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Methylation of DNA Repeats of Decreasing Sizes in *Ascobolus immersus*

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In *Ascobolus immersus*, DNA duplications are subject to the process of methylation induced premeiotically (MIP), which methylates the cytosine residues within the repeats and results in reversible gene silencing. The triggering of MIP requires pairing of the repeats, and its detection requires maintenance of the resulting methylation. MIP of kilobase-size duplications occurs frequently and leads to the methylation of all C residues in the repeats, including those belonging to non-CpG sequences. Using duplications of decreasing sizes, we observed that tandem repeats never escaped MIP when larger than 630 bp and showed a sudden and drastic drop in MIP frequencies when their sizes decreased from 630 to 317 bp. This contrasted with the progressive decrease of MIP frequencies observed with ectopic repeats, in which apparently the search for homology influences the MIP triggering efficiency. The minimal size actually required for a repeat to undergo detectable MIP was found to be close to 300 bp. Genomic sequencing and Southern hybridization analyses using restriction enzymes sensitive to C methylation showed a loss of methylation at non-CpG sites in short DNA segments, methylation being restricted to a limited number of CpG dinucleotides. Our data suggest the existence of two distinct mechanisms underlying methylation maintenance, one responsible for methylation at CpG sites and the other responsible for methylation at non-CpG sites.

The process of methylation induced premeiotically (MIP) detects DNA sequence duplications in the haploid genome of *Ascobolus immersus* and methylates the cytosine residues within the repeats (29). MIP is triggered only during the sexual reproduction cycle, most likely in the dikaryotic tissue which results from fertilization (9). Its triggering requires a pairwise interaction between the repeats, so that in a duplication both copies are always simultaneously subject to MIP (9). The search for homology involved is particularly efficient. Ectopic duplications of a 5.7-kb fragment carrying the *A. immersus met2* gene or of its subfragments underwent MIP in 64% of the postfertilization nuclei for the largest duplication and 30% for the shortest (1.2 kb) (1, 25). The process was still more efficient with tandem duplications, since they never escaped MIP (24, 25). The fact that MIP is mediated by a homology-sensing machinery raises the question of its relationship with homologous recombination. It is unlikely that the DNA breaks that have been shown to be required in *Saccharomyces cerevisiae* in the pairing process acting in meiotic recombination (23) are also involved in MIP, as discussed previously (29). This suggests that even though MIP involves a pairing step, this step proceeds through a mechanism different from that acting in homologous recombination. This view is further supported by the demonstration that repeat-induced point mutation (RIP), a process acting in *Neurospora crassa* (31) and closely related to MIP, is not associated with recombination (17). MIP is thus likely to reveal an as-yet-undescribed DNA pairing mechanism that may play an important role in eukaryotic cells. In the case of homologous recombination, the length of homology has been shown to be one of the parameters controlling the process. The minimal length required for efficient homologous recombination has been determined to be in the 250- to 300-bp range in mammalian cells (20) and in *S. cerevisiae* (18). We took advantage of the fact that MIP is amenable to analysis to test whether decreasing the size of the repeats has an effect on MIP triggering, examining whether MIP requires a minimal repeat size.

Once MIP has been triggered, methylation of kilobase-size duplications is stably maintained in vegetative cells, and its maintenance does not require the presence of repeats, in contrast with the de novo methylation of MIP (9, 25). MIP results in gene silencing because of a defect in transcription (1). Methylation was first studied by Southern hybridization experiments using restriction enzymes sensitive to C methylation (1, 11, 25) and then by the bisulfite genomic sequencing method (10) adapted to *A. immersus* (13). Two main features emerged from these studies. (i) In all duplications, whether ectopic or tandem, methylation was restricted to the duplicated segments. (ii) Methylation was particularly dense, with all C's being methylated whether or not they were part of symmetrical sequences. This contrasted with the situation in higher eukaryotes, in which most 5-methylcytosine residues belong to short symmetrical sequences consisting of CpG and CpNpG (4–6, 14). Nonsymmetrical methylation has also been reported for *N. crassa* (32) and *Petunia hybrida* (21). This raises the question of how such methylation patterns are propagated. In the case of *A. immersus*, propagation of methylation relies on preexisting methylation (25), indicating that it is mediated by a maintenance mechanism. However, methylation at nonsymmetrical sequences challenges the maintenance methylation model proposed for perpetuation of C methylation in higher eukaryotes (15, 26), in which a maintenance methylase would recognize the hemimethylated CpG (or CpNpG) sequences issued from replication and would methylate the C in the new strand. Goyon et al. (13) proposed that methylation resulting

