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9. The PBMCs of HIV$^+$ individuals or of HIV$^-$ controls resuspended at 3 × 10^6 per milliliter in RPMI 1640 (Gibco, Grand Island, NY) were either unstimulated or stimulated in vitro for 7 days with a mixture of five previously described antigenic synthetic peptides from Env of HIV-1 (T1, T2, Th4.1, P18IIIB, and P18MN) (*8*) or the Env nonantigenic peptide p23 at a final concentration of 5 μM/ml in sterile, 96-well flat-bottom culture plates (Costar, Cambridge, MA). In a second series of experiments, PBMCs were either unstimulated or stimulated for 7 days with influenza A virus (A/Bangkok/RX73 H3N2) (final concentration, 1:500), with a pool of irradiated [50 Gy (units of absorbed dose of ionizing radiation)] PBMCs from multiple donors (resuspended at 2 × 10^6 per milliliter) (ALLO), or with PHA (Gibco) (final concentration, 1:100). The PBMCs were stimulated in the absence or the presence of 20 U of recombinant human IL-12 per milliliter. For the IL-2 production assays, the IL-2 receptor monoclonal antibody TAC was added to the cultures (2 μg/ml) to prevent consumption of the IL-2 produced. Supernatants were harvested after 7 days and serially diluted, and the amount of IL-2 produced was assessed in a bioassay with the IL-2–dependent cell line CTLL as described (*5*). In the proliferation assays, the cell cultures were cultured for 5 days in 96-well flat-bottom plates, pulsed with 1 mCi of [^{3}H]thymidine, and harvested after 18 hours.
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11. PBMCs (3 × 10^6 per well) in a 24-well LINBRO plate were stimulated for 48 hours with PHA diluted 1:100 (Gibco). The PBMCs were stimulated in medium alone or in medium containing 20 U of recombinant IL-12 per milliliter. The amount of IFN-γ present in the supernatants was determined with a two-step ELISA assay. Briefly, Immulon plates (Dynatech Labs, Chantilly, VA) were coated overnight at 4°C with 100 μl per well of the A35 IFN-γ antibody (DNAX Research Institute) (final concentration, 5 μg/ml). Plates were washed five times in phosphate-buffered saline (PBS)–Tween and blocked with 150 ml of 10% fetal calf serum in PBS. Supernatants were diluted through three serial dilutions (1:2 to 1:8) in PBS-Tween, which contained the IFN-γ nitroiodophenyl-conjugated antibody B27 (final concentration, 5 μg/ml). A 100-μl aliquot of each sample was added to each well, and a standard for IFN-γ was run concurrently. Plates were washed with PBS-Tween five times. Horseradish peroxidase–conjugated antibody J4-HRP was diluted 1:3000 in PBS, and 100 μl was added to each well and incubated for 1 hour at 37°C. Plates were again washed with PBS-Tween. ABTS peroxidase substrate was diluted 1:1 with H_2O_2 (Kirkegaard-Perry, Gaithersburg, MD), and 100 μl of this mixture was added to each well. Plates were developed for 15 min and then read in an ELISA reader at a wavelength of 405 Å. Values for IFN-γ were calculated from a standard curve.
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Dense Nonsymmetrical DNA Methylation Resulting from Repeat-Induced Point Mutation in *Neurospora*

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Cytosine methylation has been implicated in epigenetic control of gene expression in animals, plants, and fungi. It has been assumed that all methylation in eukaryotes is at symmetrical sequences such as CpG/GpC, because this can explain perpetuation of methylation states. Here the bisulfite genomic sequencing method was used to examine methylation in DNA from a *Neurospora* gene exposed to repeat-induced point mutation. 5-Methylcytosine was not limited to symmetrical sites and individual molecules showed different patterns and amounts of modification. The methylation extended beyond the mutated region and even beyond the edge of the duplicated segment.

A fraction of the cytosines in the DNA of many organisms becomes methylated after replication. This methylation is not randomly distributed. Some sequences, such as the CpG islands of animals, are rarely if ever methylated (*1*). Others are methylated under certain circumstances, such as when the sequence is inherited from the mother rather than the father (*2*), and still others appear methylated in all tissues and at all times (*3*).

