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A Targeted-Replacement System for Identification of Signals for De Novo Methylation in *Neurospora crassa*

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Transformation of eukaryotic cells can be used to test potential signals for DNA methylation. This approach is not always reliable, however, because of chromosomal position effects and because integration of multiple and/or rearranged copies of transforming DNA can influence DNA methylation. We developed a robust system to evaluate the potential of DNA fragments to function as signals for de novo methylation in *Neurospora crassa*. The requirements of the system were (i) a location in the *N. crassa* genome that becomes methylated only in the presence of a bona fide methylation signal and (ii) an efficient gene replacement protocol. We report here that the *am* locus fulfills these requirements, and we demonstrate its utility with the identification of a 2.7-kb fragment from the ψ_{63} locus as a new portable signal for de novo methylation.

Certain cytosines in DNA of many fungi, plants, and animals are subject to methylation, and it is becoming increasingly clear that this DNA methylation plays important roles in eukaryotes. In animal cells, increased methylation can induce tumorigenic transformation (57), while decreased methylation can lead to death during embryogenesis (26). Numerous studies, especially in animal systems, suggest connections between DNA methylation and gene expression (see reference 18). Despite strides made in understanding the function of methylation, the factors that control DNA methylation remain mysterious. Some sequences appear prone to methylation independent of environment or circumstance (45), while methylation of others depends on chromosomal position, cell type, developmental stage, and/or history of the sequence (19, 38, 55).

Neurospora crassa provides a simple system with which to study the control of de novo methylation. A mutant with no detectable DNA methylation has been isolated, implying that methylation is not essential in this organism (12). Most of the genome of *N. crassa* is devoid of DNA methylation, but several heavily methylated chromosomal regions are known. One is the 1.6-kb zeta-eta (ζ-η) region (45, 47). This region is a naturally occurring relic of RIP (repeat-induced point mutation), a mutagenic process that acts on duplicated sequences prior to meiosis in *N. crassa* (13, 41, 42, 44). RIP makes multiple G · C-to-A · T mutations in the duplicated sequences and typically causes them to become methylated (5). Our previous work showed that de novo methylation of ζ-η sequences depends on a portable *cis*-acting signal: ζ-η sequences, stripped of methylation by propagation in *Escherichia coli*, became methylated de novo when they were reintegrated into the *N. crassa* genome at various chromosomal positions (45). Methylation of *N. crassa* sequences flanking ζ-η in the transforming DNA was not observed, but bacterial vector sequences became methylated in some cases. This observation provided an early clue that the methylation of sequences can depend on their chromosomal position.

To investigate the control of DNA methylation, we wished to dissect sequences shown to contain methylation signals and test the pieces for their potential to induce methylation in vivo. Unfortunately, as in higher eukaryotes, integration of transforming DNA in *N. crassa* occurs predominantly by nonhomologous insertion (11). This poses potential complications in analysis, because methylation of test fragments might be influenced by (i) flanking sequences in the transforming DNA (e.g., bacterial vector sequences), (ii) rearrangements created during integration, (iii) chromosomal position, and/or (iv) copy number of the transforming sequences (46). Some of the variables may be controlled by examining multiple independent transformants, but this process is very laborious and does not overcome all the problems (46). We therefore sought to develop an efficient system to precisely target single copies of potential methylation signals, without extraneous vector sequences, to a common chromosomal position where methylation might be evaluated. The location to which test fragments are targeted must neither become methylated in response to nonspecific perturbation nor suppress methylation when bona fide signals for methylation are inserted. Here we present such a system. Our technique involves selecting for replacement of the coding region of a normally unmethylated gene, *am*, which encodes the NADP-specific glutamate dehydrogenase (22).

MATERIALS AND METHODS

***N. crassa* strains.** Wild-type and spontaneous *am* mutants of *N. crassa* used in this study are listed in Table 1. Strains N261 and N408 were transformation hosts. N408 was isolated from a cross between N261 and N36 (42) and differs from N261 by carrying the theta (θ) region from N36, rather than the homologous ζ-η region from N261. Deletions in the *am* gene obtained during the course of this study were identified and confirmed by mapping with several enzymes.

Transforming DNA. To provide homologous sequences for targeting test fragments to the *am* region, we created pMS2 by subcloning a 5.2-kb *Hin*dIII-*Pst*I fragment containing the *am* gene from pJR3 (3) into pMS1, a modified version of pTZ18U (31) lacking the *Eco*RI and *Bam*HI sites in the polylinker. The *Bam*HI site 3′ of *am* in pMS2 was cut and filled in to create a *Cla*I site in pMS3. Constructs used in targeted replacement experiments are shown in Fig. 1. To make pMS4, the *Bgl*II site in *am* of pMS3 was cut and filled in, leaving tandem *Sau*3AI sites in its place. The central 1.3-kb *Bgl*II-*Eco*RI fragment of pMS3 was deleted, to generate pVM2, or replaced either with a 0.8-kb *Tth*III1-*Eco*RI fragment from pNCAR1 containing the *N. crassa* θ region (positions 174 to 955 [13]), to make

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TABLE 1. *N. crassa* strains with wild-type or spontaneous mutant alleles at *am*

Strain	Genotype	Size (kb) of insertion or deletion at *am*	Source or reference
N261	*lys-1* ζ-η	0	J. Kinsey
N408	*lys-1* θ	0	This study
N129	*lys-1 am*$^{20\text{-}4}$	+0.2–0.4	50
N131	*lys-1 am*$^{40\text{-}3}$	+0.1	50
N132	*lys-1 am*^{77s15}	+0.5	25
N700	*lys-1 am*^{v515}	−0.2	This study
N701	*lys-1 am*^{v467}	−0.5	This study
N702	*lys-1 am*^{v485}	−0.9	This study
N703	*lys-1 am*^{v471}	−1.2	This study
N704	*lys-1 am*^{v500}	−2.0	This study
N705	*lys-1 am*^{v473}	−2.2	This study

pVM1, or with a 1.9-kb BAL 31 fragment from pJS33 (32) containing the entire sequenced region of the ζ-η region reported previously (47), plus 60 nucleotides upstream, to generate pVM60F. Plasmid pMR10 was made by replacing the 1.3-kb *Bgl*II-*Eco*RI fragment of pMS2 with a 2.7-kb *Bam*HI-*Eco*RI fragment from pJS63 (32) containing the Ψ_{63} region. Ligation junctions in pVM plasmids were verified by DNA sequencing (40). A plasmid conferring resistance to hygromycin, pES200 (52), was used for primary selection during cotransformation.

