De Novo Methylation of Repeated Sequences in *Coprinus cinereus*

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ABSTRACT

We have examined the stability of duplicated DNA sequences in the sexual phase of the life cycle of the basidiomycete fungus, *Coprinus cinereus*. We observed premeiotic *de novo* methylation in haploid nuclei containing either a triplication, a tandem duplication, or an ectopic duplication. Methylation changes were not observed in unique sequences. Repeated sequences underwent methylation changes during the dikaryotic stage. In one cross, 27% of the segregants exhibited methylation-directed gene inactivation. However, all auxotrophs eventually reverted to prototrophy. C to T transition mutations were not observed in this study. Our studies also revealed one inversion that occurred in 50% of the segregants in a single triplication cross, and a single pop-out event that occurred during vegetative growth. These alterations were similar to changes reported in experiments with duplicated sequences in *Neurospora crassa* and *Ascobolus immersus*. However, significant differences were also noted. First, the extent of methylation was much less in *C. cinereus* than in the other two fungi. Second, CpG sequences appeared to be the preferred targets of methylation.

IN *Neurospora crassa*, an ascomycete fungus, a genetic mechanism has been identified which detects and alters repeated sequences. This has been named "RIP" for repeat induced point mutation and was discovered by E. U. SELKER and collaborators (SELKER *et al.* 1987; reviewed in SELKER 1990, 1991). RIP activity causes the permanent inactivation of repeated sequences due to massive cytosine methylation and the introduction of C to T transition mutations (CAMBARERI *et al.* 1989). RIP is stage specific, reported so far only in the dikaryotic phase of the *N. crassa* life cycle (SELKER *et al.* 1987). The relationship between methylation and RIP is unclear, but there is evidence that the two activities can be separated (FINCHAM 1990).

A related mechanism has been detected in another ascomycete, *Ascobolus immersus* (GOYON and FAUGERON 1989; reviewed in ROSSIGNOL and PICARD 1991). As is the case in *N. crassa*, repeated sequences are methylated during the dikaryotic stage, but gene inactivation is reversible. More recently, repeated sequences subjected to such methylation were sequenced and no C to T transitions were detected (RHOUNIM, ROSSIGNOL and FAUGERON 1992). Taken together, these results demonstrated that no transition mutations occurred. This phenomenon has been termed "MIP" for *m*ethylation *i*nduced *p*remeiotically (ROSSIGNOL and PICARD 1991; RHOUNIM, ROSSIGNOL and FAUGERON 1992).

KRICKER, DRAKE and RADMAN (1992) have provided indirect evidence for the occurrence of RIP in mammals and in other eukaryotes that exhibit postreplicative methylation of cytosine residues. These authors used DNA sequence comparisons and concluded that CpG dinucleotides within duplicated regions of at least 0.3 kb in length are substrates for C to T mutagenesis that results in sequence divergence. To date, direct evidence for the occurrence of MIP or RIP in organisms other than the ascomycetes is lacking. Since the basidiomycete *Coprinus cinereus* exhibits extensive CpG methylation (ZOLAN and PUKKILA 1985, 1986), we set out to determine if repeated DNA sequences in *C. cinereus* are also subject to the action of MIP or RIP, or a related process.

MATERIALS AND METHODS

Strains: Table 1 lists the strains used in this study. Strain T88, a triplication strain, has been described previously (BINNINGER *et al.* 1987). Basically, strain 218 (*trp1-1,1-6*) was transformed to Trp^+ with a plasmid carrying a 6.5-kb genomic fragment (pCc1002) (Figure 1). This plasmid contained the *trp1* gene, which encodes tryptophan synthetase (SKRZYNIA *et al.* 1989) plus the pUC9 vector sequence (VIEIRA and MESSING 1982). In the triplication strain T88, two such plasmids integrated in tandem into the resident *trp1* locus, creating a triplication of the *trp1* gene and a duplication of the vector sequence (Figure 2). By mating strain T88 to a near-isogenic Trp^- strain (295), we isolated Trp^+ progeny that were mating type compatible with the original triplication strain. One of these, strain T2P15, was used in the crosses described in this report. The duplication strain Th10 was created by transforming strain 218 with pDGH (FREEDMAN 1993). This plasmid was derived from pCc1003 (SKRZYNIA *et al.* 1989) by addition of 17 bp to the polylinker. Strain Th10 contains one copy of pDGH integrated at the resident *trp1* locus. Therefore, the *trp1* sequence is duplicated and the vector sequence is single copy, as shown in Figure 2. Strain Th10 was crossed with strain 295 (Trp^-) to isolate a compatible strain that contained the duplication. Such a strain, T259 (Trp^+), was mated with the

TABLE 1

***Coprinus cinereus* strains used**

Strain	Mating type	Relevant genotype	*trp1*(#)[a]	Vector (#)[a]	Source
T88	*A3B1*	*trp1-1,1-6::trp1*-Cc1002^{+} [b]	3	2	BINNINGER *et al.* (1987)
295	*A43B43*	*trp1-1,1-6*$^{-}$	1	0	This laboratory
T2P15	*A43B43*	*trp1-1,1-6::trp1*-Cc1002^{+}	3	2	This laboratory
Th10	*A3B1*	*trp1-1,1-6::trp1*-DGH10^{+}	2	1	This laboratory
T259	*A43B43*	*trp1-1,1-6::trp1*-DGH10^{+}	2	1	This laboratory
T76	*A3B1*	*trp1-1,1-6*$^{-}$ + *trp1*-Cc1002^{+}	2[c]	1	BINNINGER *et al.* (1987)
3/92-8B	*A43B43*	*trp1-1,1-6*$^{-}$	1	0	This laboratory
3/92-8A	*A3B1*	*trp1*$^{+}$	1	0	This laboratory
3/92-11A	*A43B43*	*trp1*$^{+}$	1	0	This laboratory
3/92-9C	*A3B1*	*trp1*$^{+}$	1	0	This laboratory
3/92-10A	*A43B43*	*trp1*$^{+}$	1	0	This laboratory
3/92-3A	*A43B1*	*trp1*$^{+}$	1	0	This laboratory
3/92-6B	*A3B1*	*trp1*$^{+}$	1	0	This laboratory
3/92-9B	*A43B43*	*trp1*$^{+}$	1	0	This laboratory
3/92-11D	*A3B43*	*trp1*$^{+}$	1	0	This laboratory