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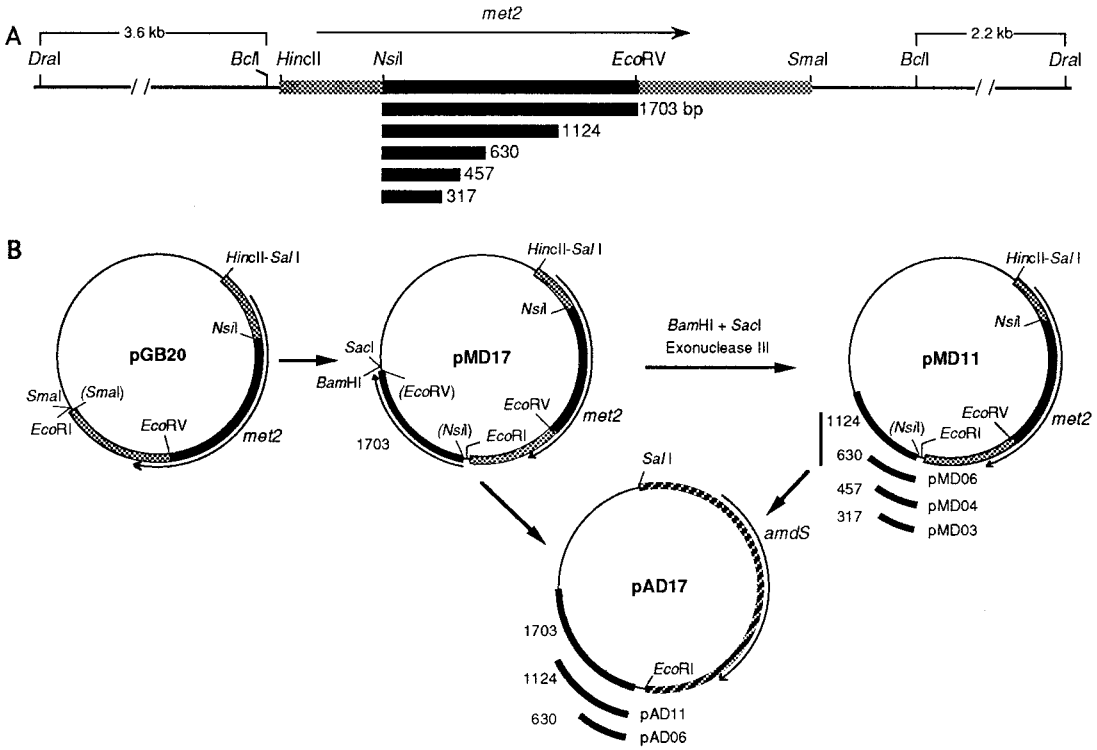

FIG. 1. Partial restriction map of the chromosomal *met2* region (A) and construction of the plasmids used for creating *met2* duplications in *A. immersus* (B). (A) The *Nsi*I-*Eco*RV fragment (black box) corresponds to the *met2* ORF. The five shorter black boxes below the map correspond to the subfragments of *met2* that have been duplicated; their sizes are indicated. The arrow indicates the transcribed region. (B) Plasmids were constructed as described in Materials and Methods. Vector pBluescriptKS (Stratagene) (thin lines), the *amdS* region (striped box), the restriction sites that have been destroyed in the cloning procedure (in parentheses), the sizes (in base pairs) of the duplicated subfragments of *met2* and the 5′-to-3′ direction of the *met2* and *amdS* transcripts (curved arrows) are shown.

from MIP was maintained by a non-sequence-specific methyltransferase activity, recognizing a hemimethylated region rather than a hemimethylated site. Hence, we questioned whether a region methylated at nonsymmetrical sites has to have a minimal size for efficient methylation maintenance.

These two questions of a minimal repeat size for methylation maintenance and for MIP triggering are interconnected, since for two repeats to undergo detectable MIP, they first must find each other in order to pair, then the pairing structure has to be de novo methylated, and finally, in order to be detected, methylation has to be maintained at each DNA replication cycle during the vegetative propagation of the mycelium. We thus addressed these questions simultaneously by assessing the minimal size required for the repeats to ensure detectable MIP (i.e., methylation) and by characterizing their methylation patterns. We created several duplication-containing *A. immersus* strains harboring one full-length copy of the *met2* gene together with a subfragment of it, whose size was decreased until MIP was barely detectable. We performed our study using tandem and ectopic repeats.