Neither the precise function nor the control of DNA methylation is understood in any eukaryote. In animals, most 5-methylcytosine (5mC) is found in the symmetrical sequence 5′CpG/3′GpC, and this is thought to be mediated by a maintenance methylase that preferentially recognizes and methylates hemimethylated sites. Such an enzyme could be responsible for propagating patterns of methylation (*4*). Indications of heterogeneous methylation in populations of cells thought to be clonally related suggest, however, that maintenance methylases cannot be solely responsible for persistence of methylation (*5–7*). There is evidence of substantial non-CpG methylation in plants (*8*) and fungi (*5, 9*) and of occasional non-CpG methylation in animals (*10, 11*). Nevertheless, it is generally assumed that all non-CpG methylation is in other symmetrical sequences, such as CpNpG (*12*), which might also be recognized by a maintenance methylase.

Results of Southern (DNA) hybridizations on DNA from the fungi *Neurospora crassa* and *Ascobolus immersus* suggested that methylation is not limited to symmetrical sites (*5, 9*). We have investigated this possibility directly in *N. crassa*. Whereas most of the *Neurospora* genome appears devoid of DNA methylation, heavily methylated chromosomal regions have been identified. At least some such regions are relics of repeat-induced point mutation (RIP), a process operating in the sexual phase of the life cycle that detects sequence duplications and riddles them with G:C to A:T mutations (*13*). We investigated the methylation of a product of RIP to explore the possible connection between DNA methylation and RIP and to further our understanding of methylation in eukaryotes.

Information about the distribution of methylated cytosines has come almost exclusively from Southern hybridizations on genomic DNA with restriction enzymes that are sensitive to 5mC. The development of several genomic sequencing methods made it possible to assess the methylation status of any cytosine in genomic

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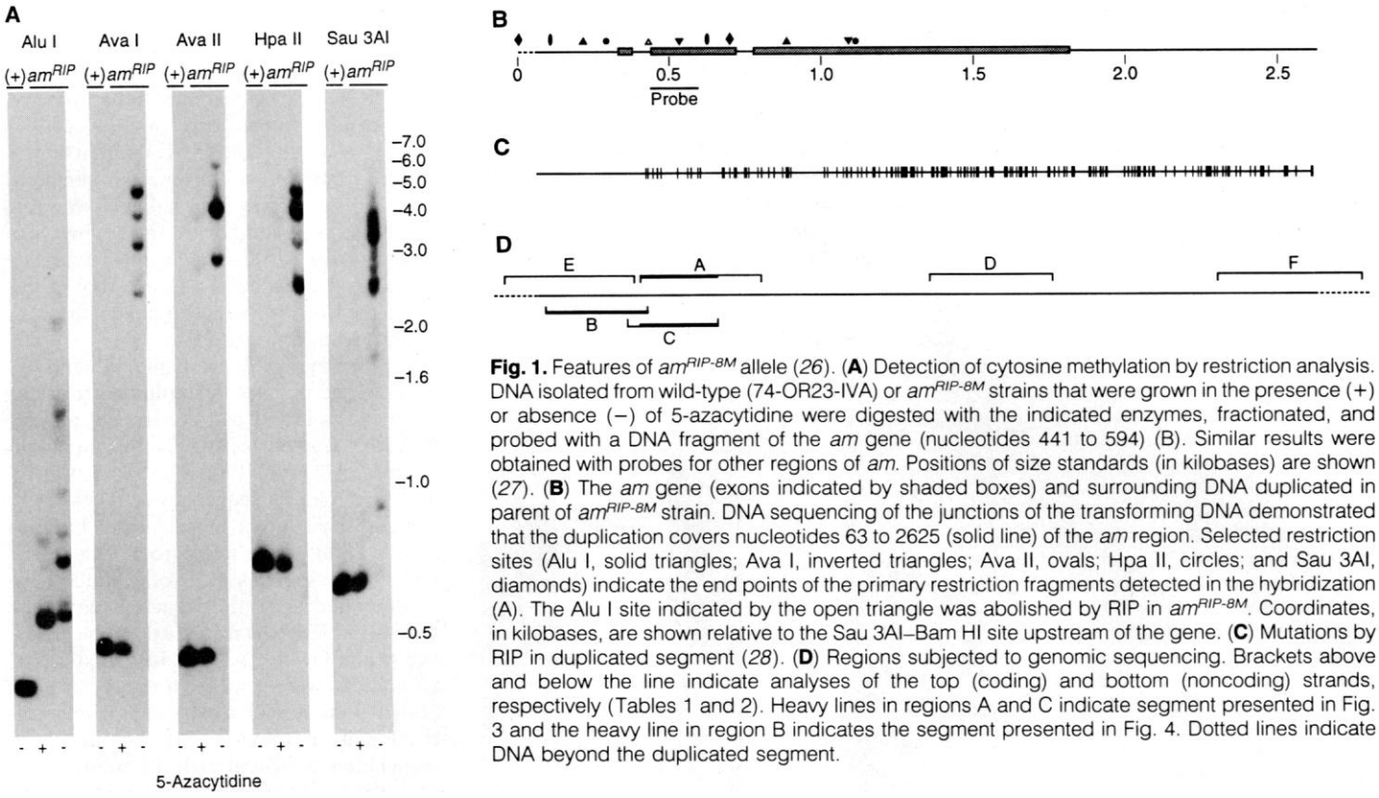