[a] The copy number of the indicated sequence is shown for each strain.
[b] Double colon indicates plasmid integration at the resident locus (*trp1*).
[c] One *trp1* copy is ectopic.

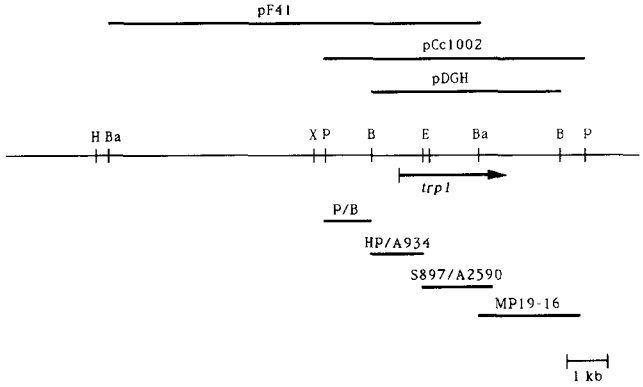

FIGURE 1.—Map of the plasmids used in this study. The top plasmid pF41 contains a *Bam*HI fragment of the *trp1* gene in a pUC9 vector. Plasmid pCc1002 is a *Pst*I fragment of the *trp1* gene in a pUC9 vector. Plasmid pDGH is a *Bgl*II fragment of the *trp1* gene in a Bluescript plasmid. The arrow below the restriction map indicates the *trp1* coding region and direction of transcription. The horizontal lines indicate the four probes used for mapping regions of methylated DNA. Abbreviations defining the probes are described in MATERIALS AND METHODS. Other abbreviations are as follows: B, *Bgl*II; Ba, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I.

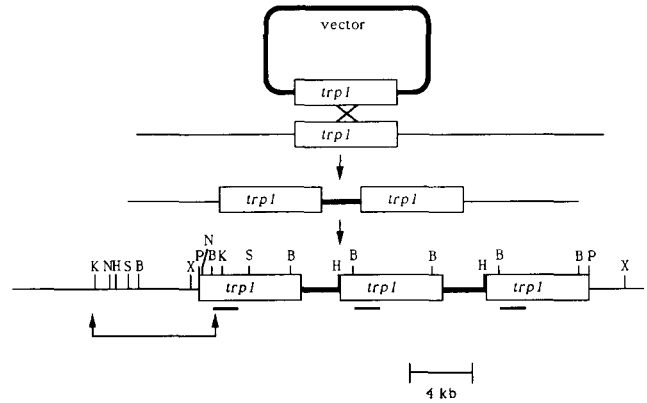

FIGURE 2.—Generation of a *trp1* duplication and triplication by homologous integration. Two arrows with line indicate the inversion described in the text. Horizontal bars indicate regions of *de novo* methylation. Abbreviations are as described in the legend to Figure 1 and also as follows: K, *Kpn*I; N, *Nco*I; S, *Sma*I. The additional *Kpn*I, *Nco*I and *Sma*I sites that are present but not relevant to mapping the inversion breakpoints were omitted for clarity.

original transformant, Th10, for the tandem duplication cross. The ectopic strain T76 has a single copy of the plasmid pCc1002 (Trp^{+}) located on a different chromosome, as well as the resident *trp1-1,1-6* sequence (BINNINGER *et al.* 1987). The ectopic strain T76 was crossed with a near-isogenic Trp^{-} strain (3/92-8B) to follow the segregation patterns of the duplication. Two single copy crosses were performed between near-isogenic compatible Trp^{+} strains. Strain 3/92-8A (Trp^{+}) was crossed with strain 3/92-11A (Trp^{+}) and strain 3/92-9C (Trp^{+}) was crossed with strain 3/92-10A (Trp^{+}), (refer to Table 1).

Culture conditions and tetrad analysis: Monokaryotic strains were serially transferred 5 times after 2 days of growth on YMGT (yeast-maltose-glucose-tryptophan) media (RAO and NIEDERPRUEM 1969; BINNINGER *et al.* 1987). Oidia (haploid asexual spores) were collected from the monokaryons after the fifth transfer and were spread on YMGT plates for germination. Oidial isolates were collected and inoculated into liquid media for DNA extraction (see below).

All strains were mated by placing two monokaryotic isolates in close proximity on YMGT plates at 37°. After 2–3 days growth of the resulting dikaryon, deep YMGT Petri dishes were inoculated with the dikaryon and incubated at 37° in the dark for 5 days. The dishes were then transferred to a room at 25° and were exposed to the alternating light and dark cycles suitable for fruiting. Gills from the fruiting bodies were split and dissected for tetrad analysis. Tetrads were isolated with a Jena micromanipulator and were transferred to 4% agar slabs supplemented with YMGT and 0.01% furfural. Individual spores were separated by hand with a glass rod. After germination, strains were transferred to YMGT at 37° for 2–3 days. Segregants were analyzed for mating types by crossing them with appropriate tester strains, (3/92-3A, 3/92-6B, 3/92-9B and 3/92-11D, Table 1). They were also analyzed for Trp^{-} auxotrophy after

transfer to minimal medium lacking tryptophan (BINNINGER *et al.* 1987).

