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Signal for DNA Methylation Associated with Tandem Duplication in *Neurospora crassa*

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Most cytosine residues are subject to methylation in the zeta-eta (ζ-η) region of *Neurospora crassa*. The region consists of a tandem direct duplication of a 0.8-kilobase-pair element including a 5S rRNA gene. The repeated elements have diverged about 15% by the occurrence of numerous CG to TA mutations, which probably resulted from deamination of methylated cytosines. Most but not all common laboratory strains of *N. crassa* have methylated duplicated DNA at the ζ-η locus. However, many strains of *N. crassa* and strains of *N. tetrasperma*, *N. sitophila*, and *N. intermedia* have one instead of two copies of the homologous DNA and it is not methylated. A cross of strains differing at the ζ-η locus produced progeny which all had duplicated, methylated, or unique, unmethylated DNA, like the parental strains. We conclude that a signal causing unprecedented heavy DNA methylation is present in the ζ-η region.

Postreplicative modification of DNA is widespread and may be ubiquitous. In procaryotes, methylation at the 5 position of cytosines or the 6 position of adenines is the basis of modification/restriction systems (1). DNA methylation also plays a central role in mismatch repair (12) and regulation of transposition (15) in bacteria. DNA methylation in eucaryotes differs from that in procaryotes in several ways: (i) only methylation of cytosine residues is generally observed; (ii) methylation is limited almost exclusively to cytosine residues preceding guanines and in many cases most of these cytosine residues are methylated; (iii) no specific recognition sequences analogous to restriction sites in bacterial DNA have been found; (iv) a given site is rarely completely methylated; that is, it is not methylated in all cells even in an apparently homogeneous tissue; (v) at least in animal cells, de novo methylation of unmethylated DNA is uncommon; and (vi) relatively nonspecific "maintenance" methylation of hemimethylated DNA is widespread (see reference 13 for reviews). There is evidence that DNA methylation affects gene expression in eucaryotes, and methylation has been invoked to help explain a variety of poorly understood biological processes such as determination and differentiation in development, aging, gene and chromosome inactivation, DNA repair, and genetic recombination (see references 6, 9, 14, and 22). It seems likely that DNA methylation plays multiple roles in eucaryotic cells.

We have recently discovered a chromosomal region of *Neurospora crassa* having an unprecedented level of cytosine methylation associated with a pair of 5S rRNA genes, zeta (ζ) and eta (η) (17, 19). The ζ-η region consists of a diverged direct tandem duplication of a 0.8-kilobase-pair (kb) segment including one 5S rRNA gene. 5S rRNA genes are generally unmethylated in *N. crassa* (18) and only approximately 2% of cytosine residues in the genome are methylated overall (16). In the ζ-η region, however, both 5S rRNA genes are methylated and most cytosine residues are subject to methylation (E. U. Selker, unpublished data); methylation is not confined to positions preceding guanines. The approximately 15% divergence between the ζ and η 0.8-kb elements can be explained as a result of numerous exclusively CG-to-TA mutations, which likely resulted by deamination of 5-methyl cytosines (17). No methylation has been detected in the unique DNA flanking the duplication. The apparent coincidence of the range of methylation and the extent of the ζ-η duplication suggested that a signal causing methylation may have been generated by the duplication event. This prompted us to look for strains lacking the tandem duplication with the aim of testing the hypothesis that a signal causing methylation is in the ζ-η region. Here we report that many *Neurospora* strains, including some common laboratory strains and distant relatives of present laboratory strains, have one instead of two copies of the DNA homologous to the ζ-η region and that, without exception, the homologous unique DNA is unmethylated. Examination of progeny from a cross of strains having duplicated, methylated, or unique, unmethylated DNA at the ζ-η locus indicates that a signal causing DNA methylation is present at or near the ζ-η region.

MATERIALS AND METHODS

Neurospora strains were generally obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City); stock numbers are listed in Table 1. Vegetative cultures were grown to stationary phase at 30°C in Vogel N medium (7), supplemented as necessary.

DNA was isolated from *Neurospora* strains as described by Stevens and Metzenberg (21). DNA samples from the cross illustrated in Fig. 4 were a gift from R. L. Metzenberg. Restriction analyses were performed by using standard techniques. For Southern hybridizations, restriction digests were fractionated by electrophoresis on 1.0 to 1.2% agarose gels and were transferred by blotting to Zetabind (AMF) nylon membranes. Hybridizations were performed overnight at 60°C in 5% dextran sulfate–0.4% sodium dodecyl sulfate–0.45 M NaCl–0.045 M sodium citrate–60 mM sodium phosphate (pH 6.6)–10 mM EDTA–0.06% bovine serum albumin–0.06% Ficoll–0.06% polyvinylpyrrolidone–50 μg of denatured salmon sperm DNA per ml. After hybridization, membranes were washed (generally at 55 or 62°C as indicated) in 50 mM NaCl–20 mM sodium phosphate (pH 6.8)–1.0 mM EDTA–0.1% sodium dodecyl sulfate. Hybrid-

