N591 and N644, and their hph^+ parents are shown in Figure 9. For both silenced strains, a decreasing proportion of spores in the population formed colonies on increasing concentrations of hygromycin, consistent with the hypothesis that there is a range of *hph* expression states within the populations.

To test the hypothesis that the various *hph* expression states revealed in Figure 9 were due to varying levels of methylation, a number of isolates of strain N644 were assessed for methylation levels and *hph* expression. Five colonies resulting from microconidia were isolated from each of three plates containing 0, 100, or 300 μg/ml hygromycin and transferred to agar slants. The resulting conidia were tested for Hyg^R by the spot test assay and were used to inoculate liquid cultures for DNA isolations and Southern analysis. In general, isolates from plates containing increasing concentrations of hygromycin showed increased *hph*

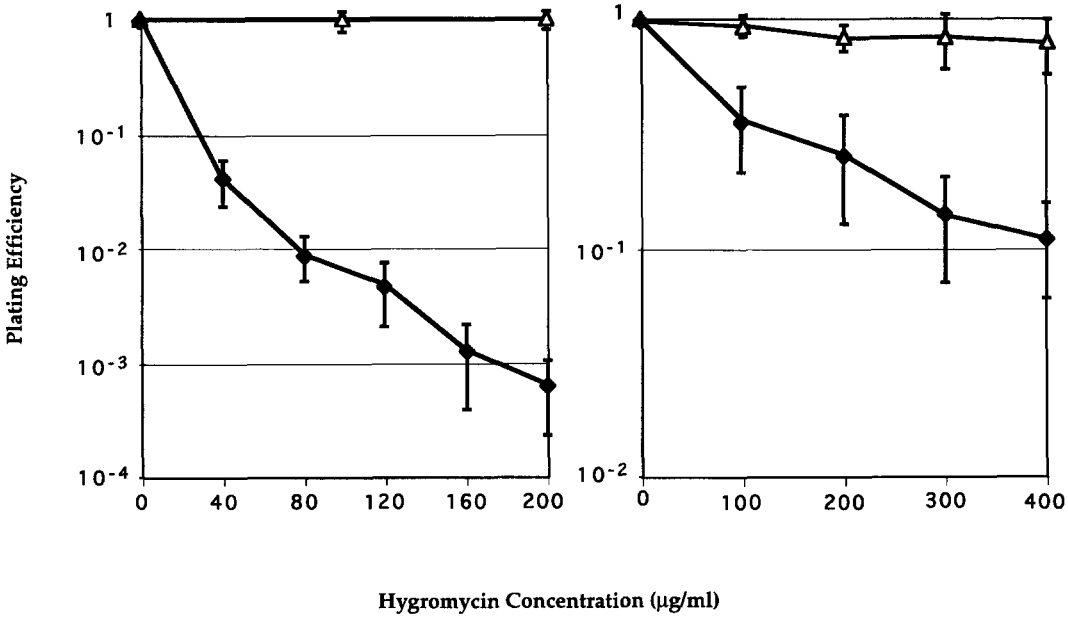

FIGURE 9.—Cell to cell variation in *hph* expression in silenced strains. Microconidia were plated on various concentrations of hygromycin. For each concentration, the plating efficiency was calculated as in Table 2 and the mean value was plotted. Error bars correspond to one standard deviation. Left: silenced strain N644 (◆) and its *hph*$^+$ parent N638 (△). Right: silenced strain N591 (◆) and its *hph*$^+$ parent N528 (△).

expression (Figure 10) and decreased methylation (Figure 11A), although there was significant variation from strain to strain. To assess the stability of these gene expression and methylation states, serial cultures were generated and assayed as described above. Different isolates showed distinct, intermediate methylation patterns that were relatively stable through serial cultures (Figure 11B), as were levels of Hyg^R (data not shown). The persistence of these distinct patterns in clonally related cultures could reflect either epigenetic propagation of methylation patterns or underlying genetic differences (*i.e.*, additional mutations). To test whether these methylation patterns were the result of an epigenetic maintenance mechanism, the third serial culture of representative strains was treated with 5AC and then grown through three additional serial cultures in the absence of 5AC as described above. After 5AC treatment, the distinct methylation patterns were all reset to the same steady-state pattern, indicating that the original patterns were propagated by an epigenetic mechanism (Figure 12).