Transformation and recovery of Am$^-$ strains. Previously published transformation (1, 8) and selection procedures (23) were modified to facilitate recovery of Am$^-$ transformants in this study (Fig. 2). *am* plasmids were digested with *Hin*dIII and *Pst*I, and the portion containing the modified *am* region was gel purified. Two micrograms of the fragment (substituted in one experiment by uncut pMR10, adjusted to provide 2 μg of the *am* fragment), mixed with 0.5 μg of pES200 (molar ratio, ≈4:1) in a total volume of 4 μl, was added to 100 μl of competent cells and incubated on ice for 20 min. One milliliter of freshly mixed 40% polyethylene glycol 3350 (Sigma)–10 mM morpholinepropanesulfonic acid (MOPS; pH 6.3)–50 mM $CaCl_2$ (PMC) was added to the cells, and the mixture was incubated at room temperature for another 20 min. The cells were further diluted with 1.4 ml of PMC, and ≈400-μl aliquots were distributed to 250-ml flasks containing 20 ml of solidified Vogel's medium (1.5% sucrose, 1.5% agar [9]) with lysine (60 μg/ml), alanine (100 μg/ml), and hygromycin (200 μg/ml; Calbiochem). Flasks were incubated at 33°C to allow regeneration and conidiation of presumptive transformants. After 4 days, conidia were harvested in water, and 10^5 to 10^6 were spread onto AAG plates (50 μg of aminoadipic acid and 500 μg of glutamate per ml [23]) and incubated for 2 days at 33°C. Fewer conidia were used in pMS4 experiments (see Results). Colonies that arose on AAG plates were transferred to nonselective medium (containing alanine and lysine). After 2 days, conidia were spot tested on selective and control media at 25°C. The test plates contained alanine and lysine (plate 1), alanine, lysine, and hygromycin (plate 2), AAG (plate 3), or glycine (150 μg/ml) and lysine (plate 4). Spots were examined microscopically after 18 h for growth of germlings. Am$^-$ strains grew on plates 1 and 3 but not on plate 4. Cultures producing a mix of Am$^+$ and Am$^-$ conidia were identified as heterokaryons and purified by spreading conidia on AAG medium and microscopically isolating germlings.

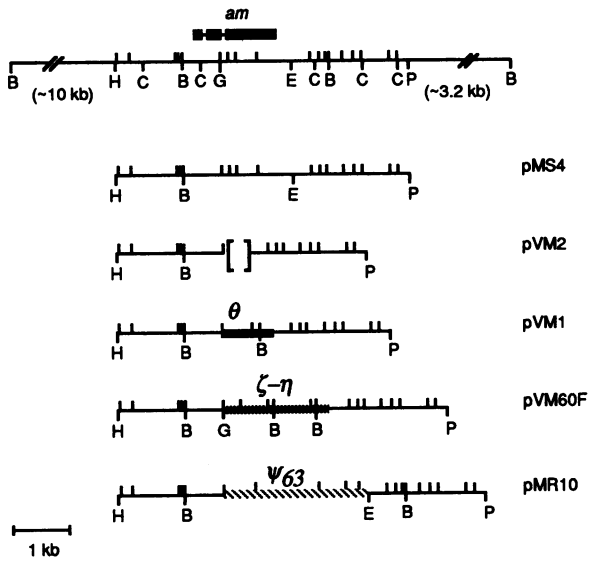

FIG. 1. The *am* locus and gene replacement constructs. A restriction map of the wild-type *am* region is shown (top), and the positions of the exons are indicated above (filled blocks). *Bam*HI (B), *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P) sites are indicated below the horizontal line, and *Sau*3AI-*Mbo*I sites are indicated by ticks above it. The coding region is contained in a 2.6-kb *Bam*HI fragment. Similar maps are shown below for each of the constructs, but for clarity, the *Cla*I sites are not indicated. Except for pMR10, all of the constructs have one more *Cla*I site than the fragment from the wild type, as a result of conversion of the right-hand *Bam*HI site to a *Cla*I site in the parental plasmid, pMS3. Inserted DNA from the θ, ζ-η, or Ψ_{63} region is indicated with different patterned bars, and the deletion to build pVM2 is indicated by square brackets.

Southern analysis of Am$^-$ strains. Genomic DNA was prepared from transformants as previously described (16) and analyzed by Southern hybridization by standard methods (29). Typically, 0.5 μg of genomic DNA was digested with 4 to 5 U of restriction enzyme to ensure complete digestion. Hybridization probes, prepared by the procedure of Feinberg and Vogelstein (10), were made from the following: the 2.6-kb *Bam*HI fragment containing the wild-type *am* gene (Fig. 1); a 1.6-kb ζ-η fragment (*Eco*RI-to-*Sau*3AI site *a* in Fig. 5); a 2.7-kb *Eco*RI-*Bam*HI Ψ_{63} fragment from pJS63; or the 2.5-kb *Eco*RV-*Bam*HI fragment containing the unmethylated *mtr* gene of *N. crassa* from pCVN2.9 (53). The nylon membranes (Zetabind; Cuno, Inc.) were washed in 50 mM NaCl–20 mM $NaHPO_4$ (pH 6.8)–1 mM EDTA–0.1% sodium dodecyl sulfate at 60°C after hybridization and stripped with 0.4 N NaOH at 42°C before reprobing.