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TABLE 1. Allelic differences at the ζ-η locus

Species	Strain	Mating type	FGSC no.[a]	ζ-η duplication
N. crassa	Abbott 4	*A*	1228	No
N. crassa	BT25	*a*	353	No
N. crassa	BT1	*A*	354	No
N. crassa	Abbott 12	*a*	351	Yes
N. crassa	E5256	*A*	424	No
N. crassa	E5297	*a*	627	Yes
N. crassa	ST73	*a*	3834	Yes
N. crassa	ST4	*A*	262	Yes
N. crassa	74-OR23-1V	*A*	2489	Yes
N. crassa	74-OR23-1	*A*	987	Yes
N. crassa	ORS	*a*	2490	Yes
N. crassa	Mauriceville-1c	*A*	2225	No
N. crassa	North Africa 1	*A*	430	No
N. tetrasperma	85	*A*	1270	No
N. sitophila	Panama (UP203)	*A*	1134	No
N. intermedia	Liberia 4	*A*	343	No

[a] FGSC, Fungal Genetics Stock Center.

ization probes were prepared either by transcription, using the SP6 promoter as described previously (17), or by the random oligomer-primer method of Feinberg and Vogelstein (8).

RESULTS

An unmethylated allele at the zeta-eta locus. A large collection of *Neurospora* strains are available, including a variety of standard "wild types" as well as numerous "exotic" strains collected from widely separated locations around the world. We screened 13 *Neurospora crassa* strains and 1 strain each of *N. tetrasperma*, *N. sitophila*, and *N. intermedia* for restriction fragment length polymorphisms (RFLPs), which might be indicative of alternate alleles at the ζ-η locus. Southern hybridizations were performed with a 0.6-kb segment of the η region cloned from strain 740R23-IVA as the probe (closely spaced vertical bars in Fig. 3C). DNA from some strains would only hybridize with this probe under relatively low stringency conditions, suggesting that substantial sequence divergence had occurred. RFLPs were readily detected and most could be simply interpreted as due to the presence of one or two copies of the 0.8-kb ζ-η repeat element. Furthermore, those strains tentatively classified as having one element based on RFLPs were also those which showed weaker hybridization. Sample data for strains Beadle and Tatum 25 (BT25) and Abbott 12, which typify the two classes observed, are shown in Fig. 1. The spectrum of fragments detected in Abbott 12 DNA, using *Eco*RI, *Hin*dIII, *Bgl*II, *Xba*I, *Bam*HI, or *Eco*RI-plus-*Bam*HI, matches the spectrum detected in a number of laboratory strains (E5256, E5297, ST73, ST4, 74-OR23-IV, 74-OR23-1, ORS) including the strain from which the ζ-η region was cloned (Table 1). Apparently these strains all have the ζ-η duplication. The restriction enzyme *Bam*HI is sensitive to methylation, and we already know that there are methylated *Bam*HI sites in both the ζ and η 5S rRNA genes of strain ORS (17). The 2.6-kb fragment detected in *Eco*RI-plus-*Bam*HI digests results from methylation of these *Bam*HI sites (see Fig. 5). That the other duplication strains also show the 2.6-kb *Eco*RI-*Bam*HI fragment suggests that the ζ-η region is methylated in all of them. The *Eco*RI, *Hin*dIII, *Bgl*II, and *Xba*I fragments detected in BT25 DNA were approximately 0.8 kb smaller than the corresponding fragments from Abbott 12, suggesting that strain BT25 has only one copy of DNA homologous to the ζ-η region. In keeping with this conclusion, hybridization to BT25 DNA was invariably less intense than hybridization to DNA from the strain known (by sequencing) to have the duplication. The fragment detected in BT25 DNA digested with *Bam*HI plus *Eco*RI was 1.0 kb rather than 0.8 kb smaller than that detected in Abbott 12 (2.6 versus 1.6 kb), consistent with the idea that the single *Bam*HI site in the DNA homologous to the ζ and η genes is unmethylated (see Fig. 5). Similar results suggested that laboratory strains Abbott 4, BT1, and E5256 and the strains from North Africa and Mauriceville as well as representatives of other *Neurospora* species also have unique, unmethylated DNA at the ζ-η locus. The results are summarized in Table 1.

