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Research Articles

A Portable Signal Causing Faithful DNA Methylation de Novo in *Neurospora crassa*

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Methylation of cytosine residues in eukaryotic DNA is common, but poorly understood. Typically several percent of the cytosines are methylated; however, it is unclear what governs which sequences eventually become modified. *Neurospora crassa* DNA containing the "zeta-eta" (ζ–η) region, which is a region of unusually heavy methylation, was tested for its ability to direct DNA methylation de novo. DNA stripped of its methylation by propagation in *Escherichia coli* was reintroduced into *Neurospora crassa* by transformation. The ζ–η region reproducibly became "properly" methylated whether inserted at its native chromosomal position or at ectopic sites. Adjacent *Neurospora* and bacterial sequences in the transforming DNA rarely became methylated. A model is presented that accounts for position-independent faithful methylation as observed in the ζ–η region, as well as position-dependent methylation, as occasionally observed, especially with sequences not native to *Neurospora*.

IN MANY AND POSSIBLY ALL ORGANISMS, DNA IS MODIFIED after synthesis by methylation of cytosine or adenine residues. Methylation alters physical properties of DNA, affects DNA-protein interactions, and has important biological consequences. In prokaryotes, DNA methylation is central to restriction-modification systems (*1*), provides for discrimination between new and old strands for DNA repair (*2*), and can be involved in gene regulation (*3*). In eukaryotes, a number of observations suggest connections between methylation and gene expression, but no clear causative relationships have been determined (*4*). The primary role of DNA methylation in eukaryotes remains to be established.

A serious gap in our understanding of DNA methylation in eukaryotes concerns what determines which sequences are destined to become methylated. In contrast to the situation in prokaryotes, eukaryotic DNA methylation does not appear limited to particular oligonucleotide sequences recognized by corresponding methyltransferases. Nevertheless, 5-methylcytosines are not randomly distributed in the eukaryotic genome. In animal DNA, for example, most of the cytosines located immediately preceding guanines (5′CpG) are methylated, and methylated cytosines at other positions are rare. This is thought to reflect a process that acts to perpetuate methylation patterns by methylating cytosines in newly replicated DNA at positions diagonally opposed to 5-methylcytosines (*5*):

(old) mCpG
(new) GpC m

Indeed, in several instances it has been found that when DNA that was methylated at every cytosine of one strand is introduced into animal cells and allowed to replicate, methylation was maintained, but only at CpG sites (*6*). Unmethylated transfecting DNA only infrequently became methylated (*7*). It has been suggested that de novo methylation of cellular DNA is also rare (*8*). Thus the distribution of 5-methylcytosine in eukaryotic DNA has been regarded as the sum of two, potentially independent, processes: de novo and maintenance methylation. In the most extreme scenario, the de novo methylase activity would make the "intelligent" decision concerning which sequences should become methylated, but only those sites recognized by the maintenance activity (possibly a subset of those methylated de novo) would remain methylated. Although this model is attractive, the distinction between de novo and maintenance methylation may be artificial; there is evidence that a purified eukaryotic methyltransferase can act on both unmethylated and methylated substrates, albeit at different rates (*9*). Whether or not most methylation found in eukaryotes is simply due to obedient maintenance, methylation remains to be established. In either case, a mechanism that depends on recognition must exist to set up the observed methylation patterns. A major unanswered question is what determines specificity of DNA methylation in eukaryotes.

We discovered a case of methylation in *Neurospora crassa* in which methylation is not limited to CpG sites or other sites of obvious

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symmetry that might be substrates for a maintenance methylation activity (*10*). The case involves the "zeta-eta" (ζ–η) chromosomal region cloned from an Oak Ridge (OR) laboratory strain of *Neurospora* (*11*). This region consists of an imperfect direct tandem duplication of a 0.8-kilobase-pair (kb) segment including one 5*S* RNA gene. Generally, *Neurospora* 5*S* RNA genes are unmethylated and dispersed in the genome (*12*), and only about 2 percent of cytosines in the *Neurospora* genome are methylated overall (*13*). In contrast, most cytosines in the ζ and η 5*S* RNA genes and in the rest of the duplicated DNA appear subject to methylation (*10*). The approximate 15 percent divergence between the ζ and η "duplicate" segments can be completely explained as a result of CG to TA mutations caused by deamination of 5-methylcytosines. Some strains of *Neurospora* have one instead of two copies of the DNA homologous to the ζ–η region, and in these the single copy is unmethylated. A genetic cross, in which one parent had duplicated methylated DNA at the ζ–η locus and the other had unduplicated unmethylated DNA at the corresponding position, produced progeny that had either duplicated methylated DNA or unduplicated unmethylated DNA, like the parental strains (*14*). This suggested that the ζ–η region originally cloned from an Oak Ridge strain includes a signal causing methylation, or lacks a signal preventing methylation. We have now tested this possibility by transforming *N. crassa* with unmethylated duplicated DNA from the ζ–η region. We have found that the DNA becomes faithfully methylated in vegetative cells, and proper methylation appears to be independent of chromosomal position. Thus, the ζ–η region contains a portable signal for DNA methylation.