RESULTS

The *am* system. The *am* gene has several advantages as a test site for assessing potential de novo methylation signals. First, the *am* gene is one of the best-characterized genes in *N. crassa* and one of only two for which a selection has been devised for loss of function (23). Thus, at least in principle, we could select for replacement of sequences at *am*. This was important since transformation by homologous recombination is rare in *N. crassa* (3, 4, 7, 11, 37). Second, DNA sequence information is available for the entire *am* coding region (21) as well as for several kilobases on each side (4, 33). Third, the *am* gene is not normally methylated, and inactivation of *am*, per se, does not induce methylation of the region (44). Finally, numerous *am* mutations are available (e.g., see reference 24). We used *am* mutants to test the potential of various types of mutations to

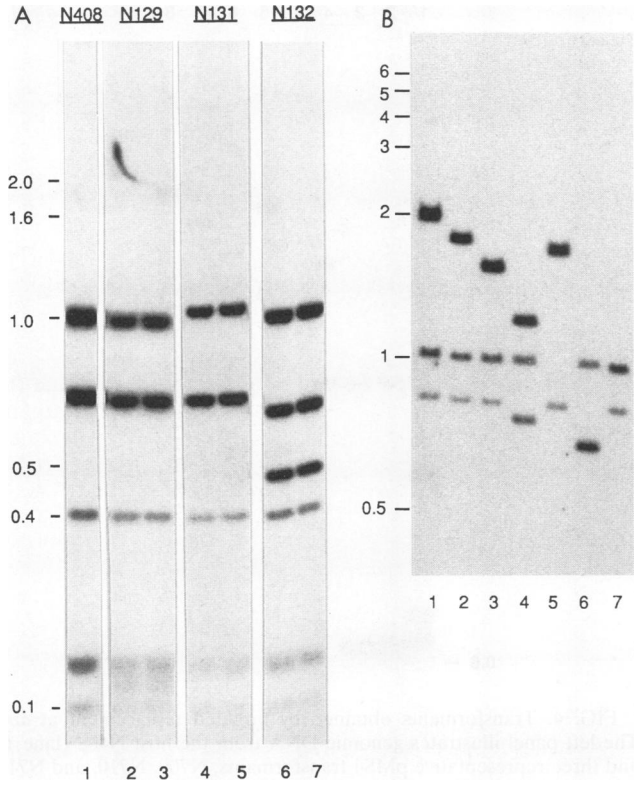

FIG. 2. Insertions and deletions at *am* do not induce methylation. (A) Samples of genomic DNA from an *am*$^+$ strain (N408) and from *am* insertion mutants (N129, N131, and N132) were digested with *Sau*3A1 (lanes 1, 3, 5, and 7) or *Mbo*I (lanes 2, 4, and 6). (B) Samples of genomic DNA from strain N408 (lane 1) and from deletion mutants N700, N701, N702, N703, N704, and N705 (lanes 2 to 7, respectively) were digested with *Cla*I. Both blots were probed with the 2.6-kb *Bam*HI fragment of *am*. Size standards are indicated in kilobases on the left.

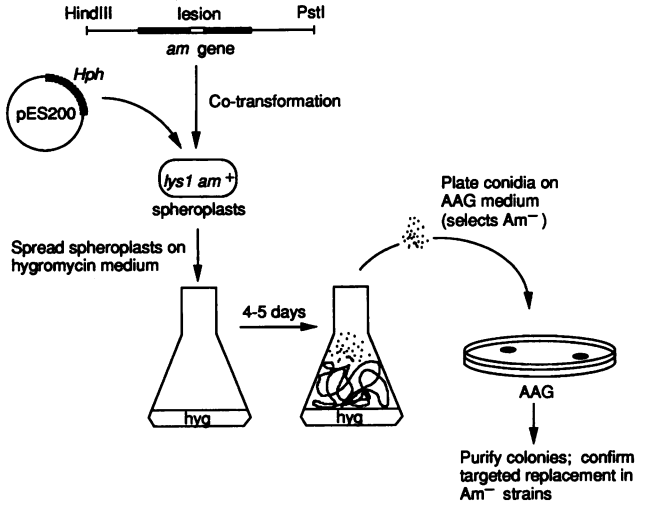

FIG. 3. Strategy for obtaining targeted gene replacements at the *am* locus. hyg, hygromycin. See text for details.

induce methylation and to provide assurance that methylation associated with a test fragment would not be due to the general chromosomal disturbance caused by transformation into *am*.

Examination of spontaneous *am* alleles. We knew from previous work that alleles of *am* such as those created by RIP are frequently, but not invariably, methylated (44, 51). Therefore, we checked whether other perturbations in *am* would induce methylation before testing the scheme for gene replacement at *am*. We were especially interested in the kind of changes that would be associated with the introduction of test fragments into this region, i.e., insertions and deletions.

We examined several *am* alleles containing insertions of ≈100 to 500 bp (Table 1). Methylation in *N. crassa* is not limited to cytosines in particular sequence motifs (43, 46, 47) and can be easily detected by Southern hybridization using the isoschizomers *Sau*3AI and *Mbo*I. Both enzymes cut unmethylated GATC sites, but only *Mbo*I will cut GATC sites in which the C is methylated (36). Results of comparing a strain having a wild-type *am* gene, N408, and three strains harboring insertions in *am* are shown in Fig. 2A. No methylation was observed. Alterations of the wild-type pattern of *Sau*3AI fragments (lane 1) were present as a result of the different spontaneous insertions (lanes 2 to 7), but *Sau*3AI and *Mbo*I digests displayed identical patterns.

We also tested six *am* deletion strains for methylation at *am*, using several cytosine methylation-sensitive restriction enzymes. Again, no methylation was observed, as illustrated by a set of *Cla*I digests shown in Fig. 2B. The wild-type profile of *am* fragments after *Cla*I digestion (lane 1) was altered in the mutants because of various deletions (lanes 2 to 7), but there was no indication this enzyme had been inhibited at any of its sites in the region; e.g., no bands corresponding to fragments that were the sum of two smaller fragments were present. Overall, these results gave us confidence that the *am* region would most likely not confound our studies by becoming methylated in response to insertions of transforming DNA per se.