To verify that the observed RFLPs resulted from differences of methylation and copy number in the ζ-η region, we examined the region in detail, using frequent cutting restriction enzymes sensitive to methylation. We took advantage of

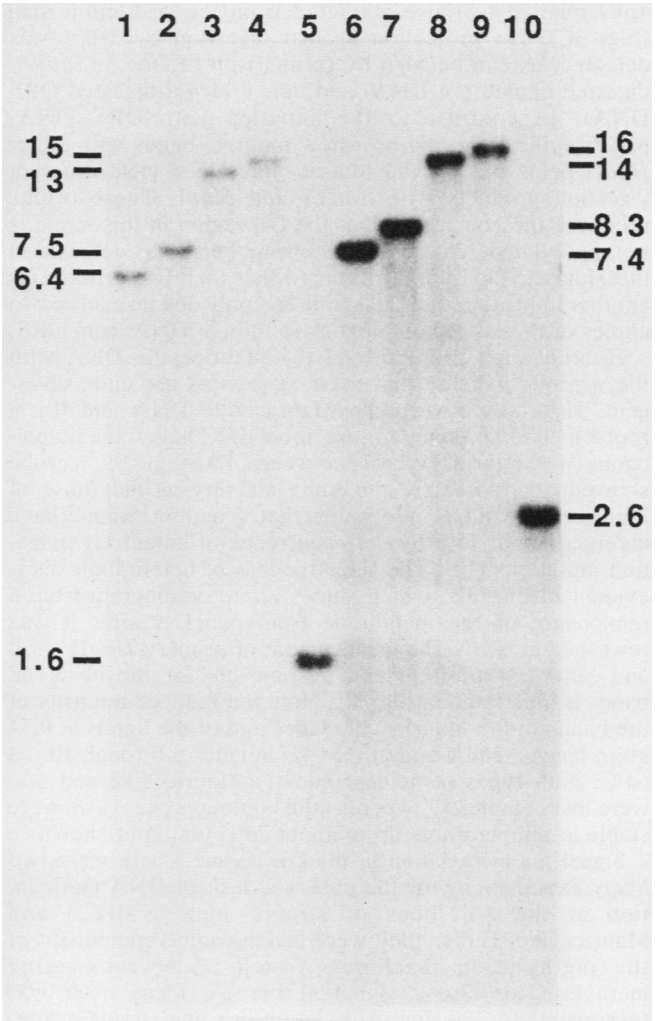

FIG. 1. RFLPs around the ζ-η region. DNA isolated from *N. crassa* strain BT25 (lanes 1 to 5) or strain Abbott 12 (lanes 6 to 10) was digested with *Eco*RI (lanes 1 and 6), *Hin*dIII (lanes 2 and 7), *Bgl*II (lanes 3 and 8), *Xba*I (lanes 4 and 9), or *Bam*HI plus *Eco*RI (lanes 5 and 10), fractionated by electrophoresis, transferred to a membrane, and probed with a 0.6-kb fragment from the ζ-η region (see Fig. 5). Fragment sizes are indicated in kilobases on the left and right for BT25 and Abbott 12, respectively.

the enzymes *Hpa*II (sensitive to methylation of the interior cytosine in the sequence CCGG), *Msp*I (sensitive to methylation of the exterior cytosine in CCGG), *Sau*3A (sensitive to methylation of cytosine in the sequence GATC), and *Mbo*I (sensitive to methylation of adenine but not cytosine in the sequence GATC) to compare a strain (ORS) having the zeta-eta duplication with one (Mauriceville) thought to have unique, unmethylated DNA at the corresponding chromosomal position. Examination of DNA from these strains, using a probe for sequences beyond the unmethylated *Bam*HI site 1.6 kb upstream of the *Bam*HI site in the ζ gene, demonstrated that these sequences are not highly polymorphic and that they lack significant methylation (Fig. 2). A sharply contrasting situation was seen with the 0.6-kb probe from the η region (which cross-hybridizes with the ζ region as illustrated in Fig. 3C). Results from one such analysis are shown in Fig. 3A. As observed previously (17), sites for enzymes sensitive to cytosine methylation (*Hpa*II, *Msp*I, and *Sau*3A) were partially protected in the ζ-η region of strain ORS, resulting in additional bands (lanes 2, 4, 7) relative to those predicted from the restriction map (Fig. 3C). No protection against digestion by *Mbo*I was detected, as can be seen by comparison of lane 6 (*Sau*3A-digested cloned ζ-η DNA) and lane 9 (*Mbo*I-digested ORS DNA). In contrast to the situation with ORS DNA, Mauriceville DNA did not show multiple bands with either *Hpa*II or *Msp*I, nor did Mauriceville DNA yield different digestion products with *Sau*3A and *Mbo*I. These results reinforce the conclusion that the ζ-η region in this strain is not methylated. That only one strong band was detected in the Mauriceville hybridizations (rather than two or more) is another indication that this strain has only one instead of two copies of the DNA homologous to the ζ-η DNA from ORS.