Fig. 1. Features of $am^{RIP\text{-}8M}$ allele (*26*). (**A**) Detection of cytosine methylation by restriction analysis. DNA isolated from wild-type (74-OR23-IVA) or $am^{RIP\text{-}8M}$ strains that were grown in the presence (+) or absence (−) of 5-azacytidine were digested with the indicated enzymes, fractionated, and probed with a DNA fragment of the *am* gene (nucleotides 441 to 594) (B). Similar results were obtained with probes for other regions of *am*. Positions of size standards (in kilobases) are shown (*27*). (**B**) The *am* gene (exons indicated by shaded boxes) and surrounding DNA duplicated in parent of $am^{RIP\text{-}8M}$ strain. DNA sequencing of the junctions of the transforming DNA demonstrated that the duplication covers nucleotides 63 to 2625 (solid line) of the *am* region. Selected restriction sites (Alu I, solid triangles; Ava I, inverted triangles; Ava II, ovals; Hpa II, circles; and Sau 3AI, diamonds) indicate the end points of the primary restriction fragments detected in the hybridization (A). The Alu I site indicated by the open triangle was abolished by RIP in $am^{RIP\text{-}8M}$. Coordinates, in kilobases, are shown relative to the Sau 3AI–Bam HI site upstream of the gene. (**C**) Mutations by RIP in duplicated segment (*28*). (**D**) Regions subjected to genomic sequencing. Brackets above and below the line indicate analyses of the top (coding) and bottom (noncoding) strands, respectively (Tables 1 and 2). Heavy lines in regions A and C indicate segment presented in Fig. 3 and the heavy line in region B indicates the segment presented in Fig. 4. Dotted lines indicate DNA beyond the duplicated segment.

DNA (*14*). Until recently, however, such methods were difficult and limited to measuring the approximate amount of methylation in a population of molecules. The development of a simple method (*15*) to determine the distribution of methylation in individual DNA molecules provided the tool needed to obtain a complete picture of DNA methylation in eukaryotic DNA. The technique uses sodium bisulfite under conditions that result in stoichiometric conversion of cytosine to uracil in single-stranded DNA, but which do not affect 5mC. To determine the positions of 5mC residues in a region of genomic DNA, we amplified the region from bisulfite-treated genomic DNA with polymerase chain reaction (PCR) and then sequenced it by standard techniques. The retention of cytosines reflects methylation, whereas the loss of cytosines reflects the absence of methylation. If the amplified DNA is cloned before the sequencing, methylation of individual molecules can be characterized to assess the heterogeneity of methylation directly.

Although RIP specifically acts on sequence duplications and occurs only in special premeiotic cells containing haploid nuclei from both parents, the methylation of affected sequences persists in vegetative cultures, even in products of meiosis that have retained only one member of the duplication (*16*). For our study we induced methylation of a well-characterized *Neurospora* gene, *am*, which encodes nicotinamide adenine dinucleotide phosphate (NADP)–specific glutamate dehydrogenase. A strain with the native copy of *am* plus one unlinked copy, which had been introduced by transformation (*16*), was crossed with a single-copy am^+ strain to induce RIP. Several Am^- strains (amination-deficient strains) were identified by growth tests and then characterized by Southern hybridization to identify single-copy segregants. We obtained preliminary information on methylation at *am* by probing digests of genomic DNA from strains grown in the presence or absence of 5-azacytidine (5AC), an inhibitor of DNA methylation, as illustrated for the allele chosen for detailed analysis, $am^{RIP\text{-}8M}$ (Fig. 1A). Analysis of $am^{RIP\text{-}8M}$ with a variety of enzymes sensitive to cytosine methylation, including Alu I, Ava I, Ava II, Hpa II, Sau 3A1, and Bgl II, revealed that most restriction sites in the previously duplicated region were completely resistant to cleavage unless the strain was grown in the presence of 5AC. These results implied that at every

Table 1. Regions subjected to genomic sequencing and primers employed.