MATERIALS AND METHODS

Plasmids (Fig. 1). Plasmid pGB20 resulted from the insertion of the 3.6-kb *Hin*cII-*Sma*I fragment carrying the entire *A. immersus met2* gene (12) into the vector pBluescriptKS (Stratagene) at the *Hin*cII site of the polylinker in the orientation regenerating a *Hin*cII site that happened to also be a *Sal*I site. Plasmids pMD17, pMD11, pMD06, pMD04, and pMD03 each contain a direct tandem duplication made of the 3.6-kb *Hin*cII-*Sma*I fragment carrying the wild-type allele of *met2* and a subfragment of this gene corresponding either to the entire 1,703-bp-long open reading frame (ORF) in pMD17 or to subfragments of the ORF decreasing in size, 1,124, 630, 457, and 317 bp, in pMD11, pMD06, pMD04, and pMD03, respectively. All of these duplications have a common border corresponding to the 5′ end of the ORF. Plasmid pMD17 resulted from the insertion of the 1.7-kb *Nsi*I-*Eco*RV fragment carrying the *met2* ORF into the *Sma*I site (the *Nsi*I ends were made blunt by removing the protruding 3′ nucleotides) of the polylinker of plasmid pGB20, in the orientation leading to a direct tandem array of *met2* sequences. Nested unidirectional deletions were constructed in plasmid pMD17 to shorten the *Nsi*I-*Eco*RV insert starting from the *Eco*RV end. This was done by controlled digestion of a *Bam*HI-plus-*Sac*I digest of plasmid pMD17 with exonuclease III, using a double-stranded nested deletion kit (Pharmacia). We thus generated plasmids pMD11, pMD06, pMD04, and pMD03, in which the extent of the deletion was determined by DNA sequencing. Plasmids pAD17, pAD11, and pAD06 resulted from the replacement in plasmids pMD17, pMD11, and pMD06, respectively, of the 3.6-kb *Sal*I-*Eco*RI fragment (*Sal*I corresponds to the regenerated *Hin*cII site at the upstream border of the *met2* insert, and *Eco*RI is in the polylinker downstream from the other border) by the 5.1-kb *Sal*I-*Eco*RI fragment carrying the *amdS* gene (encoding acetamidase) from *Aspergillus nidulans* (16). *amdS* was used as a selectable marker in transformation experiments (25).

Strains, genetic procedures, and media. All *A. immersus* strains used in this study belong to stock 28 (28). The recipient strains used for transformation, CEI1 and RL95, and the tester strains FX7, FA21, and FA24 used in crosses are of opposite mating types (*mt*− and *mt*+, respectively). Strains RL95 and FA21 carry the wild-type allele of *met2*, while the Met$^-$ strain FA24 carries the mutated allele *met2.1* (7, 11). The other Met$^-$ strains, CEI1 and FX7 (*met2*.Δ::*amdS*), harbor a deletion of *met2*, which had been replaced by *amdS*, in the following way. The 10-kb *Dra*I-*Dra*I fragment (Fig. 1A) carrying *met2* in strain RL95 was cloned. In the resulting plasmid, pAB1, the 4.2-kb *Bcl*I-*Bcl*I fragment encompassing *met2* was replaced by the 5.1-kb *Sal*I-*Eco*RI fragment carrying the *amdS* gene from *A. nidulans* (see above). The resulting plasmid, pGG2-1, was used to transform strain RL95 (which lacks the *amdS* gene [8]). Each of the 130 Amd$^+$ transformants obtained was crossed with an Amd$^-$ Met$^+$ strain to give a homokaryotic progeny (7) whose Met phenotype was tested. A substitution event in the parental transformant would result in an Amd$^+$ Met$^-$ progeny. One of the transformants gave such a progeny; the expected substitution event was confirmed by Southern hybridization. Finally, the tester strains FX7, FA21, and FA24 harbor an ascospore pigmentation marker (*b2.138*) and an ascospore shape marker (*rd1.2*). These markers were used to identify each pair of ascospores, as described previously (9), each pair corresponding to one meiotic product.