To test the effect of low temperatures on methylation in the dikaryon stage, strains T88 and T2P15 were mated on a YMGT plate at 37°. The dikaryon was stored at 4° for 22 days before inoculating deep Petri dishes containing YMGT medium for fruiting. In the control, and in all other crosses described, the dikaryons were never exposed to low temperatures before inoculation of dishes for fruiting.

To recover single vegetative cells from dikaryotic strains, dikaryotic veil cells were scraped off fruiting bodies and plated on YMGT medium where they frequently produced monokaryotic colonies. Veil cells do not undergo meiosis. Colonies were transferred to liquid YMGT medium for growth and subsequent DNA extraction (see below).

Isolation and manipulation of DNA for Southern analysis: For DNA extraction, a small amount of mycelium was inoculated into 10 ml of liquid YMGT and grown at 37° until confluent. Mycelium was collected and lyophilized, and DNA was extracted by the procedures outlined (ZOLAN and PUKKILA 1986), except that lyophilized tissue was incubated in extraction buffer for 15 min at room temperature. DNA samples were digested with appropriate enzymes and electrophoresed in gels containing 1.6–2.2% Nusieve 3:1 agarose (FMC). The extent of digestion was monitored by exploiting the observation that mitochondrial DNA is not methylated. Therefore, if enzymatic activity is adequate, bands derived from complete digestion of mitochondrial DNA are easily visible after ethidium bromide staining. We routinely confirmed that complete digestion had occurred by examining the mitochondrial DNA profiles. DNA was transferred by blotting onto Hybond N^+ nylon filters (Amersham) and Southern hybridization was performed according to the manufacturer's instructions (Amersham). ^{32}P-Labeled probes were generated by primer-extension (U.S. Biochemical primer-extension kit). Probes used for mapping experiments are described in Figure 1. Probe P/B was a 1.2-kb fragment generated by a *Pst*I/*Bgl*II double digest of the plasmid pF41 (BINNINGER 1987) recovered from a SeaPlaque gel (FMC). Probes HP/A934 and S897/A2590 were synthesized by using the polymerase chain reaction (PCR) (SAIKI *et al.* 1988) from sequence information described previously (SKRZYNIA *et al.* 1989) and recovered from 1% SeaPlaque gels. Probe HP/A934 was a 1.6-kb PCR-generated fragment of the *trp1* gene. This probe was PCR-amplified from genomic DNA (strain Th41, a derivative of strain Th10) and oligonucleotide primers, HP (a unique insertion in the polylinker) and A934 (an oligonucleotide within the *trp1* coding region). The probe S897/A2590 was a PCR-amplified 1.7-kb fragment of the internal region of the *trp1* coding sequence. The primers were S897 and A2590. Probe mp19-16 was a previously made M13 clone which spans the 3′ end and flanking sequences of the *trp1* gene (SKRZYNIA *et al.* 1989).

To locate the chromosome containing the ectopic duplication, chromosome-sized DNA was prepared and electrophoresed on a Bio-Rad CHEF apparatus (CHU, VOLLRATH and DAVIS 1986) and run according to the conditions described (PUKKILA and SKRZYNIA 1993). Preparation of plugs is described elsewhere (BINNINGER *et al.* 1991).

Statistical analysis: Statistical analysis was performed according to the method of FISHER (1948).

RESULTS

Alterations in methylation of repeated sequences: We monitored methylation in crosses containing either a triplication, tandem duplication, ectopic duplication or a single copy of the *trp1* gene. We took advantage of the fact that digestion by the restriction enzyme *Hpa*II is sensitive to methylation. Accordingly, we digested DNAs with *Hpa*II and compared the parental fragments with those obtained from meiotic progeny. An example from this study is shown in Figure 3A. Lanes P1 and P2 contain parental DNAs in the triplication cross (T88 and T2P15) and DNAs from three complete tetrads are shown. Several novel *Hpa*II fragments are apparent in the tetrad progeny ranging in size from 0.69 kb (tetrad 3) to 6 kb (tetrad 2). Every segregant had at least three new bands which were not present in the parents.

Our results are summarized in Table 2. When either or both parental strains contained more than one copy of the *trp1* gene, many new *Hpa*II fragments were observed in the progeny. Of the 46 complete tetrads observed, 40 of them contained segregants with novel *Hpa*II fragments. In contrast, when neither parent contained a duplication, alterations in *Hpa*II fragments were not observed, as illustrated in Figure 4. In Figure 4, P1 and P2 are the single copy parents (3/92-11A and 3/92-8A) and three complete tetrads are shown. No gains or loses of bands occurred in any segregant among the eight tetrads examined in the single copy crosses (Figure 4 and Table 2). The difference in methylation between tetrads from single copy and from crosses containing repeated sequences is highly significant ($\chi^2 = 14$, $P << 0.05$). We therefore conclude that *de novo* methylation within *trp1* sequences only occurs when more than one copy is present in the genome.