Region*	Length (bp)	Strand	Primer 1†	Primer 2†
A (402–796)	395 bp	Top	CACCACCCTTRCCACCACC	AAGGGTAYGTYTGAGAGAAAAA
B (86–428)	343 bp	Bottom	CCTTCCATTCCRTCTRTCT	TGTAGTGAAAAATYAAGTYAg
C (355–658)	304 bp	Bottom	AAAATRTCTAACCTTCCCTCT	GAATRGAAAGGTTGAYGGAG
D (1352–1765)	414 bp	Top	CAACCTTAACRAAACCARCAA	YTYAAGTGGATYGAGGGTG
E (−47–379)	426 bp	Top	TTTTCTCTCARA**g**TACCCTT	ATGTTYGGAGGAYGGGGAAG
F (2308–2787)	481 bp	Top	CCTCTTRTACATCCATACRC	TTTGGYAGAAATYAGGGTAATA

*Sequence coordinates are given relative to Bam HI–Sau 3AI, upstream of the *am* promoter, 62 nucleotides outside of the duplicated region (Fig. 1). †R and Y denote A/G and C/T degeneracies in oligonucleotides, respectively; lowercase signifies that the base is not complementary to the templates; boldfaced G would pair only if the C on the complementary strand did not deaminate.

site a cytosine on at least one of the strands was methylated. With each enzyme, multiple fragments were detected in the non–5AC-treated samples even when small probes were used, implying that methylation was heterogeneous in the population of molecules. With the exception of Alu I, most of the restriction fragments produced with frequent-cutting enzymes were in the 2.5- to 4.5-kb range, suggesting that the methylation extended at least the full length of the duplicated segment. There was no evidence of methylation in the corresponding region from a wild-type strain (Fig. 1A).

Evidence of mutations in the region affected by RIP was observed in the restriction analyses (Alu I digests, Fig. 1, A and B). To identify all of the mutations, we isolated the entire duplicated region of $am^{RIP\text{-}8M}$, sequenced, and compared it with the known wild-type sequence (*17*). The 2.6-kb region contained 158 mutations (Fig. 1C). All of the mutations changed cytosines to thymidines on the coding strand, decreasing the G+C content of the region from 54 to 48%. Approximately 60% of the changes occurred at CpA dinucleotides, consistent with the known site specificity of RIP (*13*). No mutations occurred in the first 359 base pairs (bp) of the duplicated region, which includes the promoter region and first exon.

In preparation for genomic sequencing, we designed primers to amplify each strand in several selected parts of the *am* region, including segments spanning the end points of the duplication (Fig. 1D). The primers were designed for sequences with relatively few sites that might be subject to bisulfite modification on the strand to be examined, and at these sites, mixtures of G and A were incorporated into the oligonucleotides if they were designed to anneal to the modified strand (Table 1). Similarly, mixtures of C and T were incorporated in primers designed to anneal to the complement of the modified strand. Results of the DNA sequencing indicated that in most or all cases the expected strand was amplified. As a control to test whether methylated cytosines were indeed resistant to modification in our bisulfite reactions, we incorporated 5mC at every position in a template (Fig. 2). Neither C to T nor G to A changes were found, indicating that none of the 5mC residues had been deaminated. In cases in which no changes occurred, it is not possible to know which strand was

