Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8114–8118, December 1985
Genetics

DNA methylation at asymmetric sites is associated with numerous transition mutations

(*Neurospora*/gene duplication/5S RNA gene/evolution/5-azacytidine)

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Communicated by Charles Yanofsky, July 29, 1985

ABSTRACT **We describe two unusual 5S RNA regions from *Neurospora crassa* that are tightly linked. Sequence analysis suggests that these genes or pseudogenes, which we designate zeta (ζ) and eta (η), arose by a 794-base-pair tandem duplication followed by hundreds of exclusively cytosine to thymine mutations. The duplication was most likely generated by nonhomologous recombination involving a DNA segment having a striking purine–pyrimidine strand asymmetry. Restriction analysis of genomic DNA from tissue grown in the presence or absence of 5-azacytidine indicates that many, and perhaps all, cytosines in the duplicated region are methylated in most cells. This is in contrast to the situation typically observed in eukaryotes, where 5-methylcytosine is found only at positions one or two nucleotides preceding guanine residues. No DNA methylation was detected in the unique DNA flanking the ζ–η duplication. Thus the "signal" for methylation may be the duplication itself. The numerous transition mutations in this region probably occurred by deamination of 5-methylcytosines. Our results suggest that DNA methylation can have important evolutionary consequences in eukaryotes.**

In all organisms 5S RNA genes are reiterated. The 5S RNA genes of *Neurospora*, unlike those of most organisms, are not tandemly arranged (1). We have studied the structure and chromosomal location of 23 of the ≈100 *Neurospora crassa* 5S RNA genes and pseudogenes. Little homology is found in the sequences flanking 5S genes except for a short consensus sequence 25–30 nucleotides upstream of the transcription initiation site (2) and a thymine-rich region on the RNA-like strand immediately downstream from the genes. The 5S RNA regions can be grouped into categories (α, β, γ, etc.) based on their nucleotide sequences. The 5S RNA from genes of different types differs in sequence by 10% or more (1, 3). Although the genes are widely dispersed on at least six of the seven chromosomes (4), their locations do not appear to be completely random. The majority of the 5S RNA genes of the most common kind, α, are on linkage group II, and several of these are closely linked. Some α 5S genes, however, are also found on linkage groups I, V, and VI. In this paper, we describe two *Neurospora* 5S RNA genes on linkage group I that lie close enough together to be on the same *Eco*RI restriction fragment. We show that these genes, which we designate zeta (ζ) and eta (η), arose by a tandem duplication followed by an accumulation of a large number of nonrandom mutations. It appears likely that ζ and η are no longer functional genes. We present evidence that the duplicated region is heavily methylated and we suggest that the numerous mutations resulted from deamination of methylated cytosines, as already demonstrated for *Escherichia coli* (5–7). Our results provide evidence for the evolutionary importance of DNA methylation in eukaryotes.

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MATERIALS AND METHODS

DNA fragments of the ζ–η chromosomal region of *N. crassa* (74OR23-IVA) (1, 4) were subcloned by standard techniques. DNA sequencing was done by the method of Maxam and Gilbert as described (1). Hybridization probes for the ζ–η region were prepared by transcription of the 0.6-kilobase (kb) *Cla* I/*Bam*HI DNA fragment cloned in pSP64 (8) (see Fig. 5*b*). For Southern hybridizations (9), restriction digests were fractionated by electrophoresis on 1.0–1.2% agarose gels and were transferred by blotting to nylon membranes. Hybridizations were performed at 37°C in 50% formamide/10% dextran sulfate/2% $NaDodSO_4$/0.45 M/NaCl/0.045 M Na citrate)/180 mM sodium phosphate, pH 6.8/10 mM EDTA/0.06% bovine serum albumin/0.06% Ficoll/0.06% polyvinylpyrrolidone) and denatured salmon sperm DNA (50 μg/ml). Filters were washed at 45°C in 50 mM NaCl/20 mM sodium phosphate, pH 6.8/1 mM EDTA/0.1% $NaDodSO_4$. *N. crassa* (74-OR8-1a) DNA was prepared from conidial cultures (≈5×10^6 conidia per ml) grown in Vogel's medium (10) containing 2% sucrose for 18 hr at 30°C with shaking (300 rpm). DNA isolated as described (11) was further purified by centrifugation in CsCl/ethidium bromide.