Methylation occurs preferentially at CpG sequences: To detect site preferences for methylation activity, several different methylation sensitive enzymes were utilized. The restriction enzymes *Hpa*II and *Msp*I both recognize the same sequence CCGG, but *Hpa*II does not cut if either C is methylated, whereas *Msp*I does not cut if the external C is methylated (NELSON and MCCLELLAND 1991). Thus, novel fragments appearing in *Hpa*II but not in *Msp*I digests indicate that methylation is occurring specifically at CpG sites. Figure 3 compares digests of the same DNAs with *Hpa*II (Figure 3A) and *Msp*I (Figure 3B). In the *Hpa*II digests, each tetrad showed a distinct pattern of bands, and no segregants were identical to the parental pattern. In contrast, in the *Msp*I digests, no changes in band sizes can be detected. Therefore, we concluded that methylation occurred at CpG sites and not at CpC sites.

It appears that the parental DNAs were methylated, since *Hpa*II and *Msp*I patterns are not identical, although the *Hpa*II patterns of P1 and P2 are identical to each other. Since P1 is a primary transformant while P2 is a compatible meiotic segregant, we con-

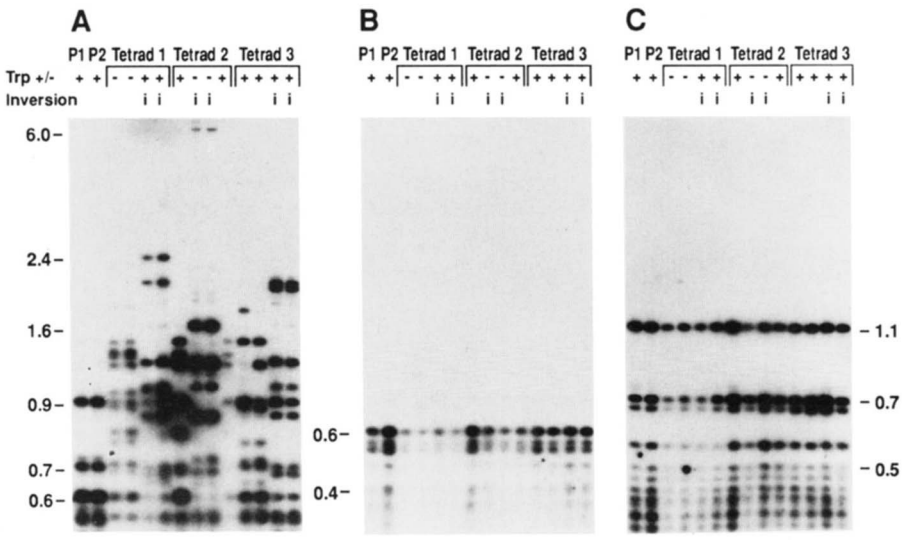

FIGURE 3.—Autoradiograms illustrating methylation in the *trp1* triplication. DNAs were digested with: (A) *Hpa*II, (B) *Msp*I and (C) *Nla*III. P1 and P2 are the two parental strains T88 and T2P15. The Trp phenotype (+/−) and presence of the inversion (i) described in the text are indicated at the top of the figure. Sizes are indicated (in kilobases). The probe was ^{32}P-labeled pCc1002.

TABLE 2

Methylation changes observed in progeny and monokaryons

Cross/Strain	No. of segregants scored	% Trp$^-$	% Tetrads with altered bands	% Segregants with altered bands	Average no. of new bands[a]	% Tetrads with only 2:2 segregation
Triplication cross #1 T88 × T2P15	44 (11 tetrads)	27[b]	100	100	5.9	55
Triplication cross #2 T88 × T2P15	24 (6 tetrads)	0	100	50	2	100
Triplication cross #3 T88 × T2P15	40 (10 tetrads)	0	100	75	3.9	60
Tandem duplication cross Th10 × T259	20 (5 tetrads)	0	100	50	1.8	60
Ectopic duplication cross T76 × 3/92-8B	56 (14 tetrads)	0	57	29	1	100
Single copy cross #1 3/92-8A × 3/92-11A	20 (5 tetrads)	0	0	0	0	100
Single copy cross #2 3/92-9C × 3/92-10A	12 (3 tetrads)	0	0	0	0	100
Veil cells T88 × T2P15	7	0	NA[c]	86	2.7	NA
Parental monokaryon T88	8	0	NA	0	0	NA
Parental monokaryon T2P15	8	0	NA	0	0	NA

[a] This refers only to segregants that were methylated.
[b] 12/12 Trp$^-$ segregants reverted to Trp$^+$ after growth on selective media.
[c] Not applicable.

cluded that the limited methylation observed in these strains took place during vegetative growth of the primary transformant. It is formally possible that the methylation occurred prior to transformation in this particular isolate, although comparison of *Hpa*II and *Msp*I digests has not revealed any methylation at the *trp1* locus in non-transformed strains (results not shown).

The novel fragments present in the *Hpa*II digests of segregants probably arose from *de novo* methylation of CpG sequences. Several other methylation sensitive restriction enzymes including *Alu*I, *Mbo*I and *Scr*FI were used to determine if methylation was occurring at sites other than CpG in the triplication. The restriction enzyme *Alu*I recognizes the sequence AGCT but does not cleave if either the C or the A residues are methylated. This would indicate the presence of GpC, CpT and adenine methylation. Similarly, the restriction enzyme *Mbo*I recognizes the sequence GATC and will not cut DNA if the A residue is methylated. This would indicate the presence of adenine methylation. And finally, the enzyme *Scr*FI recognizes the sequence CC(A/T)GG, but will not cut if the internal C is methylated. Failure to restrict this sequence would

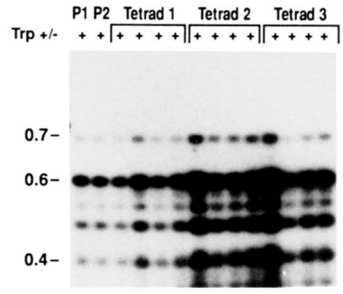

FIGURE 4.—Autoradiogram illustrating lack of methylation changes in the single copy cross. DNAs were digested with *Hpa*II. P1 and P2 are the parental strains 3/92-11A and 3/92-8A. The Trp phenotype is indicated. Sizes are indicated on the left (in kilobases). The probe was ^{32}P-labeled pCc1002.

indicate the presence of CpC methylation. No changes in banding patterns as observed with Southern analysis were detected with any of these enzymes (data not shown). This indicates that CpG sites are the preferred target for methylation.