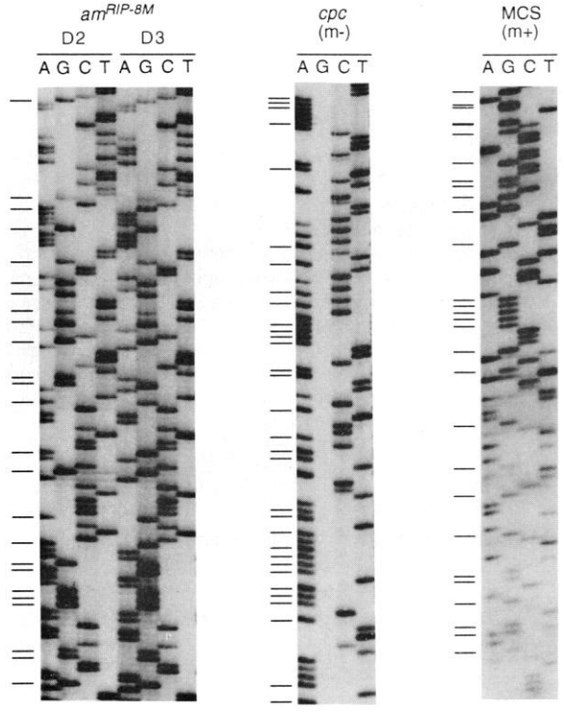

Fig. 2. Bisulfite genomic sequencing of a segment of $am^{RIP\text{-}8M}$ and methylated (m+) and unmethylated (m−) control templates. The results shown, for two molecules in region D (nucleotides 1655 to 1764) of $am^{RIP\text{-}8M}$, one molecule in the *Neurospora cpc* gene (nucleotides 219 to 313), and one molecule of a DNA segment (MCS) in which all cytosines had been replaced with 5mC's, were obtained from DNA treated in the same tube with sodium bisulfite (*29*). The resulting C:G to T:A mutations show up in these data as G to A changes because of the choice of primers. The lines to the left of the autoradiograms mark the positions of all C's (G's on strand shown) before the bisulfite reaction.

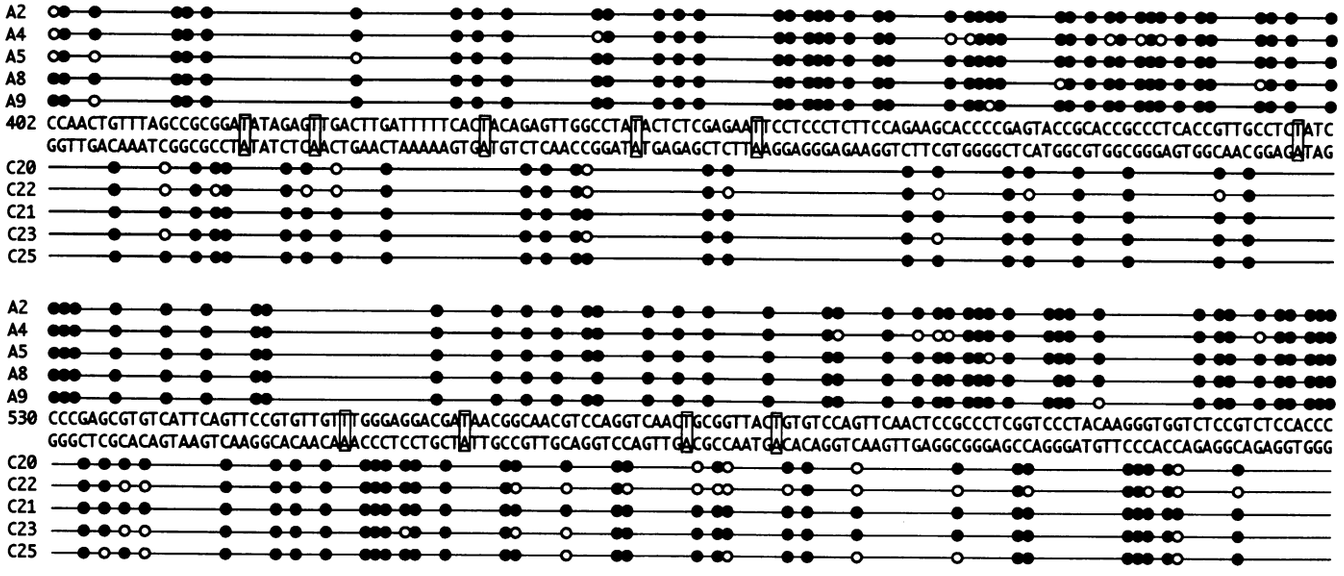

Fig. 3. Distribution of methylation on five coding (top) and five noncoding (bottom) DNA chains in a region mutated by RIP (overlap between regions A and C; Fig. 1D). Filled and open circles indicate methylated and unmethylated cytosines, respectively. The methylation status of one cytosine in molecule A8 was not ascertained. Mutations by RIP are boxed in the sequence.