RESULTS AND DISCUSSION

Unusual Pair of A+T-Rich 5S Genes: ζ and η. In a survey of 22 distinct *Eco*RI-generated clones containing 5S DNA, one was found to contain two 5S genes, ζ and η (4). We completely sequenced the ζ and η genes and their flanking DNA (Fig. 1). The ζ and η 5S RNA coding regions are separated by <1 kb. Nevertheless, they are surprisingly different from each other and from other known 5S genes. The ζ and η genes differ at 19 of 120 positions. Comparison of ζ and η with other *Neurospora* 5S RNA and 5S DNA sequences indicates that their closest known relative is γ', although the latter differs from ζ at 24 positions and from η at 15 positions (Fig. 2). For comparison, human and frog 5S RNAs differ at eight positions (13). γ' is much closer in sequence to other 5S RNAs from *Neurospora* (and to 5S RNAs from other organisms) than are the predicted 5S RNAs from either ζ or η. Thus, at positions of difference between γ' and ζ or γ' and η, the nucleotides found in γ' are probably ancestral. Curiously, all of the differences between γ' 5S RNA and the predicted ζ or η 5S RNAs are replacements of cytosines or guanines in γ' by uracils or adenines, respectively. This leads us to propose that both ζ and η evolved directly and recently from γ' by G·C → A·T mutations only.

If the ζ and η genes are expressed, they must produce very G+C-poor 5S RNAs. The ζ and η RNAs would be 33% and 40% G+C, respectively, compared with 54%, 52%, and 53%

Abbreviation: kb, kilobase(s).

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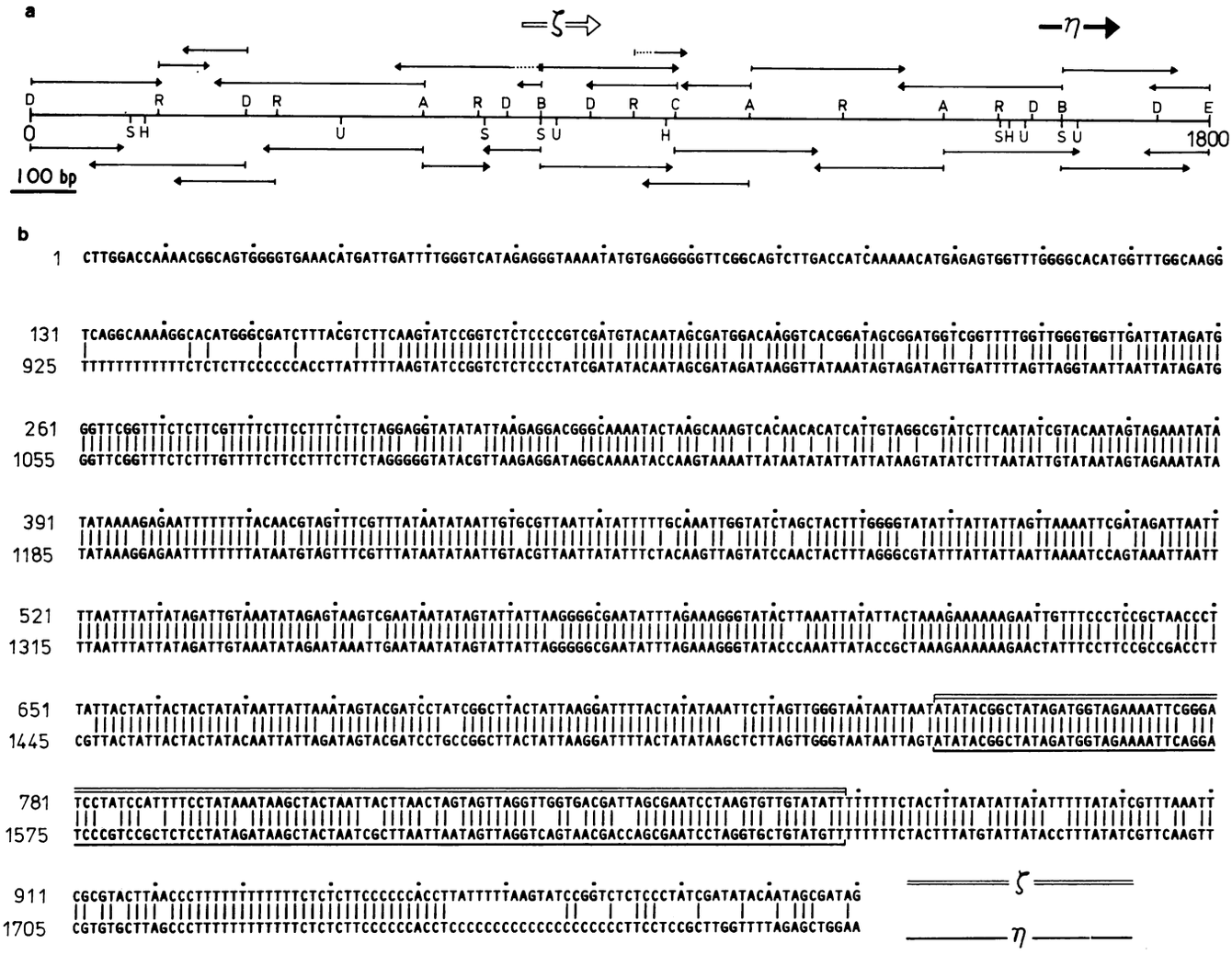