DISCUSSION

Previous studies on the cytosine methylation associated with RIP raised the possibility that single-copy genes located near sequences that had undergone RIP might be inactivated by this methylation. This methylation was observed to exceed the boundaries of the mutated sequences (SELKER *et al.* 1993; V. MIAO and E. SELKER, unpublished data) and in some cases to inhibit expression of genes located within the mutated sequences (M. ROUNTREE and E. SELKER, unpublished data). The experiments described here revealed that the methylation associated with RIP of linked duplications can silence a gene located in the intervening, single-copy sequences. The resulting epigenetically silenced alleles provide a simple model system for studying the relationship between cytosine methylation and gene silencing.

Gene silencing by cytosine methylation has been studied most extensively in animal cells (reviewed in EDEN and CEDAR 1994). Cytosine methylation may directly inhibit transcriptional activation by reducing binding affinity of transcriptional activators. Alternatively, transcription may be blocked by steric hindrance of transcription factors by proteins that bind to methylated DNA sequences (BOYES and BIRD 1991). One of these methyl-DNA-binding proteins MeCP-1 was shown to require a threshold density of methylated cytosines in order to bind (BOYES and BIRD 1992). This led to a simple on/off model in which methylated sequences are transcriptionally active unless the methylation density is sufficient to nucleate MeCP binding, which then prevents transcription (BIRD 1992). Several studies have shown intermediate transcription levels of methylated genes in animals, and in some cases there is evidence to suggest that this was the result of a mixture of expressing and nonexpressing cells within the population assayed (MCGOWAN *et al.* 1989; HSIEH 1994; MICHAUD *et al.* 1994).

We tested a prediction of the on/off model, that individual cells within a population should exist in either an active or an inactive transcriptional state. Using a simple plating assay, we found a continuum of hygromycin-resistance levels within a clonal population, suggesting that methylation can lead to intermediate levels of *hph* expression. In concluding that a continuum of expression levels are present we have assumed that the ability to form a colony is a measure of the gene expression state of the cell as it is plated. While it is formally possible that cells are capable of switching to the expressing state after a period of time on the plate, this

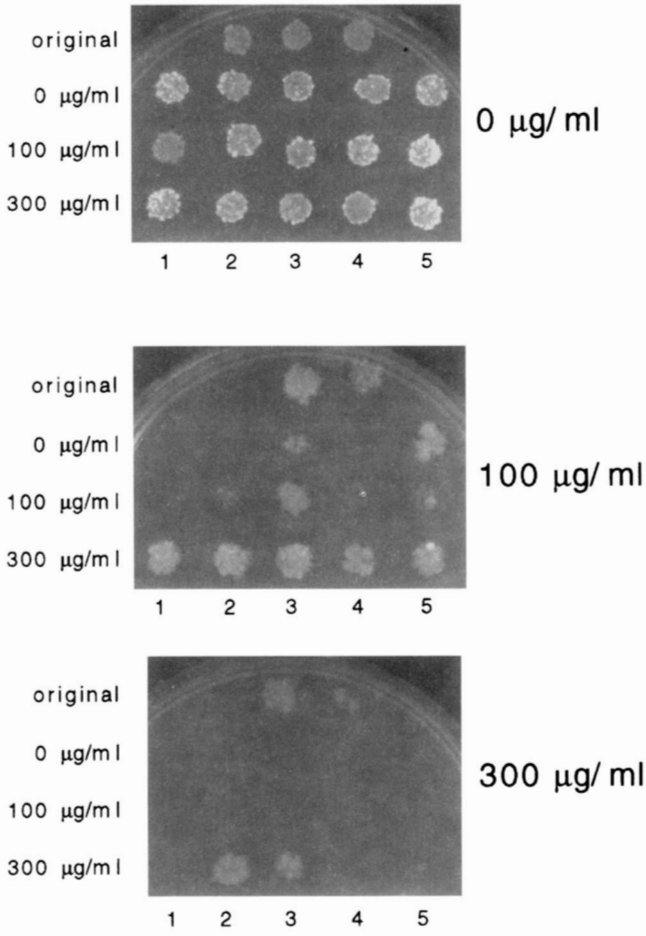

FIGURE 10.—Intermediate levels of *hph* expression after hygromycin selection. Five hundred conidia from each of various cultures were spotted on plates containing 0 (top), 100 (middle), or 300 (bottom) μg/ml hygromycin. The top row in each panel (original) shows control spots of the original N644 culture (column 2), the original culture treated with 5AC (column 3), and a culture grown after 5AC treatment (column 4). Each subsequent row shows five cultures (columns 1–5) derived from N644 by isolating independent microconidial colonies from plates containing 0 (second row), 100 (third row), or 300 (bottom row) μg/ml hygromycin.