The Met$^+$ strains TD17, TD11, TD06, and TD04 harbor 1,703-, 1,124-, 630-, and 457-bp duplications, respectively; strains TD03$^{(1)}$ and TD03$^{(2)}$ harbor a 317-bp duplication at two different chromosomal sites. The TD strains, which resulted from transformation of the Met$^-$ strain CEI1 with plasmids pMD17, pMD11, pMD06, pMD04, and pMD03, respectively, were selected for having integrated a single copy of the corresponding duplication (this was checked by Southern hybridizations; results not shown). The Amd$^+$ Met$^+$ strains TE17$^{(1)}$ and TE17$^{(2)}$, TE11$^{(1)}$ and TE11$^{(2)}$, and TE06$^{(1)}$ and TE06$^{(2)}$ harbor 1,703-, 1,124-, and 630-bp ectopic duplications, respectively, each made of the resident *met2* copy and the truncated transgenic subfragment of the corresponding size. They resulted from transformation of the wild-type Amd$^-$ Met$^+$ strain RL95 with plasmids pAD17, pAD11, and pAD06. Amd$^+$ transgenic strains that had integrated, each at a different ectopic site, a single copy of the plasmid used were selected (this was checked by Southern hybridizations; results not shown).

For the ascospore progenies, the Amd phenotype was checked on minimal medium containing acetamide (10 mM) as a nitrogen source instead of urea (9) and supplemented with methionine (20 μg/ml). The Met phenotype was checked on minimal medium: Met$^+$ progenies invaded the plates in 2 days following germination, while Met$^-$ progenies did not show any growing filament in this period. A property of Met$^-$ strains containing a *met2* gene silenced by MIP is reversion after a lapse of time when placed on selective medium (11, 25); this feature was used to discriminate the Met$^-$ derivatives in which *met2* was silenced by MIP from the Met$^-$ progeny that harbored the null *met2* allele.

Transformation was performed as described previously (7). Standard *A. immersus* genetic procedures (27) were used. Media were as described elsewhere (3, 7, 9, 27).

Isolation and manipulation of DNA. Most experimental procedures were as previously described (11). Other standard techniques were as described in reference 30. Mycelia used for DNA extraction of all TD and TE derivatives, whether Met$^-$ or Met$^+$, were grown on minimal medium plus methionine (20 μg/ml). To study methylation, the following restriction enzymes sensitive to cytosine methylation (22) were used. (i) Enzymes that are sensitive to the methylation in their target sites of a C belonging to a CpG dinucleotide; they corresponded to *Xho*I (target site, CTCGAG), *Ava*II (which has, in the tested duplicated sequences, a unique target site, GGACC, in which the external C is followed in 3′ by a G), and *Hpa*II (target site, CCGG). These enzymes are sensitive to the methylation of either C in their target sites. (ii) *Msp*I, which, like *Hpa*II, recognizes the target site CCGG but, unlike *Hpa*II, is sensitive to the methylation of the external C only (and is therefore insensitive to the methylation of the CpG dinucleotide). (iii) Enzymes whose target sites tested contained C's which were never embedded in CpG dinucleotides (and were therefore insensitive to CpG methylation): *Alu*I, *Bsm*AI, *Eco*RII, *Hph*I, and *Sau*3AI [target sites, AGCT, GTCTC, CC(A/T)GG, TCACC, and GATC, respectively]. In some studies, *Nde*II, which, like *Sau*3AI, recognizes the sequence GATC, but which is insensitive to C methylation, was used as a control. Both cleave the sequence when it is unmethylated, but *Sau*3AI will not cut it if the C is methylated, whereas *Nde*II is insensitive to cytosine methylation. To ensure that digestion of DNA by C-methylation-sensitive enzymes was complete, all Southern blots were stripped and reprobed with the unmethylated *b2* gene of *A. immersus* (5a).

Bisulfite genomic sequencing. The whole bisulfite genomic sequencing procedure was done as described previously (13). Two pairs of primers were designed to perform the strand-specific PCR amplification of a definite DNA fragment, the same in each of the duplications investigated, that encompassed the duplicated segment from the entire *met2* copy. The two primers used to amplify the transcribed strand were CG30 (5′TATGATGAGTTTGTGGATTTTGTAAT3′) and CG31 (5′CAACACCCTACCTCCCATCTCCAA3′) (positions 1388 to 1363 and 688 to 711 in the published nucleotide sequence, respectively [12]). The two primers used to amplify the nontranscribed strand were CG32 (5′TTATTAAA TGGAAATAATGTTTTA3′) and CG33 (5′TAACAAACTCCTAAATCATCC ATA3′) (positions 674 to 697 and 1408 to 1385 in the published nucleotide sequence, respectively [12]). Finally, the two primers used to amplify the ectopic truncated copy from the TD06 derivatives were CG34 (5′TATAGGGTGAAT TGGAGTATT3′) and CG35 (5′ACCCTCTCATCAATAAAATTA3′). CG34 is specific for the pBluescriptKS portion located between the *amdS* gene and the *met2* truncated copy. CG35 starts in pBluescriptKS, 4 nucleotides before the opposite junction, and ends at position 744 in *met2* (12).