Hybridization properties of the Mauriceville DNA with the η probe indicate that these sequences are quite divergent. Hybrids between the Mauriceville DNA and the η probe melted at a temperature about 15°C below the homologous (η-η) hybrid. Hybrids between ζ DNA and the η probe showed approximately the same stability as did those of Mauriceville and η. We know that ζ and η regions have diverged about 15% by the occurrence of numerous transition mutations (17). The selective loss of heteroduplexes is evident in Fig. 3B, which shows an autoradiogram from a reexposure of the membrane from panel A after it was rewashed at 62°C. The arrangement of primary *Hpa*II/*Msp*I and *Sau*3A/*Mbo*I fragments responsible for the observed bands is illustrated in Fig. 3C. Note the reduced intensity of the bands in the Mauriceville lanes and of the bands at 0.74 kb in lanes 2 and 4 and at 0.54 kb in lanes 6 through 10. At 64°C, both types of heteroduplex (η-Mauriceville and ζ-η) were lost completely, whereas the homoduplexes (η-η) were stable at temperatures up to about 76°C (data not shown).

Signal for methylation in the ζ-η region. There were two likely explanations for the observed lack of DNA methylation at the ζ-η locus of strains such as BT25 and Mauriceville. Either they were lacking some component of the methylation machinery, such as a *trans*-acting methylase, or else a signal at the ζ-η locus itself was responsible for causing methylation in some strains or preventing methylation in others. None of the 16 strains we characterized by Southern hybridization exhibited unmethylated duplicated DNA or methylated unique DNA at the ζ-η locus. This correlation between possession of the duplication and methylation made it seem probable that the methylation difference was the result of the presence or absence of a local signal. To test this possibility, we examined progeny from a cross between strains having duplicated, methylated, or unique unmethylated DNA at the ζ-η locus (Fig. 4). A convenient *Eco*RI fragment polymorphism between strain Mauriceville and Oak Ridge strains was used to follow segregation of the ζ-η alleles (the *Eco*RI sites are outside of the region subject to methylation). Digestion with *Bam*HI was used to assay methylation in the ζ-η region. The region showed linkage to *ad*-9, *nuc*-1, and mating type as

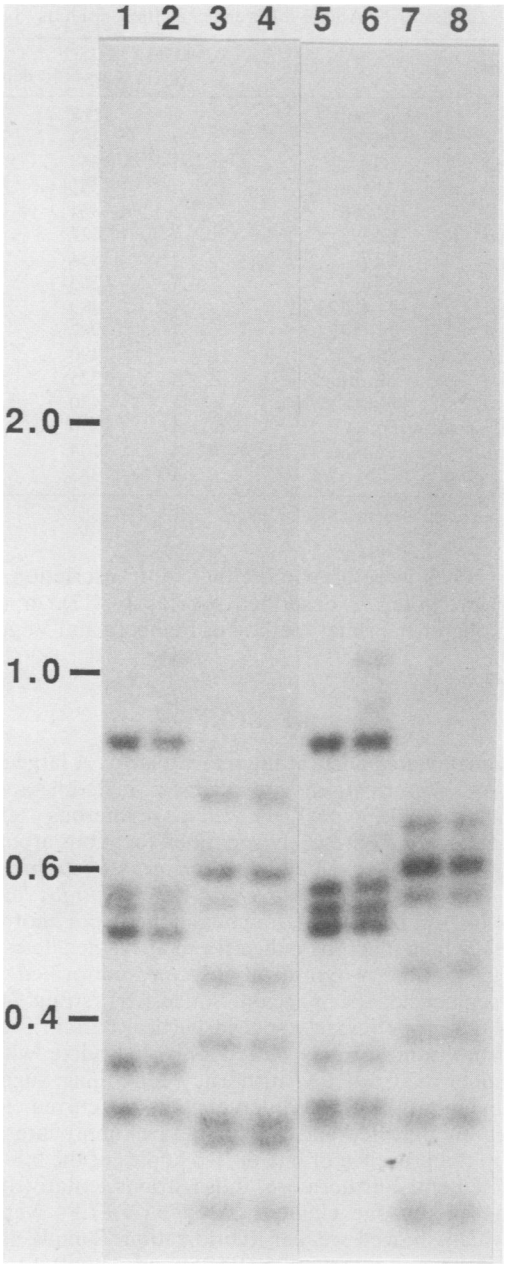

FIG. 2. Comparison of DNA from strains ORS and Mauriceville in the region adjacent to the ζ-η region. DNA samples of strain Mauriceville (lanes 1 to 4) or ORS (lanes 5 to 8) were digested with *Mbo*I (lanes 1 and 5), *Sau*3A (lanes 2 and 6), *Hpa*II (lanes 3 and 7), or *Msp*I (lanes 4 and 8). The Southern blot was probed with a 4.8-kb *Eco*RI-*Bam*HI fragment extending upstream from the first unmethylated *Bam*HI site next to the ζ-η region. Sizes are given in kilobases.