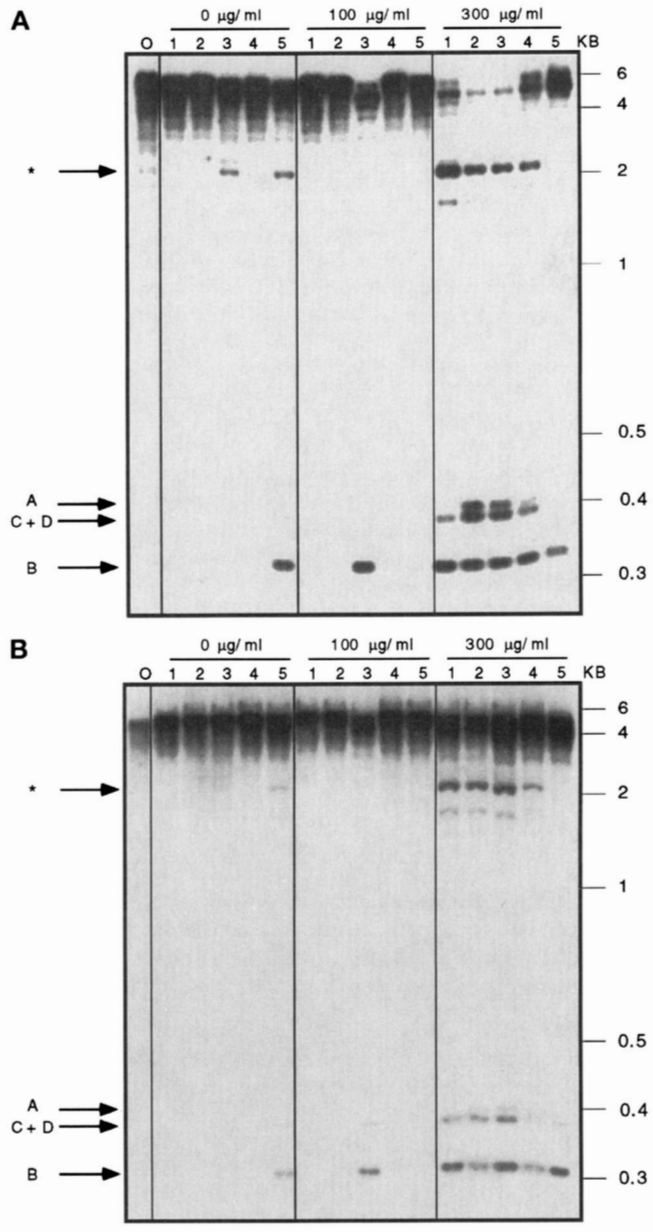

FIGURE 11.—Intermediate methylation levels after hygromycinselection. Southern analysis was conducted using the *hph* probe as for Figure 8. (A) Genomic DNA was isolated from the cultures shown in Figure 10 and digested with *Sau*3AI. Each of the five cultures derived from colonies on plates containing 0 (left), 100 (middle), or 300 μg/ml hygromycin (right) are numbered (1–5) as in Figure 10. The original N644 culture (O) is shown in the far left lane as a control. The positioning of size standards (kb) and the identity of key fragments are as in Figure 8. (B) Cultures were derived from each of the cultures shown in A through three rounds of serial culturing and were treated as in A.

possibility seems unlikely since cells that did not form colonies did not show any evidence of growth and since incubation in the presence of hygromycin quickly kills hygromycin-sensitive cells (J. IRELAN and E. SELKER, unpublished data). Furthermore, the intermediate expression levels were shown to be stable upon further culturing. Thus it appears that if Neurospora does use proteins equivalent to MeCPs to inhibit transcription, it does so in a manner that allows for intermediate levels of transcription, perhaps due to weak binding of MeCP-like proteins or binding of fewer proteins. This result is consistent with the observation that methylation of a spore pigment gene in *Ascobolus immersus* can give rise to intermediate pigment levels (COLOT and ROSSIGNOL 1995).