FIG. 1. (*a*) Restriction map and summary of DNA sequencing of ζ–η region. Arrows above and below the map represent sequencing runs on the 5S RNA-like strand and on the opposite strand, respectively. The rightward and leftward arrows above the map (and the leftward and rightward arrows below the map) show the extent of sequence information obtained using 5′ or 3′ end-labeled DNA, respectively. Sequence data were not collected for the portions of sequencing runs indicated with dotted lines. All restriction sites for the following endonucleases are shown: *Dde* I (D), *Rsa* I (R), *Acc* I (A), *Bam*HI (B), *Cla* I (C), *Eco*RI (E), *Sau*3A (S), *Hpa* II (H), and *Alu* I (U). bp, Base pairs. (*b*) Nucleotide sequence of DNA including ζ–η tandem duplication and adjacent regions. The RNA-like strand of the 1794-nucleotide DNA segment, including the ζ and η 5S genes, is presented with the homologous segments (nucleotides 160–953 and nucleotides 954–1747) aligned to demonstrate their homology. Vertical bars designate the positions of identity in this alignment. The ζ and η 5S RNA regions (nucleotides 749–868 and nucleotides 1543–1662, respectively) are marked.

G+C for α, β, and γ 5S RNAs. Compared with γ′ and other *Neurospora* 5S RNAs, ζ and η would form considerably less stable secondary structures. We have not yet detected ζ or η 5S RNA *in vivo* and it is possible that these 5S RNA regions are silent. However, η is transcribed in an *in vitro Neurospora* RNA polymerase III system (unpublished data).

The ζ and η 5S RNA Regions Resulted From a Tandem Duplication Followed by Accumulation of Nonrandom Mutations. From the sequence of the ζ–η region, we see that the 5S RNA genes are oriented in the same direction (Fig. 1). Exactly 794 base pairs separate homologous positions in ζ and η. Furthermore, unlike any of the other 21 *Neurospora* 5S RNA regions examined (refs. 1 and 2; unpublished data), the sequences flanking the ζ and η 5S RNA structural regions are very similar. The homology extends from 589 nucleotides upstream of the 5S regions to 85 nucleotides downstream of the 5S regions and includes all the DNA in between (Fig. 1*b*). Apparently, ζ and η are the result of a tandem duplication. The DNA sequences flanking the duplicated elements are not similar to each other or to sequences within the duplicated region. The duplication most likely resulted from a nonhomologous recombination event, as illustrated in Fig. 3. It may be significant that the breakpoint occurred in a region of long homopurine–homopyrimidine stretches in one crossover partner (sequence b in Fig. 3). On the RNA-like strand, 31 of 32 nucleotides preceding the breakpoint are pyrimidines and 27 pyrimidines, including 20 consecutive cytosine residues, follow the crossover position. This region of DNA may be expected to have an unusual secondary structure (14).