Methylation is not accompanied by point mutations: In *N. crassa*, the repeat-dependent RIP process leads to numerous C to T transition mutations (CAMBAREREI *et al.* 1989). We used the restriction enzyme *Nla*III to look for C to T transitions occurring in repeated sequences in *C. cinereus*. The recognition sequence for *Nla*III is CATG, which contains two copies of the dinucleotide CpA in double stranded DNA. CpA is the preferred target for C to T transitions in *N. crassa* (CAMBAREREI *et al.* 1989). We reasoned that any C to T transition mutations that occurred within this target sequence would eliminate the restriction enzyme recognition site, and alterations in hybridization patterns would be observed. However, no changes in *Nla*III digests were found in any of the 58 segregants, revertants and veil cell isolates examined in this study. Second and third triplication and tandem duplication crosses were not examined. An example of this analysis is shown in Figure 3C. Two parental strains (T88 and T2P15) and three complete tetrads from the first triplication cross are shown. No alterations in any of the 10 resolved bands were detected. This indicated that base pair changes did not occur in the *Nla*III restriction sites in the parents or their progeny.

The *Nla*III assay might fail to reveal sequence alterations in methylated *Nla*III sites, since the sensitivity of this enzyme to methylation is unknown. Consequently, we also used the restriction enzyme *Msp*I to ask if any base pair changes occurred within CCGG sequences. However, the *Msp*I patterns of every segregant we examined (106 samples) were identical to the parental patterns, indicating that no base pair changes occurred within a sequence known to be a target for *de novo* methylation (Figure 3, A and B). Although these assays are less sensitive than sequence comparison, this evidence suggests that either RIP

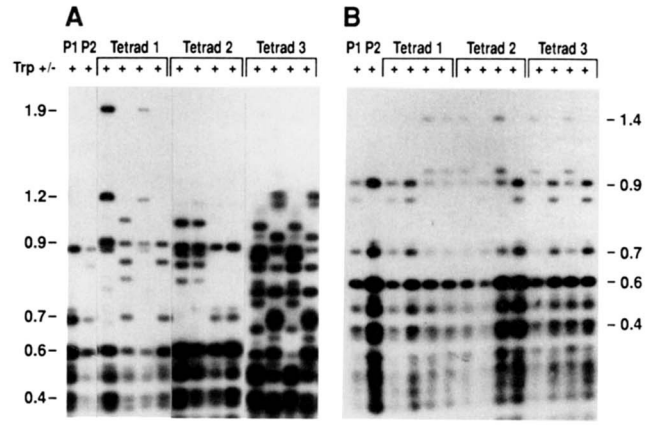

FIGURE 5.—Autoradiograms illustrating the effect of low temperatures on methylation in the triplication crosses. DNAs were digested with *Hpa*II. The Trp phenotype is indicated. (A) Triplication cross #3 exposed to low temperatures. P1 and P2 are parental strains T2P15 and T88. (B) Triplication cross #2 (control). P1 and P2 are parental strains T88 and T2P15. Sizes are indicated on the left and right (in kilobases). The probe was ^{32}P-labeled pCc1002.

does not occur in *C. cinereus* or that it occurs much less frequently than MIP.

Methylation can result in the inactivation of the *trp1* gene: To detect possible methylation-directed inactivation of the *trp1* gene, we grew segregants on minimal medium to score the Trp$^-$ progeny. In triplication cross #1, 27% of the segregants were Trp$^-$. This was a surprising result, because previous random spore analysis from this cross had not revealed extensive methylation that could result in gene inactivation (data not shown). We realized that the dikaryotic culture used for the random spore analysis had been maintained at 25°. The cultures were then stored at 4° for several days prior to inoculation of dishes for fruiting of cross #1. We wondered if incubation of the dikaryon in the cold could somehow lead to an increase in methylation levels, and hence explain the gene inactivation observed. This hypothesis was tested by comparing methylation frequencies from the same dikaryon either exposed (cross #3) or not exposed (cross #2) to low temperatures during the dikaryotic stage as described in MATERIALS AND METHODS. Although gene inactivation was not observed among segregants of the cold treated cross, the methylation levels were clearly higher in cross #3 when compared to the control. The two crosses are shown in Figure 5. In the cold-treated cross (Figure 5A), more altered bands of different molecular weights are apparent than in the control (Figure 5B; see also Table 2). The difference in methylation of segregants between the two crosses as measured by the average number of new bands per segregant is statistically significant ($\chi^2 = 4.2$, $P < 0.05$). Furthermore, there was an increase in the number of bands that failed to show 2:2 segregation in triplication cross #3 (discussed in the following section). It appeared that cold treatment during

the dikaryon stage could lead to increases the amount of methylation in a fashion that might be difficult to control. Consequently, in all other experiments, we never exposed any portion of the cultures to temperatures below 25°. We were unable to identify the conditions that allowed us to detect inactivated genes in triplication cross #1.