Gene replacement at *am*. Our scheme for targeting sequences to *am* is based on the observation that *lys-1* mutants cannot grow on a minimal medium supplemented with aminoadipic acid plus glutamate (AAG), whereas *lys-1 am* double mutants can (23). We could not directly select for gene replacement events at *am* because a large fraction of cells prepared for transformation are multinucleate and *am*$^+$ nuclei are dominant in heterokaryons. We therefore tested a two-step method based on the fact that cotransformation is very efficient in *N. crassa*. In the first step of the procedure, illustrated in Fig. 3, *lys-1 am*$^+$ competent cells are treated simultaneously with a plasmid containing the dominant selectable marker *hph* (which confers resistance to hygromycin [52]) and a 4- to 5-kb fragment that includes a defective *am* gene flanked by ≈2 kb of normal sequences on either side. Instead of plating the transformation mix to obtain individual colonies, the transformants are grown en masse in a flask containing solidified medium with lysine, alanine, and hygromycin. The purpose of this step, which selects for the cotransforming DNA, is to obtain homokaryotic conidia, some of which should have replaced the wild-type *am* allele with the defective copy in the transformation mix. These rare *am* transformants are selected by plating a large number of conidia on AAG medium.

We first tested the gene replacement system with a simple construct that preserved the overall structure of the *am* region. The *am* region of pMS4 harbors two restriction site changes that allow it to be distinguished from the wild-type allele: an extra *Cla*I site 3′ of the coding region, and a filled-in *Bgl*II site within the coding region that results in a 4-bp insertion and is sufficient to prevent *am* expression (Fig. 1). We introduced this

TABLE 2. *N. crassa* strains with simple replacements at *am*

Transforming DNA	No. of independent transformants	Host	Transformant(s)[a]
pMS4	6	N261	N706, N707, N708
		N408	N709, N710, N711
pVM1	2	N408	N712, N713
pVM2	4	N408	N714, N715, N716, N717
pVM60F	3	N261	N718
		N408	N719, N720
pMR10	5	N408	N721, N722, N723, N724, N725

[a] N706, N707, N708, N714, and N718 were obtained by an earlier protocol in which spheroplasts were embedded in 0.5% agar for regeneration. N724 and N725 were obtained by using uncut plasmids.

construct into two *lys-1* strains, N261 and N408. The significant difference between the strains is that at the native ζ-η locus, strain N261 carries ζ-η, the methylated allele, whereas strain N408 carries θ, the unmethylated allele. In each experiment, we used ≈0.5 μg of pES200, a plasmid conferring hygromycin resistance, and ≈2 μg of the modified *am* fragment; the transformation mix was distributed to approximately 10 flasks (Fig. 3). We plated 10^4 to 10^5 conidia from each flask on AAG and, with either strain, typically obtained putative *am* replacement strains from about half of the flasks. We characterized the transformants for *am* function (growth on glycine-supplemented medium) and vegetatively purified any that produced a mix of Am^+ and Am^- conidia. With both hosts, most AAG-resistant strains were Am^-, and the results from Southern analysis showed that more than half of the Am^- strains were simple replacements of the wild-type gene with the in vitro-generated allele. These replacement strains were unambiguously identified by mapping with several restriction enzymes. For example, a triple digest with *Bam*HI, *Bgl*II, and *Eco*RI proved that the wild-type restriction pattern had been changed as expected in three independent pMS4 transformants of strain N408 (Table 2; Fig. 4). In this digest, the transformation host shows fragments of ≈0.66, 0.7, and 1.3 kb (Fig. 4, lane 1). Replacement of the native locus with the pMS4 version of *am* resulted in replacement of the 0.7-kb *Bam*HI-*Bgl*II fragment by a 2-kb *Bam*HI-*Eco*RI fragment (lanes 2 to 4). In N711, the plasmid's new *Cla*I site (converted *Bam*HI site) was also integrated into the chromosome. Thus, this strain had not only the 2-kb fragment but also a 3.6-kb band representing the *Eco*RI-*Eco*RI fragment that derives from cutting at *Eco*RI sites within the central region and in the 3′ flanking DNA (lane 4). Results from mapping of replicate transformants in the N261 background were also consistent with precise substitution of wild-type sequences by their modified counterparts (data not shown).

Generation of deletion and substitution mutations by gene replacement. We built a set of three constructs to further test the *am* region as a potential assay site for methylation signals. One construct, pVM2, carries a deletion of a 1.3-kb *Bgl*II-*Eco*RI fragment near the middle of the 5.2-kb *Hin*dIII-*Pst*I *am* fragment. In the other two, pVM1 and pVM60F, the *Bgl*II-*Eco*RI fragment was replaced, respectively, by a segment with the unmethylated θ region and the homologous methylated region, ζ-η (Fig. 1). To simplify analyses later, we used N408, the strain lacking the ζ-η region, as the primary transformation host; however, we also used N261 in some experiments. We obtained two or more independent transformants with a simple targeted replacement at *am* for each construct (Table 2).