Like the ζ and η 5S RNA structural regions, the surrounding DNA is unusually A+T-rich. The duplicated DNA is 70% A+T compared with 47% A+T outside of this region and for the total genome (15). The duplicated elements have diverged ≈14% (16% in the 5S RNA region; 14% in the flanking DNA). The sequence of both elements of the duplication, and some flanking DNA, is presented in Fig. 1*b* with the repeated regions aligned. Vertical bars mark positions of nucleotides in common between the ζ and η repeats. The differences are distributed fairly evenly throughout the duplicated DNA. There are no gaps in the alignment. Remarkably, all of the 113 differences between the 794-nucleotide duplicated regions are transitions; there are 55 C-T differences and 58 G-A

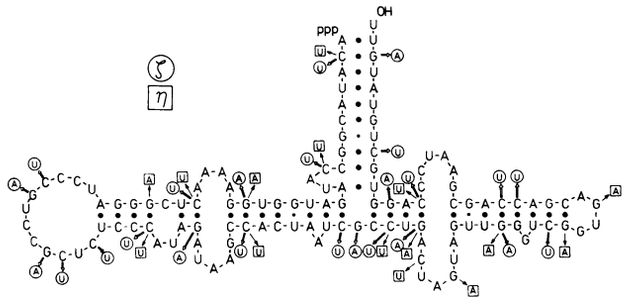

FIG. 2. Nucleotide differences in predicted ζ and η 5S RNA relative to *Neurospora* γ' 5S RNA. γ', which is the closest known relative of ζ and η, differs from the standard γ sequence at nucleotide 17 (3). The RNA is drawn in the secondary structure proposed by Nishikawa and Takemura (12). Note that all of the differences between γ' and ζ (○) and between γ' and η (□) are transitions and are polarized (G γ' → A or C γ' → U).

differences but no G-C, G-T, A-C, or A-T differences. In other comparisons of homologous genes from eukaryotes, it has been found that transition substitutions outweigh transversions, although not to anywhere near the extent reported here (16).

Explanation for Transition Mutations and Atypical Base Composition. A simple model can account for the extraordinary frequency of transition mutations and the high A+T content in the ζ–η region. We propose that most, if not all, cytosine residues in this duplicated DNA were methylated. Numerous cytosine to thymine mutations resulted from deamination of 5-methylcytosines. The guanine to adenine changes are a manifestation of cytosine to thymine changes on the other strand. Transition mutations have occurred not only at the positions where ζ and η differ, but also at numerous other positions, which now appear common to the duplicated elements. Such mutations at identical positions in the two repeats could have occurred before the duplication event or independently after the event. In the 5S regions, these positions are detected as sites where γ' differs from both ζ and η. The A+T-rich nature of the entire ζ–η region is interpreted as a reflection of hundreds of C·G → T·A mutations, including those that resulted in the 113 differences between present day repeat units.

There is a precedent for this interpretation in *E. coli*. Spontaneous deamination of cytosine occurs frequently in DNA. The resulting uracil is normally removed from DNA by the enzyme DNA-uracil glycosylase. The G·C base pair is then restored, thereby preventing occurrence of a transition mutation (5, 6). Deamination of 5-methylcytosine gives 5-methyluracil (thymine), which is not excised by DNA-uracil glycosylase. Thus, 5-methylcytosine residues are often hot spots for spontaneous transition mutations (5, 7). It has been suggested on the basis of circumstantial evidence that 5-methylcytosine is a hot spot for transition mutations in eukaryotes as well (17). Almost all 5-methylcytosine in animal DNA is in the dinucleotide CpG, and animal genomes with high levels of DNA methylation tend to be deficient in CpG and correspondingly high in TpG and CpA. Furthermore, restriction sites containing the dinucleotide CpG tend to be particularly polymorphic in animal DNA.