Of the Trp$^-$ segregants detected in triplication cross #1, 100% reverted to prototrophy after incubation on selective media. The time required for reversion varied among strains from 2 days to 1 month. No base pair changes were detected, but one pop-out event (the reverse of an homologous integration) occurred in one revertant (discussed below). We conclude that methylation can result in gene inactivation that is reversible.

Mapping experiments were performed to determine the site of methylation in the repeated sequences. Radioactive probes spanning the *trp1* gene (Figure 1) were utilized to reprobe Southern analyses, and a map of methylated regions was constructed (Figure 2, horizontal bars). In most cases, the highest molecular weight fragments in a *Hpa*II digest hybridized to the HP/A934 probe. We concluded that most of the methylation changes were confined to the 5′ end of the *trp1* gene and to upstream flanking sequences. We were particularly interested in comparing methylation patterns of the Trp$^-$ segregants to the patterns obtained in the Trp$^+$ revertants. As a rule, changes in methylation at this region of the *trp1* gene were detected when we compared the segregant and its revertant. Surprisingly, some of the Trp$^+$ revertants exhibited more high molecular weight bands in *Hpa*II digests, while others showed reduced levels of methylation (data not shown). Because there were three copies of the *trp1* gene available with which a probe can hybridize, it was not possible to determine which repeat(s) were methylated in a particular segregant.

Methylation changes occur preferentially during the dikaryotic stage: To investigate the timing of methylation changes, we analyzed cells for methylation at different stages of the organism's life cycle. Monokaryotic (unmated) parental strains T88 and T2P15 were serially transferred and oidial isolates (asexual spores) of each parent were analyzed for methylation changes by DNA extraction and Southern analysis. No methylation changes were detected in the monokaryotic strains (Table 2). After compatible strains were mated, each dikaryotic cell contained one haploid nucleus from each parent. We examined veil cells from the second triplication cross to see if such dikaryotic cells exhibited altered methylation patterns in repeated sequences. Veil cells are dikaryotic cells found on the outer surface of the fruiting body which never experience meiosis. If veil cells are removed from the fruiting body and cultured, either monokaryotic or dikaryotic hyphae are produced. Accordingly, we isolated DNA from each culture and analyzed it for methylation changes. As shown in Table 2, 86% of the veil cells exhibited altered banding patterns in a *Hpa*II digest. This value is comparable to the proportion (50%) of meiotic segregants that exhibited changes in this cross (see Table 2). This indicated that methylation can occur in the dikaryon stage and does not require karyogamy (nuclear fusion) and meiosis.

To determine if methylation changes only occurred during the dikaryotic stage prior to karyogamy, we examined tetrad segregation patterns. In Figure 3A, the three tetrads shown from the first triplication cross exhibited a 2:2 segregation pattern after digestion with *Hpa*II. However, no two tetrads were identical. These patterns are most easily explained by the recognition and methylation of the repeated sequences in the dikaryon prior to premeiotic DNA replication and by the maintenance of the new methylation patterns following meiosis and growth of the haploid segregant. We concluded that methylation probably occurred premeiotically.

Occasionally, methylation changes were detected which did not segregate 2:2. For example, in Figure 3A, tetrad #3, the first segregant has a fragment with a molecular weight of 1.75 kb that is not present in the second (sister) segregant. The second segregant has a 1.25-kb fragment that is not present in the first segregant. Tetrads that contained bands present in only one segregant were more common in the two crosses (triplication cross #1 and #3) in which the dikaryon was exposed to low temperature (43% of all tetrads) than in the other three crosses where the dikaryon was incubated at 25° or above (9% of all tetrads). This difference is significant ($\chi^2 = 7.6$, $P < 0.01$). There are two possible explanations for these 2:1:1 tetrads. First, they could have resulted from recognition of the repeated sequences and methylation following premeiotic DNA replication. Second, such patterns might arise if methylation changes arose premeiotically and were unstable during subsequent growth of the monokaryon. In a previous study, we determined that methylation patterns were faithfully maintained throughout meiosis and mitosis in *C. cinereus* (ZOLAN and PUKKILA 1986). In these experiments, we also observed faithful transmission of methylation patterns in monokaryons that were serially transferred five times prior to isolation of and growth of the colonies for DNA extraction (Table 2). However, we did observe a single change in a subculture from the original T88 stock (2.8-kb band in P2, Figure 5A and in P1, Figure 5B). In addition, we observed changes in methylation patterns in Trp$^-$ monokaryons after incubation on plates lacking tryptophan. We

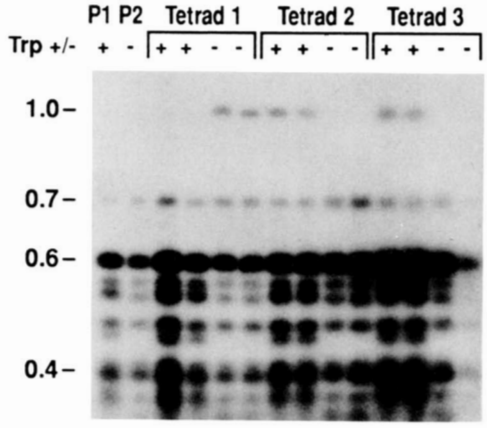

FIGURE 6.—Autoradiogram illustrating methylation in the ectopic duplication. DNAs were digested with *Hpa*II. P1 and P2 are the parental strains T76 (Trp^+) and 3/92-8B (Trp^-). The Trp phenotype is indicated. Sizes are indicated on the left (in kilobases). The probe was ^{32}P-labeled pCc1002.

conclude that methylation patterns are usually faithfully maintained in monokaryons although alterations can occur, particularly under selective conditions. Methylation changes occur most often during the dikaryotic stage.