In animal cells, gene silencing via methylation appears to be determined by both the density of the cytosine methylation and the strength of the promoter being silenced (BOYES and BIRD 1992). Methylation densities as low as one methylated cytosine per 126 base pairs (bp; BOYES and BIRD 1992) or one methylated cytosine per 300 bp (HSIEH 1994) have been shown to reduce gene expression in human cell lines, and increased densities of methylation resulted in even

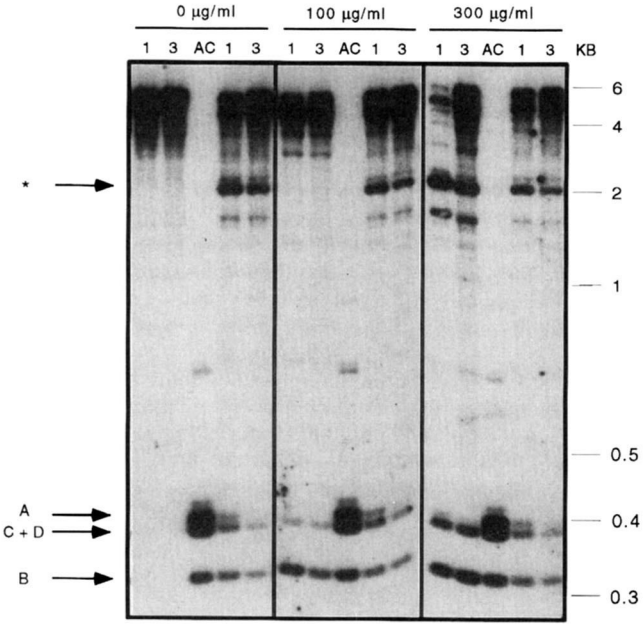

FIGURE 12.—Stability of intermediate methylation patterns in partially silenced strains. Southern analysis was conducted as in Figure 11. The strains illustrated in the three panels are representatives from the corresponding panels of Figure 10 (0 μg/ml: culture 1; 100 μg/ml: culture 3; and 300 μg/ml: culture 1). In each panel, the lanes contain, from left to right, DNA from the first (1) and third (3) serial culture after isolation of the colony, DNA from the 5AC-treated culture (5AC), and DNA from the first (1) and third (3) serial culture after 5AC treatment.

lower expression. MUMMANENI and colleagues (1995) found that placement of a *de novo* "methylation center" adjacent to the mouse *aprt* (adenine phosphoribosyltransferase) gene resulted in 5AC-reversible silencing of *aprt* only if the *aprt* promoter was truncated. The methylated alleles of *hph* described here all initially showed extremely dense methylation levels, as nearly all of the cytosines sampled were methylated in every DNA molecule. Highly variable levels of hygromycin resistance were observed in these strains, however. This imperfect correlation between methylation level and phenotype may reflect the presence of a few specific cytosines, not contained in the GATC sites assayed, that are critical for methylation-dependent silencing. Some underlying genetic differences between the *hph* alleles examined could also account for the phenotypic differences. The most heavily silenced strain examined, N644, had lost a restriction site within the *hph* coding sequences and was not completely reactivated by 5AC treatment, indicating that this allele was partly inactivated by mutation(s), most likely due to RIP. These mutations may have weakened the promoter, resulting in a greater susceptibility to silencing by methylation as described in mammalian cells (BOYES and BIRD 1992; MUMMANENI *et al.* 1995), or they may have affected *hph* expression at a later step.

Methylation levels and degree of *hph* silencing were correlated in clonally related N644 cultures that had been reactivated for *hph* expression by 5AC treatment or by selection on hygromycin. In all cases examined, an intermediate level of *hph* expression (at least at the level of the multinucleate conidia) and methylation was maintained upon subsequent growth. This result indicates that some of the methylation observed in the original cultures was dependent on preexisting methylation and hence can be regarded as "maintenance methylation," as opposed to *de novo* methylation, which occurs in the absence of preexisting methylation (SINGER *et al.* 1995b). The mechanism by which methylation is maintained is not established. The system proposed for maintenance of CpG methylation in animals, whereby the hemimethylation that results from DNA replication triggers methylation of the opposite cytosine on the newly replicated DNA chain (HOLLIDAY and PUGH 1975; RIGGS 1975), does not account for maintenance methylation in filamentous fungi since most of the methylation is not at symmetrical sites such as CpGs (SELKER and STEVENS 1985; SELKER *et al.* 1993; GOYON *et al.* 1994).