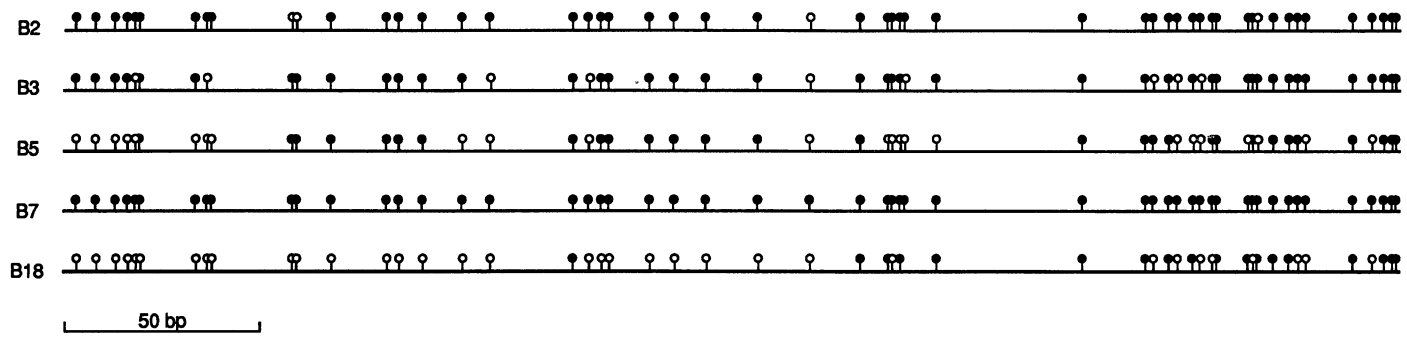

Fig. 4. Methylation beyond the region mutated by RIP. Filled and open lollipops represent methylated and unmethylated cytosines, respectively, in a 336-bp segment of region B (Fig. 1D) starting at position 86. Mutations by RIP start at nucleotide 422. The last five C/mC positions at the right end were also assessed in region C (Fig. 3). The methylation status of one cytosine in molecule B18 was not discerned.

amplified. Another control experiment was done to verify that all unmethylated cytosines were subject to modification. For this, we amplified a segment of *Neurospora* DNA in which no methylation was expected. We examined part of the *cpc1* gene (*18*). Every cytosine that was assayed was converted. This result indicated that the region was unmethylated and confirmed that the bisulfite reaction had gone to completion (Fig. 2).

Sample results of genomic sequencing in the $am^{RIP\text{-}8M}$ region are shown (Fig. 2) and additional data are summarized (Figs. 3 and 4 and Table 2). The results revealed three features of DNA methylation in *N. crassa*: (i) The pattern of DNA methylation in a region can be extremely variable. (ii) Methylation resulting from RIP can extend beyond the mutated region and even beyond the edge of the duplicated segment. (iii) 5mC is not limited to symmetrical sequences such as CpG or CpNpG. Methylation in *Neurospora* is not limited to one DNA strand or skewed toward one strand in any obvious way (Fig. 3). Most of the molecules assayed in each of the six regions of $am^{RIP\text{-}8M}$ examined showed methylation at more than 80% of the cytosines, and nearly half showed methylation at 95 to 100% of the cytosines. No particular sequences appear exempt from methylation. Although no striking pattern of methylation was apparent, the distribution of unmethylated sites does not appear completely random. As inferred from Southern hybridizations on relics of RIP (*5*), different sites appeared to vary in their amount of methylation. Moreover, some molecules showed considerably more methylation, overall, than did others. For example, the methylation of the DNA giving rise to clone B18 was low in comparison with that observed in other molecules in this border region and was distributed in a gradient with its low end toward the edge of the affected region (Fig. 4). The distribution of methylation in $am^{RIP\text{-}8M}$ did not appear closely correlated with the distribution of mutations by RIP (Figs. 1 and 3 and Table 2). For example, cytosines within a few base pairs of mutated sites were not preferentially methylated. Cytosines in the sequence contexts most prone to RIP (for example, CpA dinucleotides) (*13*) appear neither more nor less prone to methylation than cytosines in other contexts. Thus, DNA methylation in *N. crassa* does not show even limited sequence specificity comparable with the sequence preference of RIP in this organism. The absence of sequence specificity of methylation in *N. crassa* contrasts with the sequence specificity of methylation in animals and bacteria.