As was observed previously (13), the efficiency of the bisulfite treatment in converting C into U was not 100%. Thus, we assessed this efficiency with each batch of bisulfite-treated DNA by restriction cleavage of a definite PCR product, the 3′ part of the *met2* gene (named "R fragment" in reference 13) (positions 2018 to 2685 of the published sequence [12]), located outside any of the duplicated fragments sequenced in this study. The *Eco*RV site located within this fragment at position 2427 should be destroyed by the treatment. If the treatment was 100% efficient, all DNA molecules in the PCR product would be resistant to cleavage by *Eco*RV; a partial cleavage would be indicative of a lack of efficiency. The R fragment was PCR amplified, as described previously (13), from the bisulfite-treated DNA of each of the strains analyzed, which all harbored one entire *met2* copy. The PCR products were digested with *Eco*RV, and the amount of the shorter fragments resulting from the cleavage at the *Eco*RV site was measured by densitometry on an agarose gel. The completion of the *Eco*RV digestion was monitored by adding to the restriction digest mixture 300 ng of circular pBluescriptKS DNA and verifying its complete linearization. We deduced that for all of the DNA batches tested, the percentage of the PCR DNA molecules that were cleaved ranged between 0 and 3%. These values represent the percentage of C's that were not converted into U by the treatment at the *Eco*RV site. By extension to all C's, we estimate that at most 3% of unmethylated C's appeared as falsely methylated C's in this study.

RESULTS

Tandem duplications. We crossed the Met$^+$ transgenic strains TD17, TD11, TD06, and TD04, harboring 1,703-, 1,124-, 630-, and 457-bp-long tandem duplications, respectively, and TD03$^{(1)}$ and TD03$^{(2)}$, each harboring a 317-bp-long duplication (Fig. 1), with the Met$^-$ tester strain FX7. The ability of each of the duplications to trigger MIP could thus be assessed. The occurrence of MIP followed by gene silencing would lead to a loss of the Met$^+$ parental phenotype in the progeny. This loss was looked for by testing in individual asci the Met phenotype of one ascospore of each of the four pairs of ascospores (which correspond to the four meiotic products) and was thus revealed by the presence of 0 Met$^+$:4 Met$^-$ asci (Fig. 2A).

All the asci analyzed in the progeny of strains TD17 and TD11 (12 and 15, respectively) showed the segregation 0 Met$^+$:4 Met$^-$, indicating that the 1,703- and 1,124-bp-long duplications never escaped MIP, as was shown previously for the 5.7-kb-long tandem duplication (25). In the progeny of strain TD06, only 12 of the 82 asci analyzed were 0 Met$^+$:4 Met$^-$; 47 asci were 2 Met$^+$:2 Met$^-$, indicating that *met2* had not been inactivated by MIP and was thus functional in the strains issued from the two meiotic products that inherited the 630-bp duplication (Fig. 2A), and 23 asci were 1 Met$^+$:3 Met$^-$, indicating that *met2* was inactive in only one of the two meiotic products that inherited the duplication. This last result contrasted with the results obtained with kilobase-size duplications, for which inactivation always occurred in both products. In addition to this, most Met$^-$ TD06 derivatives were highly unstable, reverting to prototrophy within an unusually short time. Finally, *met2* never appeared inactivated in the progeny of strains TD04, TD03$^{(1)}$, and TD03$^{(2)}$, which gave only 2 Met$^+$:2 Met$^-$ asci among 17, 11, and 25 asci analyzed, respectively.

We first checked the methylation in TD17, TD11, and TD06 Met$^-$ derivatives by Southern blot hybridization using *Sau*3AI, a restriction enzyme sensitive to C methylation. A restriction map of the *met2* region in which methylation was searched for is shown in Fig. 3A and B.