On the hypothesis that all 113 differences between the ζ and η repeats arose by deamination of 5-methylcytosines, we can assign the mutations to one repeat or the other. For example, at a position of difference between the ζ and η repeats where a cytosine residue is in ζ and a thymine residue is in η, we would conclude that a mutation occurred in η but not in ζ. Similarly, in the case of G-A difference, the mutation would be assigned to the repeat unit having the adenine residue. Fig. 4 shows the deduced distribution of mutations resulting in the differences. Cytosine to thymine and guanine to adenine changes, which presumably represent mutations on opposite strands, are indicated, respectively, as bars above and below the central horizontal line. Apparently, the two strands have suffered an approximately equal number of mutations. Inspection of the distribution along the sequence, however, suggests that they did not occur completely randomly. Mutations are concentrated in the middle of the ζ–η region centered about the duplication junction. Note also that the middle region is particularly A+T-rich. Possibly, the outer regions were less heavily methylated and thus suffered fewer mutations.

DNA Methylation in the ζ–η Region. To explore our hypothesis that the mutations occurred by deamination of 5-methylcytosine residues, we examined the genomic DNA in the ζ–η region for methylation. We took advantage of restriction enzymes that fail to cut if particular nucleotides in their recognition sequences are methylated. If all 113 transitions observed in the ζ–η region occurred by deamination of 5-methylcytosine, methylation of cytosine residues must not have been limited to positions one or two nucleotides preceding guanine residues, as is usually the case in eukaryotic DNA (18). Less than 3% of the cytosine residues in *Neuspora* DNA are methylated, and their distribution is not known (D. Swinton and S. Hattman, personal communication).

We performed Southern hybridizations (9) to assess the sensitivity of a variety of restriction sites in the ζ–η region of genomic DNA. Protection against cleavage was observed. For example, the *Bam*HI sites in both ζ and η were resistant to digestion, whereas the *Bam*HI sites on either side of the ζ–η region were cleaved normally (Fig. 5). *Bam*HI cleaves GGATCC, GGmATCC, and GGATCmC, but not GGATmCC (19). Restriction sites for other enzymes sensitive to 5-methylcytosine, such as *Hpa* II, *Msp* I, and *Sau*3A, were also resistant to cleavage in the ζ–η region (see below). However, several other restriction enzymes, such as *Mbo* I, which cuts GAmCT but not GmACT, cut as expected based on the primary nucleotide sequence. Thus, the blockage in the ζ–η region appears specific for cytosine residues.

To determine whether the putative cytosine methylation is a general feature of *Neurospora* 5S regions, we examined the sensitivity of *Bam*HI sites in other 5S RNA regions including

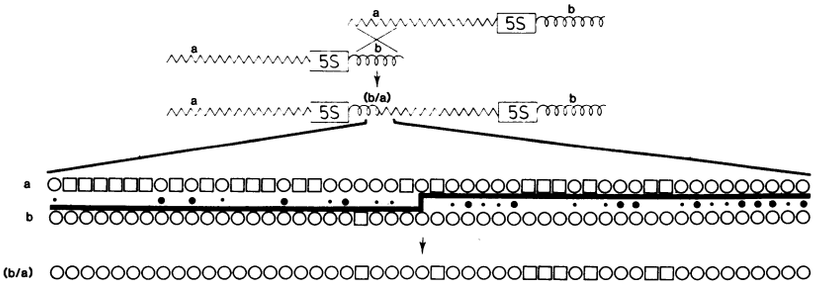

FIG. 3. Hypothetical generation of ζ–η duplication by unequal crossover at "a" and "b." Since only transition mutations have occurred subsequent to generation of the duplication (see Fig. 1), the ancestral and present day DNA would have the same sequence of purines (□) and pyrimidines (○). Only the indicated crossover would result in the observed sequence of purines and pyrimidines in (b/a). Large solid dots between sequences a and b mark positions of identity between the present day sequences. Smaller dots indicate positions of transition differences.