Transformation of *Neurospora* with DNA from the ζ–η region. To determine whether the ζ–η region contains a signal for de novo methylation, we introduced unmethylated DNA into *N. crassa* and then checked for methylation of the transforming sequences. In *N. crassa*, DNA-mediated transformation is generally associated with integration of the transforming DNA by nonhomologous recombination (*15*). Nevertheless, integration of transforming DNA by homologous recombination is also observed. We took advantage of this situation to test for de novo methylation of the ζ–η region both at its native chromosomal location and at other chromosomal positions. To facilitate analysis of transformants, we used an *N. crassa* strain having unmethylated, unduplicated DNA at the ζ–η "locus" as the host for the transformation experiments. The chosen recipient strain (N24) also has a deletion removing the entire *am* (glutamate dehydrogenase) gene (*16*), thus allowing the use of this gene as a selectable marker in the *Neurospora* transformations. We tested for methylation by performing Southern hybridizations with restriction enzymes that fail to cut if particular nucleotides in their recognition sequences are methylated.

To introduce unmethylated ζ–η sequences into strain N24, we constructed a plasmid (pES174) containing the *N. crassa am* gene, pUC8 sequences, and the complete ζ–η region along with about 6 kb of adjacent sequences (flank) which are normally unmethylated in the *N. crassa* genome (Fig. 1). We transformed strain N24 with pES174 and characterized ten transformants (*17*). All but two of the ten transformants analyzed had single copies of the plasmid integrated in the genome (*18*). The other two contained several copies of pES174. Six of the single-copy integrants appear to have the entire plasmid integrated while in the remaining two, portions of the plasmid DNA had been deleted or rearranged.

One transformant, T-ES174-1, resulted from integration of the entire plasmid by homologous recombination in sequences adjacent to the ζ–η locus. That it had integrated in this region was indicated by the disruption of restriction fragments of the recipient strain. This can only be observed with restriction enzymes lacking sites in the region of homology between the plasmid and the host genome (Fig. 2A). Results from an analysis of T-ES174-1 with Eco RV, which lacks sites in the region of pES174 homologous to host sequences, indicate that integration of the plasmid disrupted the homologous chromosomal sequences (Fig. 2B). Enzymes having sites in the region of pES174 homologous to sequences in the host strain generated fragments that appeared to be the simple sum of fragments of pES174 and the host strain, as expected for integration

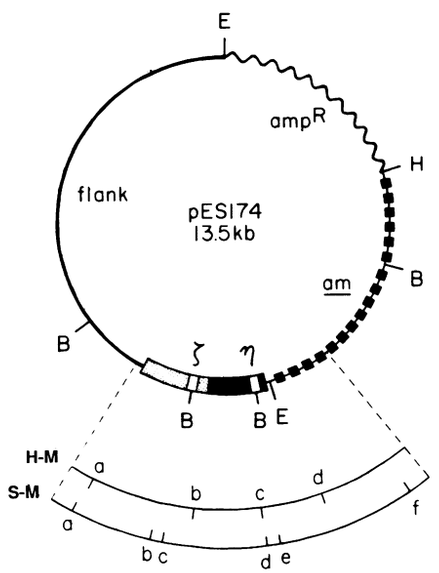

Fig. 1. Structure of pES174. Restriction sites for Eco RI (E), Bam HI (B), and Hind III (H) are indicated on the primary map; Hpa II–Msp I (H-M) and Sau 3A–Mbo I (S-M) sites are given for a critical region. The plasmid was constructed by ligation of an Eco RI fragment from pJR1 (*31*) having the *N. crassa am* gene region (heavy dashed line) and pUC8 sequences (wavy line) with an Eco RI fragment from pJS33 (*10*) containing the zeta-eta (ζ–η) region (wide lines) and flanking sequences (narrow solid line). The position of the ζ and η 5*S* RNA regions are shown as empty bars and the homologous 800-bp elements of which they are part are indicated by half- or full-tone lines, respectively. One degree equals 37.5 bp.