Methylation changes occur in both tandem and ectopic duplications: To monitor methylation changes in duplications, two types of crosses were performed. One cross (Th10 × T259) resulted in the presence of a tandem duplication in every nucleus. In the other cross (T76 × 3/92-8B), a nontandem ectopic duplication was present in only one nucleus in each dikaryotic cell. The duplication strain T76 carried a mutant allele of the *trp1* gene (*trp1-1,1-6*) on chromosome *IV* and an additional copy of the $trp1^+$ gene. To map the additional copy, DNA from strain T76 was subjected to CHEF electrophoresis to resolve the chromosomal sized DNAs. Following Southern blotting and hybridization, both a 3.4-mb band (chromosome *IV*) and a 2.2-mb band (unresolved chromosomes *X*, *XI* and *XII*) hybridized to plasmid pCc1002 (results not shown). We concluded that strain T76 contained an ectopic duplication. Tetrads from these two crosses were analyzed by Southern analysis for methylation changes using *Hpa*II, as before.

In the tandem duplication cross, methylation changes were detected in 50% of the segregants and 100% of the tetrads (Table 2). In the ectopic cross, 29% of the segregants and 57% of the tetrads exhibited methylation changes (Table 2). The difference in the number of segregants with methylation changes in the tandem cross compared to the ectopic duplication cross was slight, and not statistically significant ($\chi^2 = 3.0$, $P > 0.08$).

In the ectopic duplication experiment, a single novel *Hpa*II fragment (1 kb) was detected in 29% of the tetrad progeny (Figure 6 and Table 2). This band hybridized to probe HP/A934, which derives from

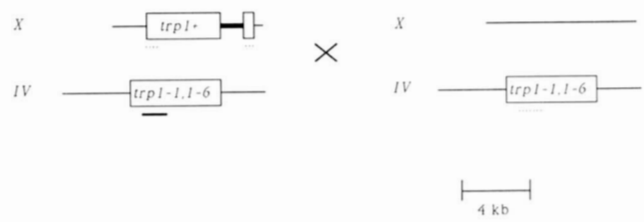

FIGURE 7.—Cartoon illustrating that the endogenous *trp1* gene is methylated when an ectopic copy is present. Thin line indicates plasmid DNA, and white box represents the *trp1* gene. Horizontal bar indicates methylation and dotted lines indicate the corresponding regions where methylation is not detected. *IV*, portion of chromosome *IV*; *X*, portion of chromosome *X*, *XI* or *XII*.

the 5′ end of the *trp1* gene. In the tandem duplication cross, only two novel *Hpa*II fragments (1.5 and 1.3 kb) were observed in the segregants with altered bands (results not shown). It is striking that the *Hpa*II banding patterns were so much more uniform in the duplication crosses than in the triplication crosses (compare Figure 6 and Figure 3a). It also appeared that the amount of methylation in both duplication crosses was remarkably similar despite the fact that in the ectopic duplication cross, one copy was located on a different chromosome.

In the ectopic duplication experiment, either no methylated segregants (0:4) or two methylated segregants (2:2) were observed, but we never found four methylated segregants in a tetrad (no 4:0 segregation patterns). All tetrads segregated 2:2 for Trp^+, indicating that the ectopic copy (Trp^+) was segregating in a Mendelian fashion. The ectopic copy segregated with the 1-kb band 50% of the time. This was taken to indicate that the endogenous copy was methylated and detected by our methods (1-kb band), whereas the ectopic copy either was not methylated or methylated and undetected (Figure 7). This was a surprising result because previous experiments using triplications had demonstrated that MIP never affects just one copy (FAUGERON, RHOUNIM and ROSSIGNOL 1990). Data from restriction maps revealed that when pCc1002 inserted into the ectopic site on chromosome *X*, the plasmid was broken within the region where methylation can occur (Figure 7). Therefore, methylation of this region may go undetected because of nearby flanking *Hpa*II sites located outside the integrated *trp1* sequence. An *Hpa*II site in close proximity to the *trp1* insertion might generate so small a fragment after *Hpa*II digestion that it would not be detected. We concluded that although we were unable to detect methylation in the ectopic copy, we did observe methylation in the endogenous *trp1* gene in response to an unlinked duplicated sequence. This was a very significant result because we have never observed *de novo* methylation of single copy endogenous sequences in either this study (see Figure 4) or in previous studies involving a different genomic region (ZOLAN and PUKKILA 1986).

Genetic rearrangements observed: To determine whether gross rearrangements or pop-out events occurred during our studies, we analyzed segregants from all crosses with repeated sequences, revertants and veil cells for possible rearrangements. Rearrangements were detected by Southern analysis using several different restriction enzymes. Figure 2 shows a rearrangement detected in 50% of the segregants in triplication cross #1. A 7.6-kb DNA fragment (see arrows in Figure 2) was inverted in these strains. This inversion was not detected in either parent and must have occurred very early after fertilization since the identical inversion was recovered in two members of every tetrad. The inversion had no apparent effect on the methylation process in this cross since methylation changes were observed in 100% of segregants containing the inversion, and also in 100% of segregants that did not contain the inversion. Furthermore, the inversion had no apparent influence on the recovery of Trp^- segregants. Among the twelve Trp^- segregants we recovered, ten harbored the inversion and two did not (see Figure 3).