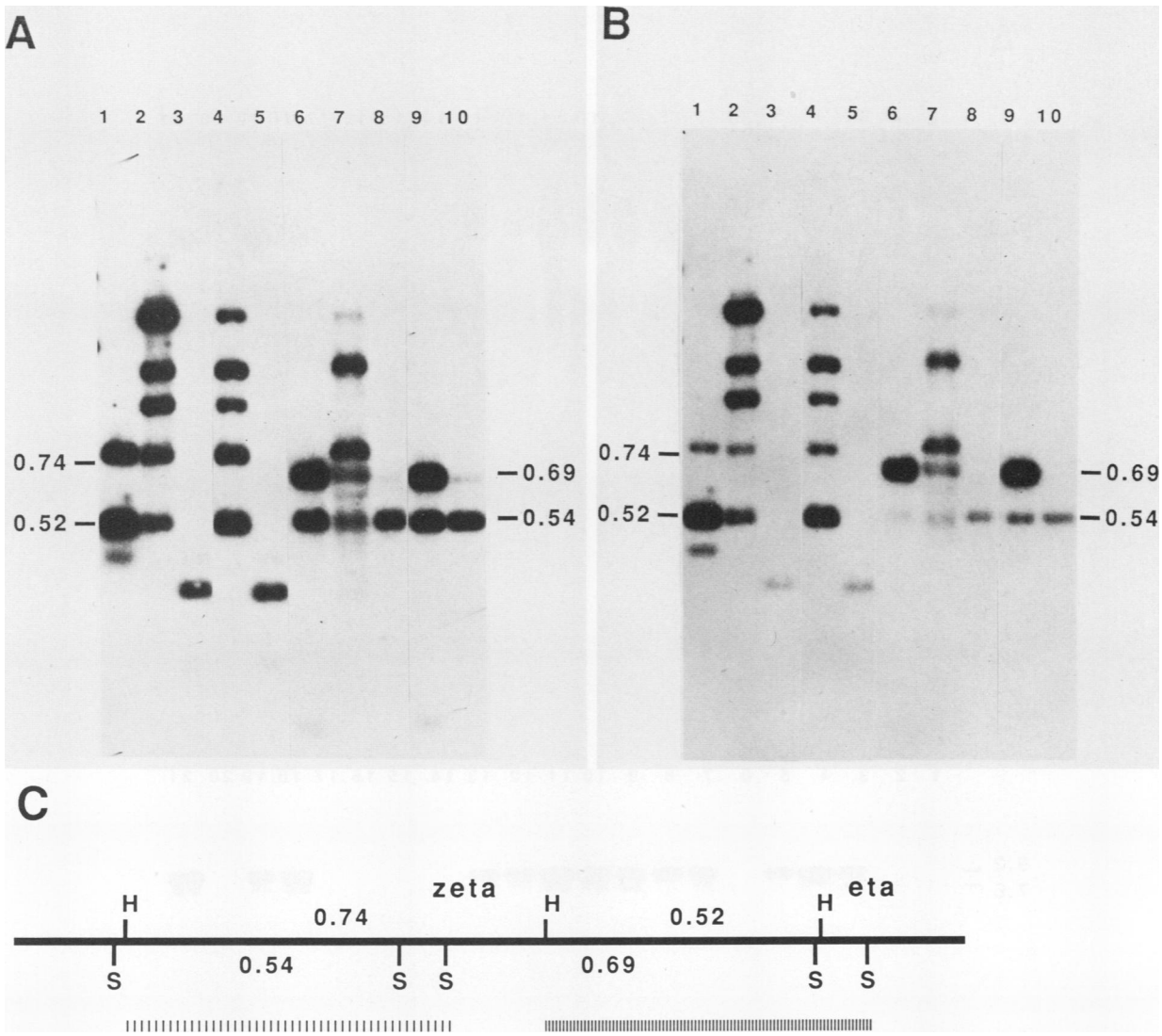

FIG. 3. Allelic differences in structure and methylation in the ζ-η region. DNA samples (0.5 μg) from *N. crassa* strain ORS (lanes 2, 4, 7, and 9) or Mauriceville (lanes 3, 5, 8, and 10) were digested with *Hpa*II (lanes 1, 2, and 3), *Msp*I (lanes 4 and 5), *Sau*3A (lanes 6, 7, and 8), or *Mbo*I (lanes 9 and 10) and fractionated on a gel next to digests of pES174 (13.5-kb plasmid including the ζ-η region from an Oak Ridge strain) DNA (0.1 ng) digested with the same enzymes (lanes 1 and 6). DNA was probed with a 0.6-kb RNA transcript made from the ζ-η region as indicated in panel C. (A) Autoradiogram of membrane washed at 55°C. (B) Autoradiogram of the same membrane after a subsequent wash at 62°C. Fragment sizes are given in kilobases. (C) Restriction map of ζ-η region of Oak Ridge strain 74-OR23-IV. Sizes are given for the primary fragments detected with *Hpa*II and *Msp*I (above) or *Sau*3A and *Mbo*I (below), using the η-region probe from 74-OR23-IV DNA (closely spaced vertical bars). The probe cross-hybridizes with DNA from the ζ region (loosely spaced vertical bars) and the homologous DNA of strain Mauriceville.

previously reported (10). Of primary interest, the methylation character segregated with the ζ-η region. None of the progeny showing the *Eco*RI fragment diagnostic of the Mauriceville allele exhibited *Bam*HI fragments larger than the 1.6-kb fragment resulting from complete digestion, and all of the progeny showing the *Eco*RI fragment diagnostic of the Oak Ridge allele exhibited *Bam*HI fragments larger than those resulting from digestion at all *Bam*HI sites. The identities of the fragments detected are shown in Fig. 5. The perfect correlation between methylation and the presence of the Oak Ridge ζ-η region indicates that the critical factor causing the methylation difference is tightly linked to the ζ-η locus (and may be at the ζ-η locus itself). It seems most likely that a structural feature of the Oak Ridge allele is responsible for its methylation.

DISCUSSION

In eucaryotes, methylation of cytosines is generally limited to positions immediately preceding guanines, and it is thought that this reflects the mechanism which maintains methylation patterns in replicating DNA. A maintenance methylase blindly methylates the unmethylated strand of newly replicated hemimethylated DNA at positions diagonally opposed to methylcytosines (3). Indeed, it has been shown that arbitrary DNA sequences introduced into animal cells after in vitro methylation of every cytosine on one strand become methylated on the other strand at CpG sites (only) and maintain this artificial methylation "pattern" through many rounds of replication (20). The methylation we have observed in *N. crassa* is qualitatively different in that