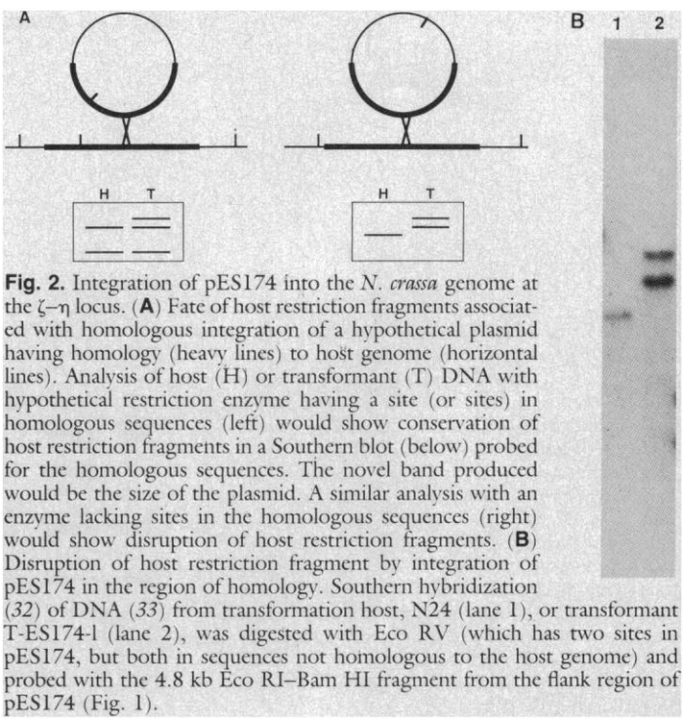

Fig. 2. Integration of pES174 into the *N. crassa* genome at the ζ–η locus. (**A**) Fate of host restriction fragments associated with homologous integration of a hypothetical plasmid having homology (heavy lines) to host genome (horizontal lines). Analysis of host (H) or transformant (T) DNA with hypothetical restriction enzyme having a site (or sites) in homologous sequences (left) would show conservation of host restriction fragments in a Southern blot (below) probed for the homologous sequences. The novel band produced would be the size of the plasmid. A similar analysis with an enzyme lacking sites in the homologous sequences (right) would show disruption of host restriction fragments. (**B**) Disruption of host restriction fragment by integration of pES174 in the region of homology. Southern hybridization (*32*) of DNA (*33*) from transformation host, N24 (lane 1), or transformant T-ES174-1 (lane 2), was digested with Eco RV (which has two sites in pES174, but both in sequences not homologous to the host genome) and probed with the 4.8 kb Eco RI–Bam HI fragment from the flank region of pES174 (Fig. 1).

by homologous recombination (*19*). Digests of the transformant DNA with either of two enzymes that cut pES174 at single sites in the flanking region (Kpn I or Mlu I) yielded a plasmid size band (13.5 kb), a result that is consistent with our conclusion that the plasmid integrated in a homologous manner (Fig. 2A).

De novo methylation of the ζ–η region at its native chromosomal position. The methylation status of transforming DNA was determined with the use of the restriction enzymes Sau 3A, Mbo I, Hpa II, and Msp I. Both Sau 3A and Mbo I recognize and cleave the sequence GATC when it is unmethylated, but Sau 3A will not cleave if the cytosine is methylated. Mbo I is insensitive to cytosine methylation but will not cleave if the adenine residue is methylated (*20*). Adenine methylation has not been observed in *N. crassa*.

Analysis of the plasmid sequences in T-ES174-1, the transformant that has one copy of pES174 integrated at the ζ–η locus, indicated that cytosines in the ζ–η region became faithfully methylated, de novo (Fig. 3). Plasmid DNA grown in *E. coli* was completely digested by Sau 3A (Fig. 3A, lane 1) demonstrating that plasmid sequences were not methylated prior to transformation of *Neurospora*. DNA samples from the wild-type strains ORS (lanes 2 and 5) and Mauriceville-1 (lanes 3 and 6) were included as controls. Oak Ridge strains (for example, ORS) have duplicated methylated DNA at the ζ–η locus, whereas the Mauriceville strain has unmethylated unduplicated DNA at this position (*14*). The transformation recipient (N24) has the unmethylated unduplicated allele derived from Mauriceville. The Mbo I digest of T-ES174-1 DNA, when probed with ζ–η, revealed just the bands expected from complete digestion of the ζ–η region of pES174 and the recipient strain (compare lanes 1, 6, and 7). This is evidence that the ζ–η allele was not rearranged in this transformant and confirms lack of adenine methylation at these sites. In contrast to the results with Mbo I, *incomplete* digestion of the ζ–η region of the transformant was observed with Sau 3A. Most hybridization was to fragments spanning one or more Sau 3A sites (fragments $\bar{a}f$, $\bar{b}f$, $\bar{a}d$, $\bar{d}f$, $\bar{b}d$). Relatively little hybridization was observed at positions corresponding to fragments resulting from complete digestion; indeed, most of the observed hybridization to fragment $\bar{a}b$ is from the unmethylated unduplicated allele of the host (*21*). Other sequences in the same samples were completely digested. Thus, cytosines in the ζ–η region became methylated de novo.

What is particularly striking is that the pattern of methylation in the ζ–η region introduced in pES174 is indistinguishable from that observed in the homologous DNA in the wild-type strain ORS. In both cases, the most prominent Sau 3A band came from the 1329-bp fragment a-d and the second strongest band resulted from the fragment extending from Sau 3A site a to the first site downstream of the ζ–η region (*22*). Fragment b-d also produced a strong band in Sau 3A digests of both ORS and T-ES174-1 DNA. In contrast, some other possible Sau 3A fragments were not represented. For example, the 624-bp a-c fragment, the 702-bp c-d fragment, and the 1704-bp c-f fragment did not appear, presumably as a result of complete blockage of Sau 3A site c, since the corresponding fragments extending to site b (530-bp fragment a-b, 794-bp fragment b-d, and 1796-bp fragment b-f) or site d (1329-bp fragment a-d and 1002-bp fragment d-f) were observed. Similarly, absence of the 1421-bp fragment a-e and the 886-bp fragment b-e reflects total methylation of Sau 3A site e. We conclude that in both the native