The distribution of various types of transformants in a group of experiments is illustrated in Table 3. Using the three transforming fragments, we recovered a total of 17 strains with the Am^- phenotype on AAG medium. Eleven of these carried transforming DNA integrated at *am*. Among the 11, 7 (64%) were of the desired simple-replacement type and were identified by diagnostic changes in the overall size of the target region, as illustrated in Fig. 4 with *Hin*dIII-*Pst*I digests (lanes 5 to 8). Digests with other enzymes (*Bam*HI, *Cla*I, and *Hin*dIII-*Eco*RI as appropriate) also gave results consistent with clean replacement (data not shown). Curiously, these seven, as well as all other simple-replacement strains obtained in this study,

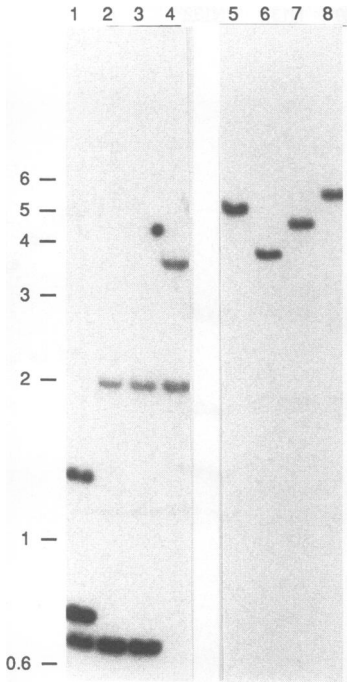

FIG. 4. Transformants obtained by targeted replacement at *am*. The left panel illustrates genomic DNA from the host N408 (lane 1) and three representative pMS4 transformants, N709, N710, and N711 (lanes 2 to 4), digested with *Bam*HI-*Bgl*II-*Eco*RI. The right panel shows a comparison of N408 (lane 5) and representative pVM2 (N717; lane 6), pVM1 (N712; lane 7), and pVM60F (N719; lane 8) transformants digested with *Hin*dIII-*Pst*I. DNAs were probed with the 2.6-kb *Bam*HI fragment of *am* to reveal changes at *am* and were verified for complete digestion by reprobing with the *mtr* gene (not shown). Size standards are indicated in kilobases on the left.

TABLE 3. Alterations of the *am* region in Am^- strains[a]

Change in *am* region	No. of strains obtained with:		
	pVM2	pVM1	pVM60F
Simple replacement	3	2	2
Complex replacement	1	1	2
Other mutations			
Deletion	0	0	1
No obvious change at *am*	0	2	3

[a] All Am^- strains obtained in the same experiment are included in order to show an unweighted distribution of the different types of changes at *am*. The pVM2 transformants were obtained from one experiment. The pVM1 and pVM60F transformants listed were accumulated from two experiments each. One pVM2 and one pVM60F simple replacement (N714 and N718 from Table 2) obtained with an earlier version of the current protocol are not included here.

were hygromycin sensitive (Hyg^s). In contrast, only one of four strains harboring a complex replacement at *am* was Hyg^s. Six other strains, which were Hyg^s, showed at the Southern analysis level either no change or a deletion at *am*. These were most likely spontaneous mutants of *am*, considering the large number of conidia (10^5 to 10^6) plated on AAG. With one exception, none of these strains had any detectable transforming DNA. The *am* gene was isolated from two putative spontaneous mutants and shown to be defective by a transformation assay (27). One strain showing no obvious change at *am* had additional hybridization to the *am* probe. A spontaneous rearrangement may have duplicated *am* sequences, or the strain may have integrated transforming *am* DNA at an ectopic site. Since our criterion for targeted replacement required verification at the Southern analysis level, such strains were readily identified and excluded at an early stage of analysis.

Methylation in targeted sequences. All transformants obtained for each construct were assessed for methylation using several enzymes. No evidence of methylation was observed in pMS4, pVM2, or pVM1 transformants, harboring the 4-bp insertion, the 1.3-kb deletion, or the substitution of θ for *am*, respectively (Fig. 4 and 5 and data not shown), and all replicate transformants for the same construct gave the same results. The targeted, but complex, replacement transformants of pVM1 and pVM2 were also examined for methylation to evaluate whether the requirement for simple replacement in our system was needed. This requirement proved to be justified, as illustrated by strains transformed by pVM2. We observed that while single-copy, simple-replacement strains were not methylated (e.g., Fig. 5A, lanes 15 and 16), complex-replacement strains like N726 were extensively methylated (e.g., lanes 19 and 20).

Simple-replacement transformants obtained with the ζ-η construct pVM60F all showed obvious methylation. Thus, *am* did not suppress the ability of the ζ-η sequences to reestablish their normal methylation pattern. Methylation characteristic of the ζ-η region was most obvious by comparing *Mbo*I- and *Sau*3AI-digested DNA from pVM60F transformants (Fig. 5A, lanes 1 to 4) with the same digests of DNA from the host strain that carries a native copy of the ζ-η region (N261; lanes 9 and 10). *Mbo*I digests showed the approximately 0.5- and 0.7-kb internal fragments expected from a ζ-η probing of DNA from these strains (Fig. 5B). *Sau*3AI digests showed little of the bands observed with *Mbo*I. Instead, higher-molecular-weight bands indicative of methylation were prominent. Fragments of 1.3 and 1.8 kb characteristically result from methylation at the native ζ-η region (Fig. 5A, lane 10). The 1.3-kb fragment (sites a to d in Fig. 5B) results from methylation of two sites (b and c) and was detected in both transformants (Fig. 5A, lanes 2 and 4). The 1.8-kb native fragment results from methylation at four sites (b to e); the corresponding fragment in the transforming DNA is the 1.9-kb fragment (sites a to f in Fig. 5B). That the 1.9-kb fragment was the largest prominent fragment indicated the methylation was confined chiefly to the ζ-η region. Interestingly, very low intensity bands corresponding to fragments of about 2.8 and 3.6 kb were also detected in *Sau*3AI digests. These bands, detected again by reprobing the lanes with *am* (Fig. 5A, lanes 11 and 12), are indicative of methylation 5′ of the ζ-η insert.

Most transformants were obtained with N408, which has the unmethylated θ allele, but an N261 transformant was also obtained with the ζ-η-containing plasmid pVM60F to check for a copy number effect on the methylation of ζ-η sequences (Table 2). Methylation at the native locus of ζ-η did not appear to affect methylation of ζ-η at *am*, or vice versa. The intensity of the 1.9-kb fragment at *am* was the same whether or not there was a methylated allele at the native locus (Fig. 5A, lanes 2 and 4). In addition, the θ insert failed to become methylated in the θ-containing host N408 (lanes 5 to 8), indicating that duplication of this sequence does not induce methylation. Thus, copy number does not noticeably affect methylation of these sequences. The contrast between the methylation states of the ζ-η and θ alleles can be readily seen in the different intensities of the 0.54-kb fragment that the two constructs have in common (Fig. 5B and C). In an *am* probing, the band representing this junction fragment shifted to a larger size only in ζ-η transformants, because site e is methylated in ζ-η but not in θ (Fig. 5A, lanes 11 to 14).