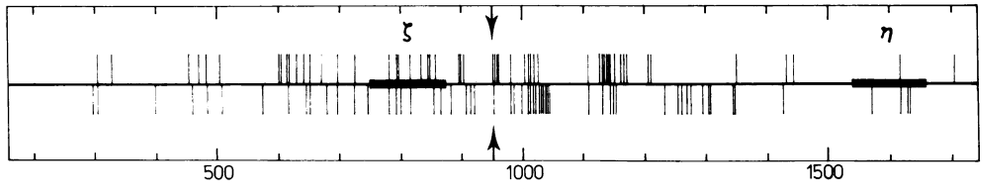

FIG. 4. Positions of presumptive mutations that gave rise to differences between ζ and η regions. Arrows (position 954) mark the junction between the duplicated segments. Differences were assigned to one or the other of the duplicated elements based on the assumption that the differences resulted from mutations of C to T or G to A and not vice versa. Upper vertical bars represent C to T changes and lower vertical bars represent G to A changes. The locations of the ζ and η 5S RNA regions are indicated.

α, β, γ, and δ 5S genes, and one pseudogene, N5SP1 (20). All were completely digested by *Bam*HI (unpublished data).

5-Azacytidine Relieves Blockage of Restriction Sites in the ζ–η Region. The cytidine analogue 5-azacytidine has been shown to prevent cytosine methylation in many systems (21). If the restriction site blockage observed in the ζ–η region is due to cytosine methylation, then DNA from cells grown in the presence of 5-azacytidine should be more digestible. We therefore isolated DNA from *Neurospora* grown in the presence of 5-azacytidine and assessed the sensitivity of restriction sites in the ζ–η region. No growth inhibition was observed up to 6 μM 5-azacytidine, and even at the highest concentration tested (48 μM) only ≈30% inhibition of growth occurred. Yet this drug efficiently abolished the blockage observed at the *Bam*HI sites in both ζ and η, as exemplified by the disappearance of the 15-kb *Bam*HI fragment and 2.6-kb *Eco*RI/*Bam*HI fragment and appearance of 1.6-kb and 0.8-kb *Bam*HI/*Bam*HI fragments (Fig. 5). Even at the lowest concentration tested (3 μM), increased cleavage by *Bam*HI was observed. Cleavage was further enhanced with increasing doses of the drug. When 5-azacytidine was present at 24 or 48 μM, cleavage was >90%. 5-Azacytidine also effectively abolished blockage at other restriction sites, including *Hpa* II, *Msp* I, *Sau*3A, and *Alu* I (Fig. 6).

These results establish that the ζ–η region is heavily methylated at numerous sites. In fact, it is possible that every cytosine residue in the duplicated region is subject to methylation. This sharply contrasts with the situation observed in other eukaryotes, where only cytosine residues present as CpG or CpNpG are reportedly methylated. Most of the sites examined with enzymes sensitive to 5-methylcytosine were predominantly blocked, indicating that these sites were methylated in most of the cells. Less heavily methylated positions were detected, however. For example, the *Alu* I sites were >50% digested. *Msp* I cleaved to a greater extent than did its isoschizomer *Hpa* II. Apparently the