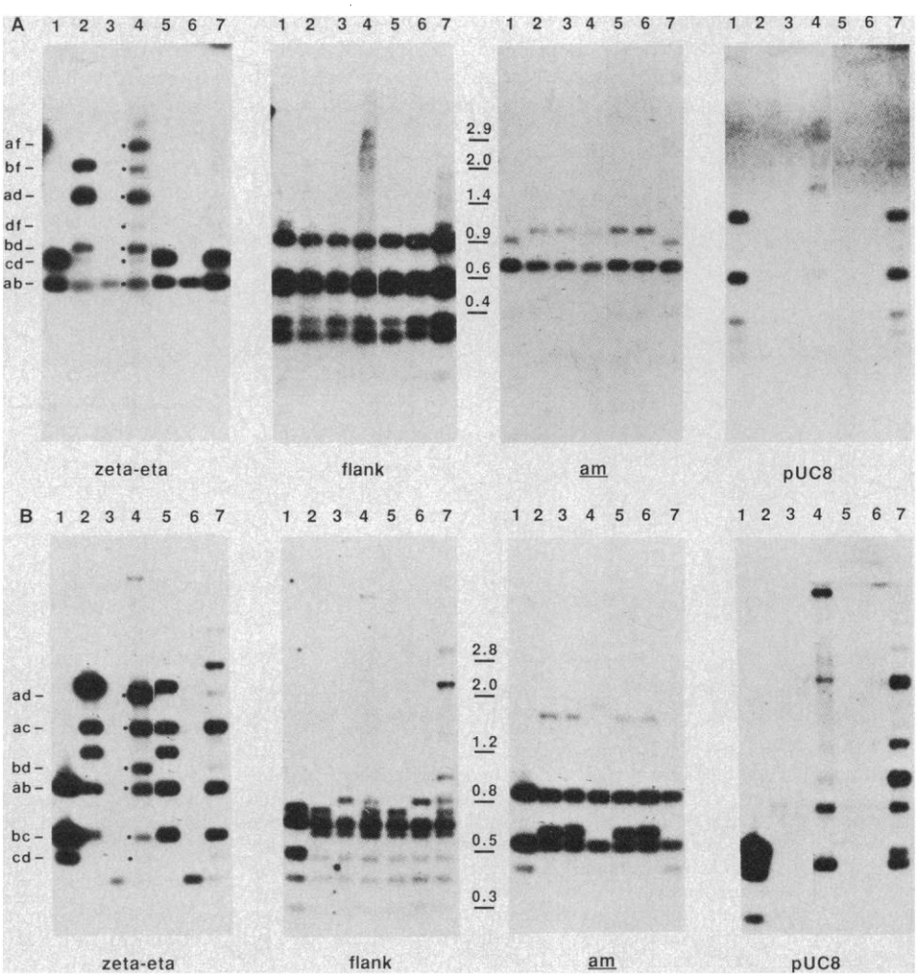

Fig. 3. Methylation of pES174 DNA integrated in the *Neurospora* genome by homologous recombination. DNA samples of pES174 (lane 1), wild-type *N. crassa* strains ORS a (FGSC 2490; lanes 2 and 5), and Mauriceville-1c A (FGSC 2225; lanes 3 and 6), or transformant T-ES174-1 (lanes 4 and 7) were digested with Sau 3A (part A, lanes 1 to 4), Mbo I (part A, lanes 5 to 7), Hpa II (part B, lanes 1 to 4), or Msp I (part B, lanes 5 to 7). Digests of *Neurospora* (0.5 μg) or plasmid (0.2 ng) DNA were fractionated on agarose gels, transferred to membranes, and probed for the regions indicated below the autoradiograph (*32*). Key fragments detected in the transformant DNA (asterisks, lane 4) are identified (left) by their position in the plasmid (Fig. 1). The sizes indicated are in kilobase pairs.

ζ–η region of strain ORS and the structurally homologous introduced copy in the transformant, cytosines at Sau 3A sites c and e (see Fig. 1) are methylated in essentially every cell, sites a and f are completely unmethylated, and sites b and d are methylated in an intermediate fraction of the cells.

A parallel situation was observed at Hpa II–Msp I sites (Fig. 3B). Hpa II and Msp I both recognize the sequence CCGG and cleave at this sequence if the interior or exterior cytosines are unmethylated, respectively. Hpa II sites b and c (Fig. 1) in the ζ–η region, which are methylated in Oak Ridge wild-type strains, were specifically methylated in the transforming DNA. Site a was completely sensitive to Hpa II digestion, as was site d, in the adjacent *am* region. Consequently, Hpa II fragment a-d was the predominant fragment detected with the ζ–η probe (Fig. 3B, lane 4). Also notable are fragments a-c and b-d, which are indicative of methylation of Hpa II sites b and c, respectively.

Additional evidence that the methylation observed in the ζ–η region was not due to nonspecific methylation of transforming DNA came from reprobing the blots sequentially with other regions of pES174. In wild-type strains (for example, Mauriceville and ORS), sequences adjacent to the ζ–η locus (flank) are normally unmethylated (Fig. 3, A and B, flank, lanes 2, 3, 5, and 6). In transformant T-ES174-1 this region stayed largely, although not completely, unmethylated (lanes 4 and 7). The flanking regions of ORS and Mauriceville include restriction fragment length polymorphisms that allow us to verify that both copies of the flanking region are represented in transformant T-ES174-1. This is most evident in Hpa II or Msp I digests (Fig. 3B, flank).