Table 2. DNA methylation in regions of $am^{RIP\text{-}8M}$

Clone	5mC's	C's	5mC (%)
	Region A		
2	110	1	99
3	111	0	100
4	99	13	88
5	107	5	96
8	107	3	97
9	109	3	97
	Region B		
2	51	4	93
3	44	9	83
5	31	24	56
7	55	0	100
18	20	34	37
	Region C		
20	55	17	76
21	71	1	99
22	41	31	57
23	60	12	83
25	61	11	85
	Region D		
1	50	18	64
2	69	16	81
3	83	2	98
5	81	4	95
9	84	16	81
	Region E		
1	139	10	93
2	143	7	95
3	130	19	87
4	146	3	98
5	130	19	87
	Region F		
2	93	0	100
6	93	0	100
10	87	6	94

Extensive methylation was found in sizable regions with no mutations (Fig. 4) and even beyond the ends of the duplication (*19*). This is consistent with the results of Southern hybridizations which suggested that methylation extended at least several hundred base pairs beyond the edge of the duplicated DNA (Fig. 1) (*20*). Methylated relics of RIP stand out because most of the *Neurospora* genome is unmethylated. We knew from previous work that the methylation of sequences altered by RIP that is observed in vegetative cells is somehow induced by the mutations. This conclusion came from transformation experiments in which mutated or pristine sequences were tested for their potential to induce DNA methylation in *Neurospora* de novo. This test has been done both with DNA fragments from a natural relic of RIP (*21–23*) and with several DNA sequences altered by RIP in the laboratory (*24*), including the $am^{RIP\text{-}8M}$ allele used in this study (*20*). Genomic sequencing of region C in a strain transformed with $am^{RIP\text{-}8M}$ confirmed that the methylation was equivalent to that in the original strain (*20*). We do not know how many of the 158 mutations are needed to induce methylation nor how such mutations can do so. It is clear from the genomic sequencing results, however, that the methylation signal created by one or more of the mutations can act at a distance of more than 400 nucleotides. This supports the idea that eukaryotic DNA methyltransferases do not take their cues directly from the sequences on which they act.

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19. Segments E and F (Fig. 1) include 105- and 100-bp segments upstream and downstream of the duplicated region, respectively. The amount of methylation detected by genomic sequencing in these two border regions was large (~95% and 99%, respectively), as in the interior regions. Caution must be exercised in drawing quantitative conclusions because of the small number of molecules examined and possible biases in the procedures.
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26. The $am^{RIP\text{-}8M}$ allele was isolated as a single-copy segregant from a cross of an am^{+} *lys1* strain (N261) and a strain (N276) that has the native *am* gene and an ectopic copy of *am* from transformant T-510-5.6 (*16*).
27. *Neurospora* DNA was isolated from cultures grown 16 hours in supplemented Vogel's minimal medium [1.5% sucrose, lysine (0.6 mg/ml), and alanine (1 mg/ml)] at 33°C with shaking from an innoculum of 2×10^6 conidia per milliliter. DNA methylation was prevented in some cultures by including 24 μM 5AC initially and adding an equal amount of 5AC after 4 hours. Procedures for DNA isolation and Southern hybridizations were as described (*21*). The blot was stripped and reprobed for the *qa-2* gene [E. Selker, D. Y. Fritz, M. J. Singer, unpublished results; (*25*)] to confirm that the digests had gone to completion.
28. We identified mutations by direct sequencing of amplified DNA with primers ~20 bp from the end points of the duplicated region. Amplification was achieved by 28 cycles of PCR (1 min at 50°C, 2 min at 72°C, and 1 min at 94°C) with ~0.5 μg of crude genomic DNA in 100 μl of reaction buffer (Promega) with 200 μM of each nucleotide triphosphate and ~0.5 μM of each primer. DNA sequencing was mostly done by cyclic sequencing {Promega *f* mole kit; [^{35}S]2′-deoxyadenosine triphosphate (dATP) incorporation method} with a single internal *am* primer and DNA purified from a low-melt agarose gel with Magic PCR Preps (Promega). Our sequencing of the wild-type and mutant *am* alleles revealed four differences relative to the published sequence (*17*) of this gene. We suggest the following modifications, none of which affect the protein coding region: change C at position 62 to G, add C at position 261, insert G between positions 2027 and 2028 (ATTGGTG), and remove G at 2498 (to give a run of only three G's).
29. In preparation for sodium bisulfite modification, 10 μg of DNA from *Neurospora* $am^{RIP\text{-}8M}$ plus 0.4 ng of a 0.3-kb segment of DNA in which 5mC was introduced at every C position were denatured in 235 μl of 0.1 M NaOH and 1 mM EDTA at 22°C. After 15 min the solution was neutralized by adding 50 μl of 1 M tris-HCl (pH 7.2). The DNA was ethanol precipitated, rinsed, dried, resuspended in 1.2 ml of 3.3 M sodium bisulfite, 0.5 mM hydroquinone (pH 5.0), incubated at 50°C for 23 hours, and dialyzed three times each (2 liters in degassed water; 4°C) against (i) 5 mM sodium acetate (pH 5.2), 0.5 mM hydroquinone, (ii) 0.5 mM sodium acetate (pH 5.2), and (iii) water. The DNA was then concentrated, treated with base, and worked up as described (*15*) except that the DNA was reprecipitated twice with ammonium acetate and ethanol. PCR reactions (generally 28 cycles of 1 min each at 52°C, 72°C, and 94°C) were carried out on 1 μl of modified DNA in 100 μl of reaction buffer (Promega) with 200 μM of each nucleotide triphosphate, ~0.5 μM of each primer (Table 1), and 5 U of Taq DNA polymerase (Promega). Poor yields were obtained when we attempted to amplify fragments larger than ~450 bp from the bisulfite-treated DNA. The product was gel purified with NA45 paper (Schleicher & Schuell), ligated into a "T-vector" (pT7-blue; Novagen), and introduced into DH5αF′ *Escherichia coli* cells by electroporation with a Bio-Rad apparatus and following their protocol. The methylated control template was made by PCR with deoxy-5-methylcytidine triphosphate, deoxyguanosine triphosphate, dATP, and deoxythymidine triphosphate (200 μM each) with M13-40 (NEB) and M13 reverse (U.S. Biochemical) primers and pBluescript SK^{+} plasmid (Stratagene) as a template. This template was reamplified from the modified DNA with the same primers. The 98-bp Kpn I–Sac I MCS fragment, which includes 32 C's on one strand and 30 on the other, was isolated and cloned into pDY1, a pBluescript-based plasmid with a readily identifiable insert in the polylinker. The cloned Kpn I–Sac I fragments and the various fragments derived from *Neurospora* DNA were sequenced on both strands with T7 and T3 primers (Stratagene). To test for completeness of the bisulfite modification, we amplified a segment of the *Neurospora cpc1* gene (nucleotides 168 to 481) (*18*) containing 79 C's on the relevant strand with primers of the following sequences: 5′-GATAGGGTGGATTTGGTTG and 5′-ACTCATACTTCCCCCTTTRRC. Sequencing of both unmethylated and methylated control templates was done for each batch of bisulfite-treated DNA. Several regions of $am^{RIP\text{-}8M}$ allele were sequenced with more than one batch of bisulfite-treated DNA, but all data presented came from a single reaction.
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Initiation at Closely Spaced Replication Origins in a Yeast Chromosome