Ψ_{63}, a new methylation signal. The success of the replacement scheme and the faithful reproduction of the methylation pattern of ζ-η in the context of *am* prompted us to test another methylated region from *N. crassa*, the Ψ_{63} region (12, 32). Methylated regions are rare in *Neurospora* species (49), and we wished to learn whether Ψ_{63}, like ζ-η, would serve as a portable methylation signal. We substituted a 2.7-kb *Bam*HI-*Eco*RI fragment containing the Ψ_{63} region for the native *Bgl*II-*Eco*RI fragment of pMS2 to create pMR10 and obtained five independent simple replacement transformants of strain N408 (Fig. 1). All five transformants showed de novo methylation of the Ψ63 sequences inserted at *am* (Fig. 6). No differences were observed among the independent transformants. Figure 6A shows representative results from *Mbo*I and *Sau*3AI digests of two transformants, N721 and N724, and their transformation host, N408. All *Mbo*I bands (lanes 1, 3, and 5) detected were expected, and as anticipated, bands representing fragments that were duplicated in N721 and N724 appeared approximately twice as intense as those from the host, which has just the native Ψ_{63} region. Digestion with *Sau*3AI (lanes 2, 4, and 6) resulted in either reduced intensity or loss of bands representing three internal fragments (z-a, a-b, and b-c) both from the native copy of Ψ_{63} and from the homologous sequence inserted into *am*, indicating that some or all of these sites became methylated. The presence of the 1-kb (w-a), 1.8-kb (z-b), 2.2-kb (w-b), 2.2-kb (z-c), and 2.6-kb (y-c) fragments in lane 2 showed that sites z, a, and b were partially methylated in the native copy of Ψ_{63}, and the presence of the 3.8-kb (x-c), 2.9-kb (y-c), 2.5-kb (y-b), 2.2-kb (x-a), 2.2-kb (z-c), 1.8-kb (z-b), and 1.6-kb (a-c) fragments in lanes 4 and 6 indicated that sites z, a, and b within the 2.7-kb Ψ_{63} region targeted to *am* had become methylated de novo.

Interestingly, like ζ-η, Ψ_{63} induced methylation of *am* sequences at least 0.7 kb upstream of the insert. The absence of a band corresponding to the 0.7-kb 5′-junction fragment (y-z) in the *am* probing and the coincident appearance of the 3.8-kb fragment (x-c) confirmed the partial methylation seen at site y in the Ψ_{63} probing (Fig. 6A, lanes 10 and 12). Other fragments expected from the partial methylation were also detected in lanes 10 and 12 upon longer exposure. The 0.4-kb 3′-junction fragment (d-f) was detected with equal intensity in both *Mbo*I (lanes 9 and 11) and *Sau*3AI (lanes 10 and 12) digests, confirming that site d in the targeted copy of Ψ_{63} and site f in the 3′-flanking *am* DNA were unmethylated. Reprobing with the unmethylated *mtr* gene demonstrated that restriction enzyme digests were complete (lanes 13 to 18).

DISCUSSION

Although there has been keen interest in *cis*-acting signals for de novo methylation (15, 28, 34, 45, 46, 54, 55), it has not been easy to unambiguously attribute an observed effect to a particular test insert. To dissect methylation signals rigorously, it is necessary to test candidate signals in a way that avoids the