Sequences on the opposite side of the ζ–η region, that is, in and around the *am* gene, remained completely unmethylated. The faint bands in Sau 3A and Hpa II digests of T-ES174-1 DNA (lane 4), not seen in corresponding digests of the plasmid (lane 1), represent fragments spanning the *am*-η junction and are due to methylation of sites in the ζ–η region. In addition to the ζ–η region, the bacterial-derived sequences (pUC8 region) also showed significant methylation in T-ES174-1. Although the bacterial sequences became methylated de novo in this transformant, this is not the general case.

The ζ–η methylation signal is portable. The ζ–η region became faithfully methylated, de novo, when it was inserted at its native chromosomal position. Most likely, some feature of the ζ–η region triggers its methylation. However, because the observed methylation may have been dependent on some local feature of the integration site, we analyzed transformants that resulted from nonhomologous integration of the plasmid at various sites. Hybridizations of transformant DNA digested with restriction enzymes having 6-bp recognition specificities suggested that each of the transformants resulted from distinct integrations of the transforming DNA. An example of a diagnostic Southern hybridization on the six transformants with single copies of the entire plasmid integrated is shown (Fig. 4). Samples of each DNA were digested with the enzymes Hpa I or Bam HI. The plasmid pES174 lacks Hpa I sites, and in strain N24 the sequences homologous to the probe (flank) are in an approximately 30-kb Hpa I fragment. Restriction digests with Hpa I were performed to determine the number of integration sites of the plasmid in each transformant. T-ES174-5, -8, -9, and -10 each show one band in addition to the host band (H_h), reflecting one site of integration in each of these transformants (*23*). The absence of new bands in Hpa I digests of T-ES174-1 and -3 suggests that the plasmid integrated into large Hpa I fragments (the ~30-kb fragment at the ζ–η locus in the case of T-ES174-1). Southern hybridization analyses with probes representing all parts of the plasmid, and with a variety of enzymes, established that all of the transformants except T-ES174-1 resulted from integration of pES174 by nonhomologous recombination. Results with Bam HI provide an example of this analysis (Fig. 4). In each case, one plasmid-derived band (B_p) was replaced by two new bands representing novel junction fragments. Host strain restriction fragments from the ζ–η and flanking regions were conserved, consistent with the conclusion that, in these transformants, the plasmid had integrated at various ectopic sites. Further analysis allowed us to conclude that in three of five transformants resulting from integration of entire pES174 at ectopic sites (T-ES174-3, -5, and -8) integration disrupted the flank region, one (T-ES174-9) disrupted the 220-bp Bam HI–Eco RI fragment at the junction between ζ–η and *am*, and one (T-ES174-10) disrupted the *am* region (*24*).

All of the transformants that we analyzed, including several not illustrated, showed faithful methylation of the ζ–η region (Fig. 5). In sharp contrast, neither the *am* region nor the sequences upstream of the ζ–η region (flank) became methylated (*25*). The pES174 sequences derived from pUC8 remained unmethylated in most, but not all cases (Fig. 5). To determine whether absence of methylation in the *am* region was due to the selection for function of this region, we isolated and analyzed another set of pES174 transformants obtained without selection for *am*. These were obtained by cotransformation, selecting only for a marker (*Bml*) on the cotransformed plasmid, pBT6 (*26*). None of three cotransformants analyzed showed methylation in the *am* region, whereas methylation in the ζ–η region was normal (*27*).

What triggers DNA methylation? Although there is considerable information on the distribution of DNA methylation in eukaryotes, essentially nothing is known about what governs which

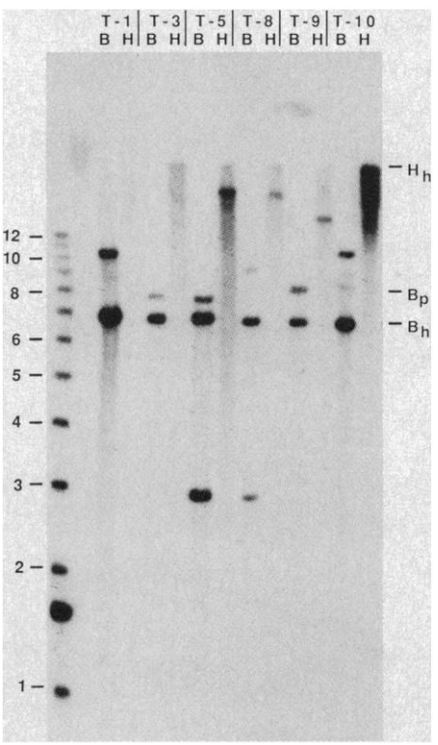

Fig. 4. Integration of pES174 in six single-copy transformants. DNA samples of the transformants (T-ES174-1, -3, -5, -8, -9, and -10) were digested with Bam HI (labeled B) or Hpa I (H), fractionated on a 0.7 percent agarose gel and probed with the 4.8-kb Eco RI–Bam HI fragment from the flank region of pES174 (Fig. 1). Positions of Bam HI and Hpa I fragments from the host strain (B_h and H_h, respectively), and the Bam HI fragment from the plasmid (B_p) detected with the flank probe, are indicated on the right; sizes (kilobases) of markers are given on the left. The very large fragments (for example, the ~30-kb Hpa I fragment) appear faint (Fig. 2A) because their sizes are similar to that of undigested *Neurospora* DNA.