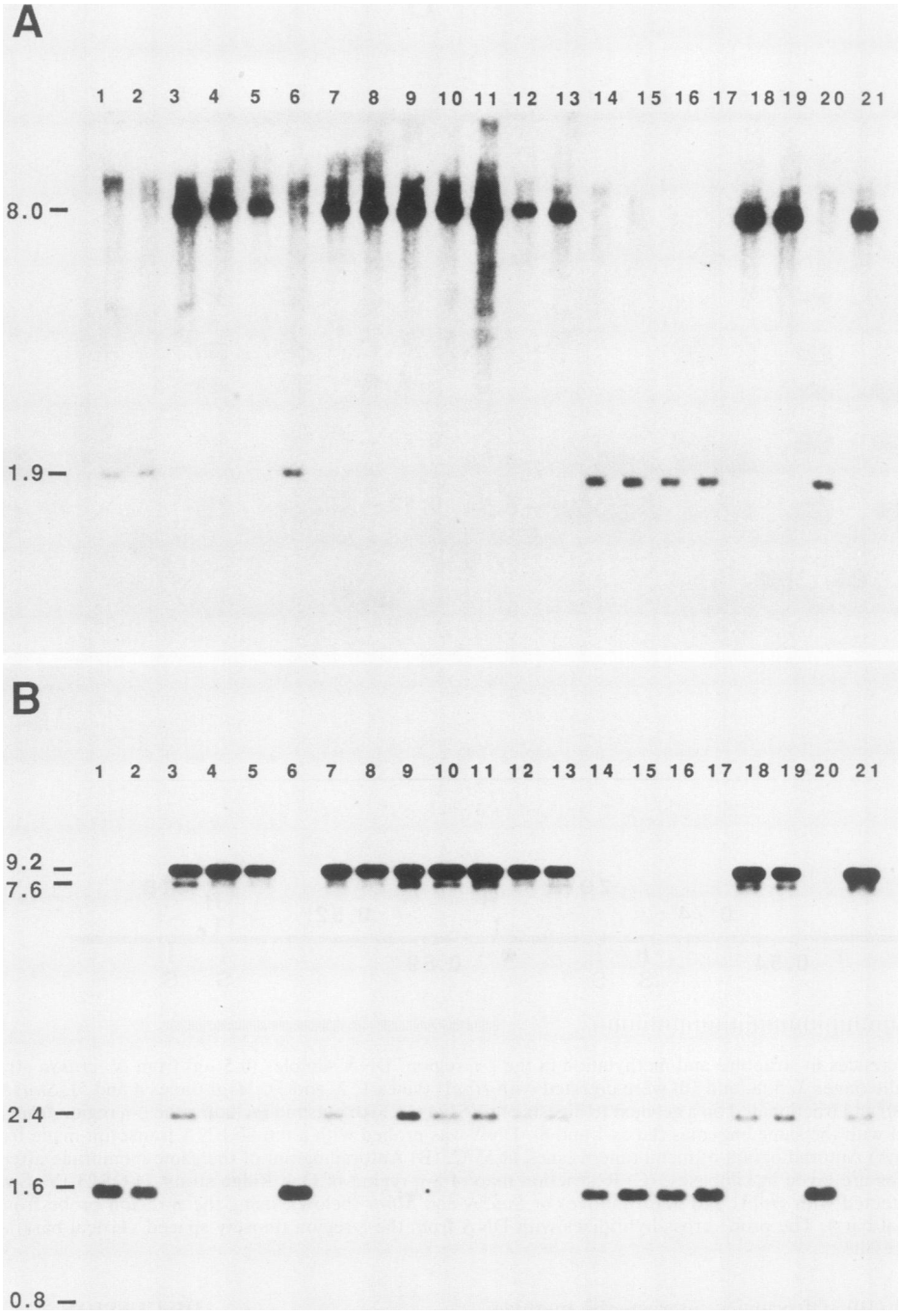

FIG. 4. Linkage of methylation status and RFLPs at the ζ-η locus. DNA from 21 progeny of a cross of an Oak Ridge background strain *nuc-1*, *ad-9*, *al2p*, *nic-1*, a) having duplicated, methylated DNA at the ζ-η locus and Mauriceville-1c-A, which has unique unmethylated DNA at the ζ-η locus, was isolated and analyzed by Southern hybridization, using either *Eco*RI (A) or *Bam*HI (B). None of the progeny showing the 1.9-kb *Eco*RI fragment (diagnostic of the Mauriceville allele) exhibited *Bam*HI fragments larger than the 1.6-kb fragment resulting from complete digestion, whereas all progeny showing the approximately 8-kb *Eco*RI fragment (diagnostic of the Oak Ridge allele) exhibited *Bam*HI fragments larger than those resulting from complete digestion (see Fig. 5).

methylation is not limited to symmetrical sites (17). Nevertheless, it appears that CpG sites are more heavily methylated (methylated in a larger fraction of the cells) than other possible sites (e.g., compare *Hpa*II and *Msp*I digests in Fig. 3). Thus symmetrical sites may be "preferred" in *N. crassa*. That a given site is never totally