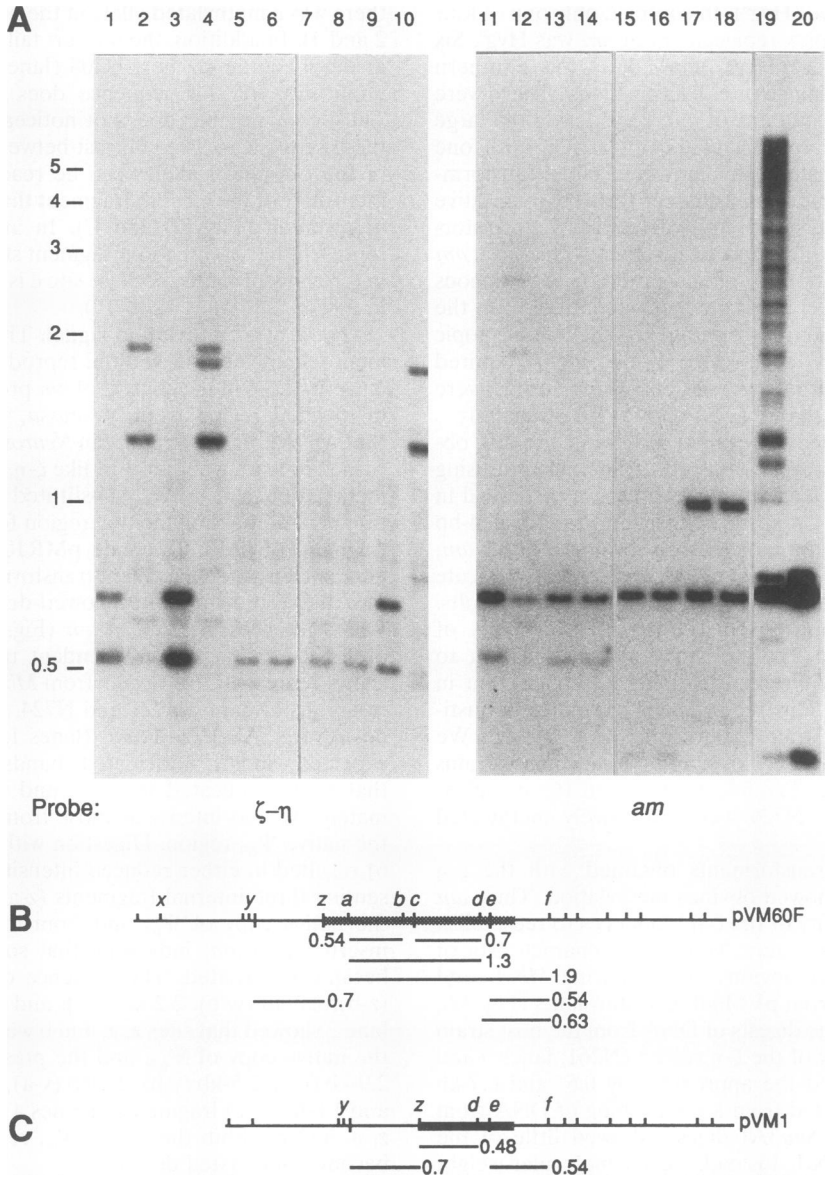

FIG. 5. Methylation associated with ζ-η. (A) Genomic DNAs from both host strains and strains transformed with fragments from the ζ-η plasmid pVM60F (N719 and N718), the θ plasmid pVM1 (N712), or the deletion plasmid pVM2 (N717 and N726) were digested with *Mbo*I (odd-numbered lanes from lane 1 to 17 plus lane 20) or *Sau*3AI (even-numbered lanes from lanes 2 to 18 plus lane 19). Lanes 1 and 2, N719; lanes 3 and 4, N718; lanes 5 and 6, N712; lanes 7 and 8, N408; lanes 9 and 10, N261; lanes 11 and 12, N718; lanes 13 and 14, N712; lanes 15 and 16, N717; lanes 17 and 18, N408; lanes 19 and 20, N726. Lanes 1 to 10 show hybridization to the 1.6-kb ζ-η region; lanes 11 to 18 show hybridization to the 2.6-kb *Bam*HI fragment of *am* (lanes 11 to 14, 17, and 18 are reprobings of lanes 3 to 6, 7, and 8, respectively). Faint bands present in both *Mbo*I and *Sau*3AI digests are due to hybridization to 5S rRNA genes (the ζ-η region includes two 5S rRNA pseudogenes [32]). Size standards are indicated in kilobases on the left. Digests were verified to be complete by reprobing with the *mtr* gene (not shown). (B) Map identifying major bands shown by hybridization of N718, N719, or N261 DNA with the ζ-η probe. (C) Map identifying major bands shown by hybridization of N712 DNA with the ζ-η probe.

extraneous variables associated with random integration of transforming DNA. In most eukaryotic transformation systems, control cannot be exerted over where and in how many places the transforming DNA integrates or over the number of copies that become integrated. Furthermore, there is normally no way to ensure that the transforming sequences do not rearrange, and thus potentially destroy a methylation signal or create one fortuitously. Vector and/or other foreign sequences typically integrate with the DNA of interest, further complicating interpretation. Researchers must be willing to assume that integration is relatively unbiased and that pooled data will give a good measure of methylation potential. To circumvent this problem, we developed an efficient targeting system to obtain *N. crassa* transformants that each contain a single, unrearranged copy of a test fragment, without extraneous DNA, at the same, defined place in the genome.

The *am* region was chosen as a site to test methylation signal candidates primarily because of the opportunity that it provided for rapidly selecting targeted replacements. Selection for replacement events is desirable because integration by homol-