sequences that are to become methylated. If it is true that most of DNA methylation in eukaryotes is due to an undiscerning "maintenance" methylation activity, this would be expected to complicate identification of primary signals earmarking particular sequences for methylation. Nevertheless, as shown by our results, at least in the case of *N. crassa*, it is possible to identify segments of chromosomal DNA containing sequences that reproducibly act as specific cues for DNA methylation. In particular, we have demonstrated that the ζ–η region includes a portable signal resulting in faithful methylation de novo and that the signal is active in vegetative (haploid) cells. This rules out methylation models in which recognition of the methylation signal depends on comparison between homologous chromosomes. Whether the signal is a discrete nucleotide sequence or a global feature of the region is not yet clear. The methylation might be directed by the DNA sequence itself or else by a higher order structure, as discussed below.

The ζ–η methylation signal could act either positively or negatively. That is, the region may include a feature causing methylation, or it may lack a feature preventing methylation. As an example of the latter, one could imagine that absence of transcription could lead to methylation, although this particular possibility is unlikely since we know of transcriptionally inactive regions that are not methylated (*28*). We cannot yet distinguish between these possibilities; nevertheless, a positive-acting signal seems more likely, since transforming DNA from sources other than *Neurospora* (for example, pUC8 sequences) do not consistently become methylated.

A critical distinction between DNA methylation in prokaryotes and eukaryotes is that, in the former, any given site is normally either methylated in every cell or else unmethylated, whereas in the latter it is not uncommon to find methylation in some percentage of the cells examined. In animals and other complex organisms, an intermediate level of methylation may simply reflect a mixture of cell types in a heterogeneous tissue. However, in *Neurospora* we have observed intermediate levels of methylation in germinating conidia, a "tissue" which would normally be regarded as homogeneous. It is unlikely that the observed heterogeneity reflects minor physiological differences among the cells since apparently identical methylation "spectra" are found in cultures younger than those used in these studies (*27*). That sites in the ζ–η region are rarely, if ever, methylated in all cells was a clue to the existence of a "discriminating" de novo methylase activity in *Neurospora* (*14*).

The numerous methylated sites in the ζ–η region each exhibit a characteristic level of methylation. An important observation from our experiments is that the characteristic pattern of methylation in the ζ–η region occurs regardless of position of the region in the genome. This pattern may simply reflect substrate preferences of a limiting methyltransferase, or it may be due to more subtle regulation. In any case, the observed heterogeneity with respect to