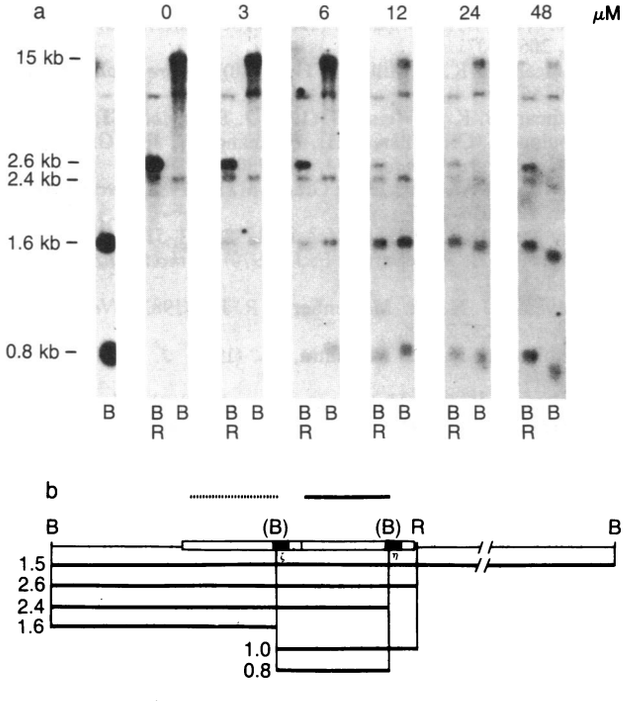

FIG. 5. (*a*) Methylation at *Bam*HI sites in ζ–η region. One-microgram samples of *N. crassa* DNA from cultures grown with the indicated concentrations of 5-azacytidine were digested with a 10- to 30-fold excess of *Bam*HI (B) or *Bam*HI and *Eco*RI (R). About 0.1 ng of pJS33 (a 15-kb plasmid containing the ζ–η region) digested with *Bam*HI was included as a control (leftmost lane). The digests were fractionated and probed with ^{32}P-labeled RNA from the region indicated in *b*. *Bam*HI cleaves at GGATCC, GGmATCC, and GGATCmC but not at GGATmCC (19). (*b*) Restriction map of ζ–η region showing *Bam*HI (B) and *Eco*RI (R) cleavage sites and location of DNA fragments detected or expected in hybridization in *a*. The ζ and η 5S RNA regions are shown as heavy lines in the boxed segments, which represent the tandemly duplicated 794-base-pair elements. The probe was synthesized from the region indicated by the solid line above the restriction map. The region indicated by the dashed line is ≈90% homologous to the probe (see Fig. 1). Restriction sites blocked by methylation in DNA from tissue not treated with 5-azacytidine are shown in parentheses. Sizes are in kb.

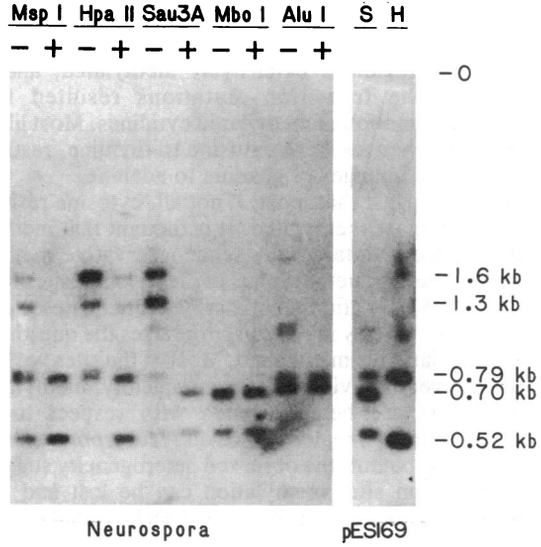

FIG. 6. Widespread methylation of ζ–η region. One-microgram samples of *N. crassa* DNA isolated from tissue grown with (+) or without (–) 24 μM 5-azacytidine were digested with the indicated restriction enzyme, fractionated, and hybridized with the ζ–η probe illustrated in Fig. 5*b*. Samples (≈0.1 ng) of pES169 (a 5.7-kb plasmid containing the ζ–η region) digested with *Sau*3A (S) or *Hpa* II (H) were included as controls. *Msp* I cleaves CCGG and, generally, CmCGG (19, 22, 23) but not mCCGG. Its isoschizomer, *Hpa* II, cleaves CCGG and mCCGG but not CmCGG. *Sau*3A cleaves GATC and GmATC but not GATmC. Its isoschizomer, *Mbo* I, cleaves GATC and GATmC but not GmATC. *Alu* I cleaves AGCT but not AGmCT (19).