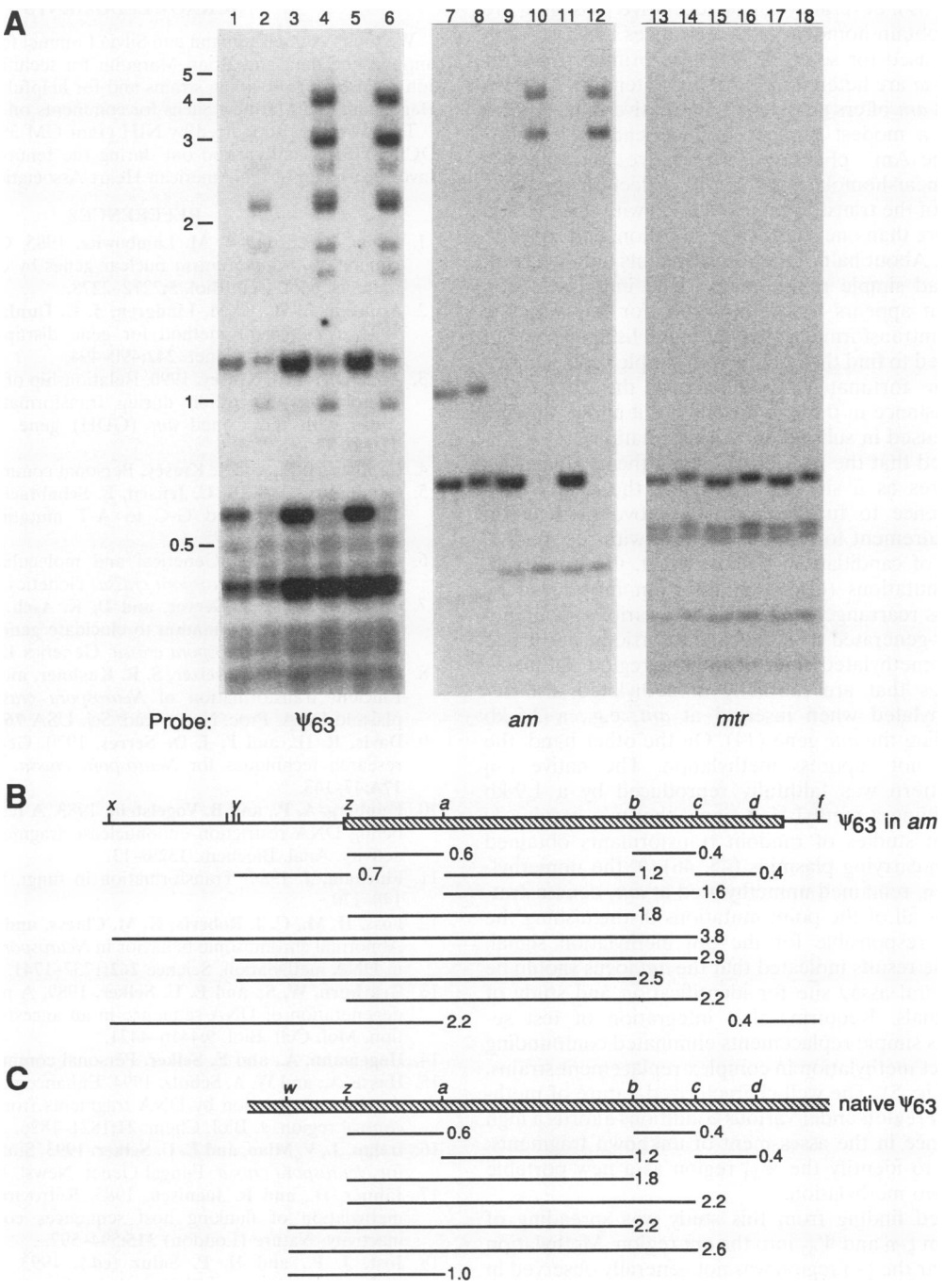

FIG. 6. DNA methylation of Ψ_{63} sequences inserted into the *am* gene. (A) Genomic DNA from two representative pMR10 (see Fig. 1) transformants (N721 [lanes 3, 4, 9, 10, 15, and 16] and N724 [lanes 5, 6, 11, 12, 17, and 18]) and the corresponding host strain (N408 [lanes 1, 2, 7, 8, 13, and 14]) were digested with *Mbo*I (odd-numbered lanes) or *Sau*3AI (even-numbered lanes) and hybridized to a 2.7-kb Ψ_{63} probe (lanes 1 to 6) or a 2.6-kb *am* probe (lanes 7 to 12). Probing of the blot with a 2.5-kb *mtr* region fragment (lanes 13 to 18) demonstrated that the restriction digests were complete. Size standards are indicated in kilobases at the left. (B) Map identifying major fragments detected from the *am* locus in N721 and N724. (C) Map identifying major fragments detected from the native Ψ_{63} locus in N408, N721, and N724 (30).

ogous recombination in *N. crassa* normally accounts for less than 1/10 of transformants, and precise replacement of sequences is even less common. For example, Paietta and Marzluf found that none of 10 targeted insertions at *am* selected from 117 primary Am^+ transformants were precise replacements (37). Some workers have suggested that the homologous integration of transforming DNA may be affected by strain background or marker effects (20, 35), but the strong tendency toward ectopic integration has been observed with most genes tested (2, 3, 6, 45). In some instances, special vectors requiring homologous integration for expression of a selectable phenotype have been used to select for targeted

insertions (3, 7, 39). Several cycles of vegetative passaging are still needed to obtain homokaryotic derivatives when a dominant marker is used for selection, because primary transformants of *N. crassa* are heterokaryotic. Our system for selecting replacements at *am* offers an efficient alternative that requires screening only a modest number of independent transformants. Since the Am^- phenotype is recessive, and only homokaryons or near-homokaryons survive selection on AAG medium, many of the transformants obtained with this method do not need more than one round of purification, and some do not require any. About half of the transformants obtained with our protocol had simple replacements. The initial selection with hygromycin appears to be important for reducing the background of untransformed cells (our unpublished data), but we were surprised to find that strains with simple replacements were Hyg^s. One fortunate consequence of the absence of hygromycin resistance in these strains is that it allows the *hph* marker to be reused in subsequent transformations.

We established that the region containing the *am* locus has desirable features as a site for evaluating the ability of a candidate sequence to function as a de novo methylation signal. The requirement for noninterference with the methylation potential of candidate signals was met. On one hand, neither small mutations (e.g., 4-bp insertion introduced by pMS4) nor gross rearrangements, including various spontaneous and in vitro-generated deletions and insertions, perturbed the normally unmethylated state of the *am* region. Other *N. crassa* sequences that are normally unmethylated also remained unmethylated when inserted at *am*, e.g., a 2.5-kb fragment including the *mtr* gene (14). On the other hand, the *am* region did not suppress methylation. The native ζ-η methylation pattern was faithfully reproduced by a 1.9-kb fragment containing a methylation signal that was previously recognized from studies of random transformants obtained with various ζ-η-carrying plasmids (45, 46). θ, the unmethylated allele of ζ-η, remained unmethylated at *am*, demonstrating that some or all of the point mutations distinguishing the two alleles are responsible for the ζ-η methylation signal. Altogether, these results indicated that the *am* locus should be useful as a general assay site for identification and study of methylation signals. Requiring that integration of test sequences occur as simple replacements eliminated confounding factors that affect methylation in complex-replacement strains, such as N726 (Fig. 5). The well-characterized nature of methylation in the *am* region under various conditions affords a high level of confidence in the assessment of unknown fragments, and allowed us to identify the Ψ_{63} region as a new portable signal for de novo methylation.

An unexpected finding from this study was spreading of methylation from ζ-η and Ψ_{63} into the *am* region. Methylation of sequences near the ζ-η region was not generally observed in other studies (45, 47, 48) but was noted in a few transformants in which ζ-η had cointegrated with bacterial sequences (46). It was unclear in those strains if methylation of pUC was dependent on ζ-η, or if it was dependent on the nature and/or site of integration. Spreading of methylation has been documented in several other instances (15, 17, 34, 55, 56).

Developing the *am* locus for assessing potential methylation signals has also resolved the question of whether local chromosomal rearrangements create signals for methylation in *N. crassa* (45). We found no evidence supporting this idea. The availability of the targeted-replacement system at *am* will allow us to proceed with dissection of methylation signals such as those in the ζ-η and Ψ_{63} regions. Results of these studies, in turn, should bring us closer to our goal of understanding the control of DNA methylation in *N. crassa*.

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