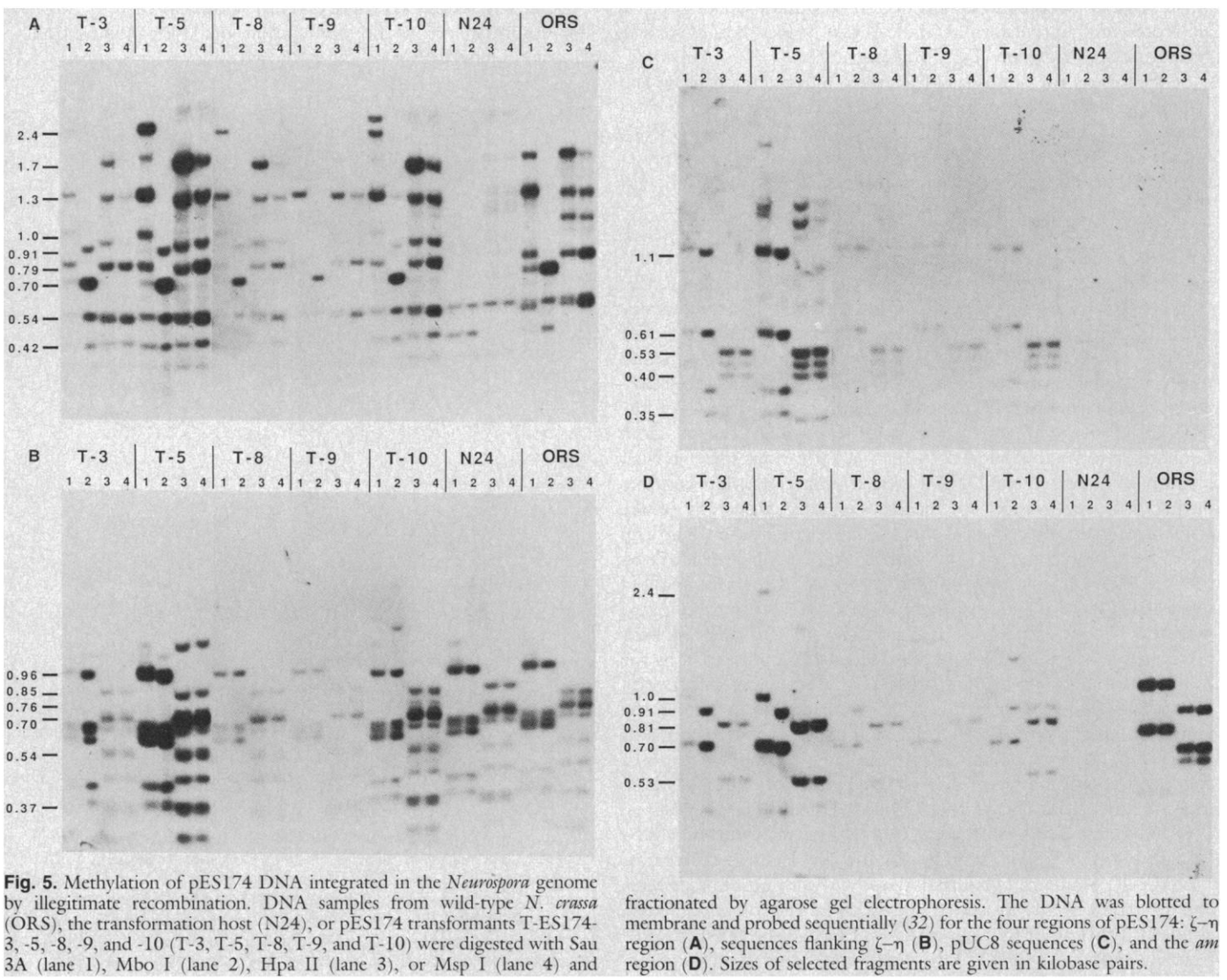

Fig. 5. Methylation of pES174 DNA integrated in the *Neurospora* genome by illegitimate recombination. DNA samples from wild-type *N. crassa* (ORS), the transformation host (N24), or pES174 transformants T-ES174-3, -5, -8, -9, and -10 (T-3, T-5, T-8, T-9, and T-10) were digested with Sau 3A (lane 1), Mbo I (lane 2), Hpa II (lane 3), or Msp I (lane 4) and fractionated by agarose gel electrophoresis. The DNA was blotted to membrane and probed sequentially (*32*) for the four regions of pES174: ζ–η region (**A**), sequences flanking ζ–η (**B**), pUC8 sequences (**C**), and the *am* region (**D**). Sizes of selected fragments are given in kilobase pairs.

methylation may provide a clue to the role of DNA methylation in eukaryotes (*10*). In particular, it seems unlikely that methylation of any individual site would be critical. Findings suggesting that 5-methylcytosines are hot spots for mutation in eukaryotes lends support to this idea since any phenomenon dependent on a single 5-methylcytosine should be doomed.

Operationally, we have observed two types of de novo DNA methylation in *Neurospora* transformation experiments. In the case of the ζ–η region, consistent position-independent methylation was observed, indicative of a portable methylation signal. In the case of the pUC8 sequences, position-dependent methylation was observed, as evidenced by occasional and inconsistent methylation of these sequences. We and others have found that position-dependent methylation of transforming DNA is not uncommon in *Neurospora* (*29*). We propose that position-independent and position-dependent methylation are manifestations of a common mechanism that has the effect of methylating a class of rearranged sequences. *Neurospora* chromosomes may be arranged in domains defined by hypothetical elements occurring periodically along the DNA such as origins of DNA replication or scaffold attachment sites (*30*). If the spacing of these elements were radically perturbed by an insertion or other rearrangement, the distorted region would become methylated. According to this model, inserted DNA lacking the hypothetical element (possibly pUC8 sequences, for example) would likely trigger methylation, depending on the size and position of the insertion. In contrast, inserted DNA including a copy of the hypothetical element (possibly the *am* region, for example) would rarely if ever become methylated. Finally, sequences containing abnormal or inappropriately spaced elements would function as portable signals for DNA methylation, as exemplified by the ζ–η region.