Southern analysis also revealed an additional rearrangement in a single Trp^+ revertant. In this revertant, the internal repeat was excised, leaving a duplication of the *trp1* gene. Apparently, this pop-out event occurred vegetatively during selective growth for Trp^+ revertants.

DISCUSSION

We have identified the hallmark characteristics of MIP activity in *C. cinereus* (ROSSIGNOL and PICARD 1991; RHOUNIM, ROSSIGNOL and FAUGERON 1992): only repeated sequences are methylated, methylation occurs during the dikaryotic stage, no C to T transitions are detected, and gene inactivation due to methylation is reversible. These observations allow us to conclude that MIP occurs in *C. cinereus*. Two differences were detected in MIP activity between *C. cinereus* and *A. immersus*: in *C. cinereus*, there is a lower level of methylation, and CpG methylation preference has been detected. By visual comparison of *Hpa*II digests of Southern gels, it is apparent that more altered bands are visible in *A. immersus* than in *C. cinereus*. There is such extensive methylation in *A. immersus* that the bands were densely clumped together in Southern analyses of *Hpa*II digests (see GOYON and FAUGERON 1989), whereas discrete bands were easily resolved in *C. cinereus*. In *N. crassa*, repeated sequences are also more methylated compared to *C. cinereus* (SELKER *et al.* 1987).

The frequency of methylation-directed gene inactivation in *A. immersus* is much higher than in *C. cinereus*. For example, in *A. immersus*, all segregants with tandem duplications were inactivated (RHOUNIM, ROSSIGNOL and FAUGERON 1992), whereas no inactivation of segregants with duplications was detected in *C. cinereus*. In ectopic crosses, 64% of the repeated genes were inactivated in *A. immersus*, and no inactivation was observed in C. cinereus in a similar cross. Methylation-directed gene inactivation was observed in only one triplication cross in *C. cinereus*.

To date, *C. cinereus* is the only organism in which CpG methylation specificity associated with MIP has been found. In *A. immersus* and *N. crassa*, cytosine methylation is not specific for CpG sites although a preference for CpG sequences have been detected in *N. crassa* (RHOUNIM, ROSSIGNOL and FAUGERON 1992; SELKER and STEVENS 1987). This CpG specificity probably reflects the type of methylase activity associated with MIP in *C. cinereus*. An inversion was detected in 50% of segregants in triplication cross #1. This rearrangement probably occurred early after fertilization since it is detected in all tetrads. An intrachromosomal recombination event was also detected in triplication cross #1 during a reversion experiment. This pop-out event occurred during vegetative growth under selective conditions. Pop-out events have been reported in *N. crassa* (SELKER *et al.* 1987; CAMBARERI, SINGER and SELKER 1991) and in *A. immersus* (GOYON and FAUGERON 1989) in response to repeated sequences. In *N. crassa*, pop-out frequencies are much higher than those observed in *C. cinereus*. Vegetative pop-out events have been reported in two of six vegetative reisolates studied in *N. crassa* (SELKER 1990). In asci, a 65% pop-out frequency was observed in crosses (SELKER 1990). In this study, no pop-out events were detected during the sexual phase of *C. cinereus*.

What is the function of MIP? It has been postulated that MIP might confer a selective advantage for its bearer by inactivating repeated sequences potentially lethal in higher doses (FAUGERON, RHOUNIM and ROSSIGNOL 1990). It could halt the spread of viruses and transposons.

In *C. cinereus*, the detection of repeated sequences appears to occur efficiently, although they are seldom inactivated. It was particularly striking to observe methylation of the endogenous *trp1* gene in 57% of the tetrads examined when an unlinked copy was present. We concluded that the premeiotic genome-wide homology search necessary for such events occurs as efficiently in *C. cinereus* as is does in *A. immersus* and *N. crassa*. However, it is not clear why MIP activity persists in *C. cinereus* since it is obviously so inefficient in inactivating repeated sequences compared to its activity in other organisms. It could be that our experimental conditions did not fully permit MIP activity. Perhaps yet unidentified environmental factors such as temperature, growth medium, or exposure to competitors or viruses are required to fully activate MIP. It could be that MIP levels in *C. cinereus*

are much higher in nature. It may be that the strains used in this experiment contained an unusually low MIP activity. Alternatively, it may be that the MIP process plays other roles in *C. cinereus*. For example, even low levels of methylation might be sufficient to "mark" ectopic repeats prior to meiosis so as to avoid ectopic recombination that would otherwise produce translocations. It will be important to unravel the biochemistry of those enzymatic processes involved in MIP and RIP, and in particular to define the mechanisms responsible for the genome-wide homology search before meiosis. A promising future study would be to monitor MIP levels in mutants defective in chromosome pairing, such as *rad3* (ZOLAN, TREMEL and PUKKILA 1988; PUKKILA, SKRZYNIA and LU 1992).

C. cinereus is a member of the highest evolutionary group so far studied to possess MIP activity, raising the question as to how widespread this process might be. In plants, transgenic DNA has been found to be methylated and reversibly inactivated only when repeated genes are present (MATZKE *et al.* 1989; SCHEID, PASZKOWSKI and POTRYKUS 1991). DNA methylation is known to be essential for viability in the mouse (LI, BESTOR and JAENISCH 1992).

DNA methylation also occurs in many fungi (MAGILL and MAGILL 1989; MOOIBROEK *et al.* 1990; PENG, SINGH and LEMKE 1992), although systematic studies that would reveal MIP or RIP activity have not yet been undertaken in many of these systems.

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