internal cytosine of the *Hpa* II/*Msp* I recognition sequence (CCGG) is preferentially methylated. In addition, it appears that the *Bam*HI site in η is not as fully blocked as is the *Bam*HI site in ζ. In Southern hybridizations using DNA from cultures grown without 5-azacytidine, a faint band was observed at 2.4 kb resulting from cleavage of the *Bam*HI site in η, but no hybridization was detected at positions corresponding to 1.6-kb, 1.0-kb, and 0.8-kb fragments, which would be expected from cleavage of the *Bam*HI site in ζ (Fig. 5). This is consistent with the idea that the center of the ζ–η region is more heavily methylated than the border regions (see above).

CONCLUSIONS

Unlike other 5S genes in *Neurospora*, ζ and η lie close together. These genes and their flanking sequences apparently arose by a tandem duplication followed by an accumulation of a large number of transition mutations. The fact that no transversion mutations are found in the repeated DNA suggests that the duplication was quite recent. The duplication probably was formed by a nonhomologous unequal crossover. Assuming that only transition mutations have occurred in this region, as proposed, we can infer from the present day DNA sequence the exact position of the postulated crossover (Fig. 3).

Comparison of ζ and η with other *Neurospora* 5S sequences suggests that transition mutations occurred at many positions in the ζ–η region in addition to those recognized as C-T or G-A differences between the present day ζ and η repeats. It is also clear that the transition mutations were polarized; C·G to T·A but not T·A to C·G changes occurred. We interpret the A+T-rich nature of the ζ–η region as a manifestation of these mutations. Based on the number of nucleotide differences between the ζ and η regions and the base composition of the duplicated region relative to that of the flanking DNA and to total *Neurospora* DNA (both of which are 53% G+C), we estimate that $\approx$40% of the ancestral guanines and cytosines have been converted to adenines and thymines. This would correspond to >800 transition mutations. The ζ–η region is extensively methylated, and we propose that the transition mutations resulted from unrepaired deamination of methylated cytidines. Most likely, all the primary changes were cytosine to thymine, resulting in partner-strand changes of guanine to adenine.

Our results suggest that most, if not all, cytosine residues in the ζ–η region are methylated. It is thought that methylation patterns are maintained by semiconservative methylation based on the symmetry typical of methylation sites (CpG and CpNpG) (18). Our finding of methylation at sites that do not share this symmetry in *Neurospora* raises the question of how the methylation is maintained. Most of the sites we have examined are very heavily, but not completely, methylated. Thus, the cells are heterogeneous with respect to the methylation in the ζ–η region. Since all *Neurospora* cells are thought to be totipotent, the observed heterogeneity suggests that, at any given site, methylation can be lost and then regained. We do not know if remethylation is truly a *de novo* process or if it is the result of a novel "maintenance methylase" having the property that it can methylate cytosine residues at a distance from preexisting "signal" methyl groups. The base composition and distribution of transition mutations in the ζ–η region (Fig. 4) supports the idea that at least at one time, the middle of the region was more heavily methylated than the outlying regions. Outside of the duplication no methylation was detected and the base composition appears normal. This tempts us to speculate that the methylation signal may be the duplication itself. Whether methylation of tandemly duplicated DNA could confer some advantage on an organism is an open question. It is clear from this work, however, that DNA methylation can have important evolutionary consequences. At least in the absence of selection, heavily methylated sequences drift toward a preponderance of adenine and thymine residues as is commonly seen in intergenic regions.

We thank R. L. Metzenberg and C. Yanofsky for valuable discussions and E. A. Craig, E. Lund, R. L. Metzenberg, J. M. L. Selker, O. Smithies, and M. Wickens for comments on the manuscript. This work was supported by a fellowship from the Helen Hay Whitney Foundation to E.U.S. and by Grant GM-08995 from the National Institutes of Health to R. L. Metzenberg.

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