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17. Strain N24 (am_{132}, *inl*, A), which has the unmethylated unduplicated allele at the ζ–η locus [E. U. Selker and J. N. Stevens, *Mol. Cell Biol.* **7**, 1032 (1987)] was transformed with the use of standard techniques [R. A. Akins and A. M. Lambowitz, *Mol. Cell Biol.* **5**, 2272 (1985); M. E. Case, M. Schweizer, S. R. Kushner, N. H. Giles, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5259 (1979)]. Transformants were selected at 25°C on medium containing 20 m*M* glycine. Approximately 1000 transformants were obtained from 1 μg of pES174 DNA.
18. Transformants of *N. crassa* are typically heterokaryotic in that not all nuclei contain the transforming DNA. This may be due to instability of transforming sequences or to propagation of untransformed nuclei in transformed cells (or both) [D. M. Grant, A. M. Lambowitz, J. A. Rambosek, J. A. Kinsey, *Mol. Cell Biol.* **4**, 2041 (1982); M. E. Case, *Genetics* **113**, 569 (1986); E. U. Selker, E. B. Cambareri, B. C. Jensen, K. R. Haack, *Cell*, in press]. Whenever possible, we isolated homokaryotic derivatives of our transformants to simplify their characterization. Homokaryotic derivatives were identified by scoring *am* expression in "colonies" obtained by plating conidia of vegetative reisolates. Cultures producing exclusively am^+ conidia were considered homokaryotic. Crossing the strains to obtain homokaryons was avoided since it has been observed that transforming DNA frequently becomes deleted or rearranged when *N. crassa* transformants are crossed [M. E. Case, *Genetics* **113**, 569 (1986); E. U. Selker, E. B. Cambareri, B. C. Jensen, K. R. Haack, *Cell*, in press]. Slot-blot [P. C. Brown, T. D. Tlsty, R. T. Schimke, *Mol. Cell Biol.* **3**, 1097 (1983)] and Southern hybridizations were performed on DNA from transformants to determine the copy number, location, arrangement, and state of methylation of pES174 sequences. Southern hybridizations of undigested DNA of the transformants indicated that in each case plasmid sequences were associated with the chromosomal DNA. No evidence was found for stable autonomously replicating DNA homologous to the plasmid.
19. Junction fragments were not detected with any of the 17 restriction enzymes tested in agreement with the conclusion that the plasmid had integrated by homologous recombination. For many of these analyses we used DNA from T-ES174-1 grown in the presence of 5-azacytidine (which inhibits DNA methylation) since methylation of portions of the plasmid obscured sites (see below).
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21. Hybridization of Bam HI digests (Fig. 4) with a ζ–η probe, indicated that the (unduplicated) host allele remained unmethylated in all the transformants.
22. Because of the juxtaposition of *am* sequences next to the ζ–η region in pES174 (Fig. 1), different sized fragments are generated from wild-type and transformant DNA samples. Thus, the fragment extending from Sau 3A site a to the first site downstream of the ζ–η region (Sau 3A–Mbo I site f in the case of pES174) (Fig. 1) is 1840 bp in the wild type (Fig. 3A, lane 2) and 2330 bp in the transformant (lane 4).
23. Reprobing of the membrane with a probe for pUC8 sequences revealed a distinct Hpa I band in T-ES174-10 (at a position corresponding to an approximately 20-kb fragment); this band was apparently obscured by the dark smear resulting from the probing with the flank fragment.
24. Transformant DNA was digested with Bam HI and other enzymes to determine what sequences of the plasmid, if any, were disrupted because of integration by nonhomologous recombination. In the case of T-ES174-3, -5, and -8, integration disrupted the flanking region of the plasmid (revealed in Bam HI, Mbo I, and Hpa II digests). T-ES174-9 showed integration into the 220-bp Eco RI–Bam HI fragment spanning the η-*am* junction (revealed by Mbo I, Hpa II, and Eco RI plus Bam HI digests), and in T-ES174-10, integration occurred near the Bam HI site in the *am* region (determined from Bam HI, Apa I, and Hpa II digests).
25. Methylation of sites adjacent to the *am* region (in the ζ–η region) is responsible for the minor differences between Mbo I and Sau 3A digests detected with the *am* probe.
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28. For example, the 5*S* RNA pseudogene, ψ4, is not methylated; E. U. Selker, J. N. Stevens, R. L. Metzenberg, in *Molecular Genetics of Filamentous Fungi*, W. Timberlake, Ed. (Liss, New York, 1985), pp. 309–319.
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30. See for example, S. M. Gasser and U. K. Laemmli, *EMBO J.* **5**, 511 (1986); P. N. Cockerill and W. T. Garrard, *Cell* **44**, 273 (1986).
31. J. A. Kinsey and J. A. Rambosek, *Mol. Cell Biol.* **4**, 117 (1984).
32. Probes were prepared by the random oligomer-primer method [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)] and hybridizations were performed as described [E. U. Selker and J. N. Stevens, *Mol. Cell Biol.* **7**, 1032 (1987)]. Nylon membranes were routinely probed again after being stripped with 0.4*M* NaOH (42°C; 30 minutes) and neutralized in 0.1× SSC, 0.5 percent SDS, 0.2*M* tris, *p*H 7.5 (42°C; 30 minutes).
33. *Neurospora* DNA was isolated by a modification of a procedure by M. G. Murray and W. Thompson [*Nucleic Acids Res.* **8**, 4321 (1980)]. Lyophilized tissue from a 50-ml culture grown in supplemented Vogel's medium [R. H. Davis and F. J. DeSerres, *Methods Enzymol.* **17A**, 47 (1970)] was ground to a fine powder, suspended in 2 ml of extraction buffer (0.7*M* NaCl, 1 percent hexadecyltrimethylammonium bromide, 50 m*M* tris-HCl, *p*H 8.0, 10 m*M* EDTA, 1 percent 2-mercaptoethanol) and held at room temperature for 10 minutes. The mixture was then extracted twice with a mixture of phenol, $CHCl_3$, and isoamyl alcohol (50:49:1), and the nucleic acids were precipitated with isopropanol, rinsed with ethanol, and resuspended in 200 μl of TE (10 m*M* tris, 1 m*M* EDTA, *p*H 7.5) containing ribonuclease (100 μg/ml). After 30 minutes at 37°C, the solution was extracted with chloroform and isoamyl alcohol (24:1) and the DNA was precipitated twice with ethanol, rinsed, dried, and resuspended in TE. DNA concentrations were determined by a fluorometric method [J. E. Puzas and D. B. P. Goodman, *Anal. Biochem.* **86**, 50 (1978)].

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