It is possible that the maintenance of methylation observed here might be an indirect effect of competition between DNA binding factors involved in chromatin structure and/or transcriptional activation and factors required for methylation. For example, the initial methylation might have prevented transcriptional activators from binding to the DNA, but when these factors were allowed to bind as a result of hypomethylation (spontaneous or 5AC induced) of critical sites, they then directly prevented access by the methylation machinery. Alternatively, binding of transcriptional activators may have led to a regional remodeling of chromatin to a structure that is resistant to methylation (SELKER 1990). The partial methylation pattern in these strains extended throughout the entire duplicated region and was not confined to the sequences containing promoter elements, consistent with a regional effect. In either case, the distinct methylation patterns observed in different strains (Figure 11) could be accounted for by invoking subtle differences in the pattern or constellation of proteins bound to different DNA molecules. However, these simplistic models for maintenance of the methylation patterns would predict that the DNA-bound proteins would direct reestablishment of the original methylation pattern after removal of methylation, which was not observed (Figures 8 and 12). Thus maintenance methylation in Neurospora may involve a complex interplay between DNA-binding proteins that bind differentially to methylated and unmethylated sequences, and a methyltransferase that is influenced by the presence of these proteins.

Epigenetic silencing of *hph* was detected at significant frequencies in each of several strains and depended on the stringency of the assay. At low hygromycin concentrations, only heavily impaired alleles of *hph* showed a reduction in growth. Strains containing such alleles

occurred at moderate frequencies (~1–10%). A retest of 10 progeny from one of these crosses at higher hygromycin concentrations, however, revealed that eight had detectable reductions in *hph* expression. Even these less silenced *hph* alleles showed dramatic, methylation-dependent reductions in mRNA levels. Thus it is likely that other genes located near sequences that have undergone RIP would also show reduced expression due to methylation. Since only low levels of *hph* expression are required to exhibit hygromycin resistance, similar effects on expression of other genes might have more obvious phenotypic consequences. Evidence of several natural cases of RIP has been reported (GRAYBURN and SELKER 1989; CENTOLA and CARBON 1994; KINSEY *et al.* 1994), and RIP of engineered duplications is commonly used to knock out genes of interest in Neurospora. Although no previous case has been documented in which methylation due to RIP directly affects expression of a nearby, single-copy gene, this might be common and could result in unexpected phenotypes. Thus when using RIP to knock out genes of unknown function, care must be taken to establish that the observed phenotype is not the result of inactivation of a nearby gene.

Cytosine methylation is probably not essential in Neurospora, as complete or nearly complete removal of methylation in vegetative tissues by 5AC (SELKER and STEVENS 1985) or mutation (FOSS *et al.* 1993; ROBERTS and SELKER 1995) does not lead to gross phenotypic consequences observable in the laboratory (FOSS *et al.* 1995). This observation raises the question, why does Neurospora have cytosine methylation? One clue to the possible function for cytosine methylation in Neurospora comes from studies of the LINE-like transposable element *Tad*, derived from an exotic Neurospora strain (KINSEY and HELBER 1989). RIP can inactivate this transposable element when it is introduced into a lab strain, and cryptic *Tad* elements are present in the genomes of laboratory strains, suggesting that RIP can provide an effective defense against replication of this transposable element (KINSEY *et al.* 1994). An insertion of *Tad* just upstream of the *am* gene, called *Tad 3-2*, has been shown to inhibit *am* expression in a manner that is reversible and dependent on methylation of the transposon (CAMBARERI *et al.* 1996). While the mechanism by which *Tad 3-2* influences *am* expression is not known, it is possible that methylation inhibits transcription originating in *Tad 3-2* (which in turn inhibits *am* expression) in a manner analogous to the silencing of *hph* described here. Thus the role of methylation in Neurospora may be to silence genes in and around repetitive sequences that have undergone RIP. Cytosine methylation may prevent transcription of genes in autonomous transposable elements that have been subjected to RIP but not fully inactivated, or it may prevent *trans* activation of defective elements that have been subjected to RIP by activator elements that have escaped RIP.

The epigenetically silenced alleles of *hph* described here represent a model system for elucidating the mechanisms by which cytosine methylation inhibits gene expression. The strain with the most heavily silenced allele may be useful for isolating mutants defective in *trans*-acting factors required for methylation and silencing of *hph*. While there are some important differences between fungi and higher organisms with regard to the patterns and consequences of DNA methylation, it is likely that some fundamental aspects of methylation-induced gene silencing have been conserved.

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