methylated suggests that, if there is a maintenance system for methylation in *N. crassa*, it is not highly effective and some form of "de novo" methylation must act to maintain the equilibrium state. Indeed, a possible explanation for the somewhat heavier methylation at CpG sites in the ζ-η region is that methylation of the region results from the sum of two processes: maintenance methylation at symmetrical sites and de novo methylation at both symmetrical and asymmetrical sites.

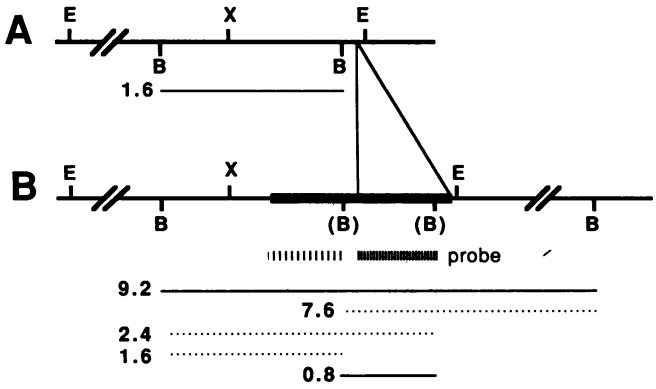

FIG. 5. Restriction maps of ζ-η region of Mauriceville (A) and Oak Ridge (B) strains of *N. crassa*. Sites for *Eco*RI (E), *Bam*HI (B), and *Xho*I (X) are shown. Parentheses around sites in the ζ-η duplication region (heavy line) indicate methylated sites responsible for the higher-molecular-weight *Bam*HI fragments (dotted lines) seen in Fig. 4. Due to divergence between the ζ and η regions and between these and the homologous DNA in other strains (17), the η probe (closely spaced vertical bars) hybridized to a lesser extent with the ζ region (loosely spaced vertical bars) and the corresponding region in Mauriceville DNA (see text).