Methylation in two Met$^-$ derivatives, one from each of the transgenic strains TD17 and TD11, was dense, involving both the truncated and the full-length copies, and was coextensive with the repeat length, as previously described for the 5.7-kb tandem duplication and other kilobase-size ectopic duplications (1, 25). For both derivatives, the pattern of hybridization of *Sau*3AI digests indicated that all GATC sites were methylated within the repeats and were unmethylated outside the repeats (Fig. 3B and C).

Seven TD06 Met$^-$ derivatives which came from asci showing the segregation 0 Met$^+$:4 Met$^-$ and two Met$^-$ derivatives which came from 1 Met$^+$:3 Met$^-$ asci also showed methylation of both the truncated and the full-length *met2* copies of the 630-bp duplication (results for one example are shown in Fig. 3C).

To analyze the methylation status of TD06, TD04, and TD03 Met$^+$ derivatives, we used, together with *Sau*3AI, three other C-methylation-sensitive enzymes: *Ava*II, *Hpa*II, and *Xho*I. Five TD06 Met$^+$ derivatives which came from 1 Met$^+$:3 Met$^-$ asci and 11 TD06 Met$^+$ derivatives which came from 2 Met$^+$:2 Met$^-$ asci all showed methylation in both the truncated and the full-length copies. However, not all sites were methylated

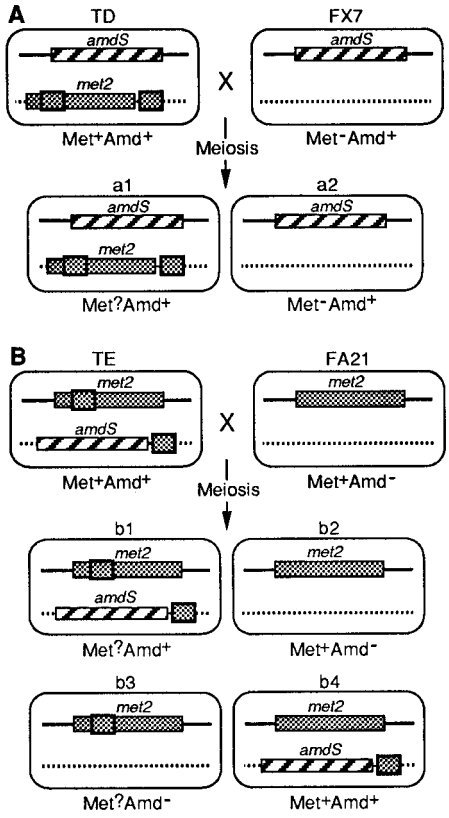

FIG. 2. Met and Amd phenotypes of the progeny of TD and TE transgenic strains. (A) Each TD strain contains a tandem duplication of a subfragment of *met2* and lacks the resident *met2* gene that had been deleted from the recipient strain and replaced by *amdS*: each exhibits a Met^+ Amd^+ phenotype. For the *met2* sequences, the full-length *met2* copy (bar) and the duplication (two squares) are indicated. The Met^- Amd^+ tester strain FX7 harbors the same deletion of *met2*. The only two possible meiotic products are shown. The a1 progeny strains that have inherited the *met2* duplication are Met^- if *met2* was silenced or Met^+ in the absence of silencing. Their phenotypes are indicated by question marks. (B) Each TE strain contains an ectopic duplication of a subfragment of *met2* and exhibits a Met^+ Amd^+ phenotype. All TE strains harbor the wild-type resident *met2* gene (bar) and one transgenic subfragment of this gene (square) integrated at an ectopic site together with *amdS*. The wild-type Met^+ Amd^- tester strain FA21 harbors the resident *met2* gene. The four possible meiotic products are shown. As for the a1 progeny strains of panel A, the b1 and b3 progeny strains that have inherited the *met2* gene from the TE parent can be either Met^- or Met^+, depending on *met2* silencing. Their phenotypes are indicated by question marks.

in all derivatives, and some sites showed partial methylation. Further analyses of some of these derivatives will be detailed below. Altogether, these results indicated that the 630-bp duplication never escaped MIP. The presence of methylated Met^+ derivatives therefore indicated that methylation could occur without inactivating *met2*. Interestingly, methylation may repress *met2* in only one of the two sister meiotic products, as shown by the presence of 1 Met^+:3 Met^- asci.