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Replication of eukaryotic chromosomes involves initiation at origins spaced an average of 50 to 100 kilobase pairs. In yeast, potential origins can be recognized as autonomous replication sequences (ARSs) that allow maintenance of plasmids. However, there are more ARS elements than active chromosomal origins. The possibility was examined that close spacing of ARSs can lead to inactive origins. Two ARSs located 6.5 kilobase pairs apart can indeed interfere with each other. Replication is initiated from one or the other ARS with equal probability, but rarely (<5%) from both ARSs on the same DNA molecule.

Eukaryotic origins of replication are activated at different times during the period of DNA replication of the cell cycle (S phase). DNA fiber autoradiography experiments have suggested that adjacent origins are activated at about the same time (*1*, *2*). Such experiments do not examine specific origins, and they only detect adjacent origins when both are active in the same S phase. As part of a study of the temporal regulation of origin activation, we previously inserted *ARS1*, an origin activated early in S phase, next to a late activated origin, *ARS501*, located about 30 kb from a telomere. The *ARS1* sequences are late replicating in this new context (*3*). Using two-dimensional (2D) agarose gel electrophoresis, we analyzed the replication of this region to assess the level of origin activity at each ARS.

Three yeast strains were used in this study. They are isogenic except for the ARS content on the right arm of chromosome V. RM14-3a (*4*) has the normal chromosome structure, which includes *ARS501*, a single-copy origin approximately 30 kb from the telomere (Fig. 1). The 1.45-kb Eco RI *TRP1ARS1* fragment has been inserted at the Eco RI site, 6 kb telomere proximal to *ARS501*, to create the second strain, BF14-3a::*ARS1* (*3*), which now contains two ARSs, 6.5 kb apart. This strain was used to create a third strain in which *ARS501* was deleted by replacement of the Sna BI–Xho I ARS fragment with *URA3*

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