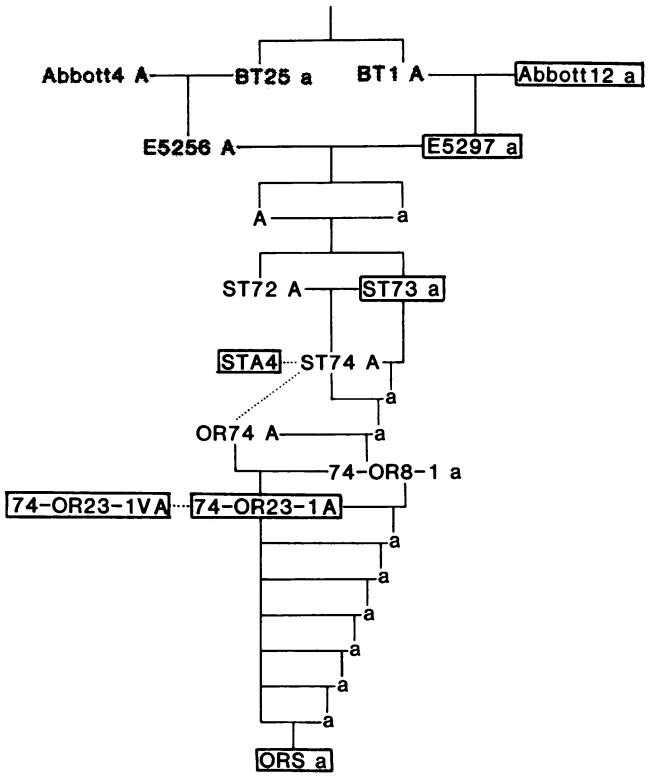

FIG. 6. Distribution of ζ-η alleles among laboratory wild-type strains of *N. crassa*. The pedigree was derived from published information (2, 5, 11) with the generous help of D. D. Perkins. Dotted lines indicate vegetative reisolates. Strains indicated by shading have unique unmethylated DNA at the ζ-η locus, whereas those indicated by open rectangles have duplicated, methylated DNA at this locus. The authenticity of existing stocks of Abbott 12a has been questioned based on *scot* genotypes (Perkins, personal communication). Strain stock numbers are listed in Table 1.

However, no maintenance methylation activity comparable to that observed in animal cells has been found in *N. crassa* (E. Cambareri and E. U. Selker, unpublished data). De novo methylation of non-homologous amplified transforming DNA has been reported for *N. crassa* (4), and we have observed rapid de novo methylation of the ζ-η region after demethylation by treatment with 5-azacytidine (unpublished data).

Results from this study indicate that there is a signal at or near the ζ-η locus causing cytosine methylation in that region. Some strains but not others have the signal; those that lack it have one instead of two copies of a 0.8-kb element homologous to sequences duplicated in tandem at the ζ-η locus of Oak Ridge strains. A survey of historically important laboratory strains suggests that the duplication (and methylation signal) entered the pedigree in strain Abbott 12 (Fig. 6). The association of a methylation signal with the duplication and lack of methylation in the unique flanking sequences leads us to speculate that the signal was created by the duplication; in fact, the duplication itself may be both the signal and the target of methylation. There are other cases of methylation in fungi which bear resemblance to that observed at the ζ-η locus. We have recently discovered another region in *N. crassa*, associated with a 5S pseudogene, which is methylated in some strains but not others, and preliminary data suggest that the methylated allele is associated with a local rearrangement. In addition, Zolan and Pukkila (23) discovered a locus which is methylated in certain strains of the basidiomycete *Coprinus cinerus* and showed that the methylation is associated with an insertion (or that lack of methylation is associated with a deletion). This case apparently differs from what we have observed in *N. crassa* at the ζ-η locus in two respects: there is no evidence for non-CpG methylation in *C. cinerus* (no protection of *Msp*I sites was detected), and unlike the situation at the ζ-η locus of *Neurospora*, the size of the methylated patch was variable in the example from *C. cinerus*. Another case of abnormal methylation associated with a rearrangement involves the nucleolar organizer region of *N. crassa*. This region, which is composed of about 180 tandem repeats of an approximately 9-kb rDNA element, is normally lightly methylated. When a small array of rDNA repeat units are moved to another chromosome, this ectopic cluster exhibits much greater methylation (11a). Whether the increased methylation is a result of the abnormal chromosomal position or the low copy number of the ectopic rDNA is not yet known. The same methylation system which methylates rDNA might be responsible for methylation of the ζ-η region. It seems possible that *Neurospora* cells have a general mechanism for recognizing and methylating "new" or rearranged DNA resulting from illegitimate recombination events. It will be interesting to learn what function this methylation serves and to define what causes certain sequences to become methylated while others are not.

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