We showed in the same way that in the eight TD04 derivatives analyzed (from eight asci), the 457-bp duplication had undergone MIP without inactivating *met2*. In each of the derivatives, methylation, either complete or partial, was found in both copies at least with one of the enzymes used (not shown).

Eight of the 10 TD03 derivatives analyzed [five $TD03^{(1)}$ and five $TD03^{(2)}$, from 10 asci] did not show any methylation. Only two of them (T3p2 and T3n6; Fig. 3B and C), both issued from $TD03^{(2)}$, showed methylation at only the *Hpa*II site and only in the truncated copy.

To assess the susceptibility to MIP of the 317- and the 457-bp duplications under strict comparison conditions, we placed the two nuclei harboring each duplication under identical environmental conditions, in the same cytoplasm, within the fruiting body where MIP occurs. This was done by crossing $TD03^{(1)}$ with TD04 [in the cross, we used one of the $TD03^{(1)}$ derivatives that had a mating type opposite to that of TD04]. The integration sites of the plasmids harboring the 317-bp duplication of $TD03^{(1)}$ and the 457-bp duplication of TD04 were nonallelic and genetically independent. Among the 52 asci analyzed, we found 12 4 Met^+:0 Met^-, 12 2 Met^+:2 Met^-, and 28 3 Met^+:1 Met^- asci, proportions close to those expected if *met2* was not inactivated. Methylation was searched for in $TD03^{(1)}$ and TD04 derivatives from eight 4 Met^+:0 Met^- asci, in which two meiotic products had inherited the 317-bp duplication while the other two had inherited the 457-bp duplication. The same four enzymes previously used were used to analyze all four meiotic products in each ascus. Methylation was undetectable in any of the $TD03^{(1)}$ derivatives present in the eight asci (not shown). In three asci, methylation was also undetectable in the TD04 derivatives (not shown). In five asci, the 457-bp duplication appeared methylated at least at the *Hpa*II sites (methylation never occurred at the *Sau*3AI sites). In two of them, both elements of the duplication were methylated in the two sister derivatives (i.e., derivatives issued from two meiotic products from the same ascus). In the other three asci, while one sister derivative had both elements methylated, only the truncated copy was found methylated in the other derivative. Examples are shown in Fig. 3C. This experiment indicates either that the 457-bp duplication is more prone to MIP than the 317-bp duplication or that methylation is hardly maintained in the latter.

Ectopic duplications. The Amd^+ Met^+ transgenic strains $TE17^{(1)}$ and $TE17^{(2)}$, $TE11^{(1)}$ and $TE11^{(2)}$, and $TE06^{(1)}$ and $TE06^{(2)}$ harbored the 1,703-, 1,124-, and 630-bp ectopic duplications, respectively. Each duplication was made up of the resident *met2* copy and the corresponding transgenic subfragment integrated at an unlinked site. These strains were crossed with the Met^+ tester strain FA21 in order to assess the ability of each of the duplications to trigger MIP. The inactivation of the resident *met2* copy belonging to the duplication would result in the presence of Met^- derivatives in the ascus progeny (Fig. 2B). This was observed in the progenies of the TE17 and TE11 strains. Among the 132 asci analyzed from crosses involving the TE17 strains, 47 showed the segregation 2 Met^+:2 Met^- [9 of 22 for $TE17^{(1)}$ and 38 of 110 for $TE17^{(2)}$]. This type of segregation was observed for 21 of the 88 analyzed asci issued from crosses involving the TE11 strains [7 of 31 for $TE11^{(1)}$ and 14 of 57 for $TE11^{(2)}$]. All other asci showed the segregation 4 Met^+:0 Met^-, indicating that *met2* had not been inactivated. Only the 4 Met^+:0 Met^- segregation was observed in the 18 and 12 analyzed asci issued from crosses involving strains $TE06^{(1)}$ and $TE06^{(2)}$, respectively.

Since we had observed that small tandem duplications were able to trigger MIP without inactivating *met2*, we decided to search for methylation in TE06 Met^+ derivatives, and also in TE11 Met^+ derivatives, by Southern hybridization experiments. In order to facilitate the analysis, we crossed the TE11 and TE06 strains with a Met^- strain (strain FA24, which carries the mutated allele *met2.1*). In asci showing the segregation 2 Met^+:2 Met^-, the Met^+ products would have inherited the noninactivated *met2* resident copy, and if in addition they displayed the Amd^+ phenotype, they would have also inherited the transgenic truncated copy. We analyzed 87 and 88 asci in

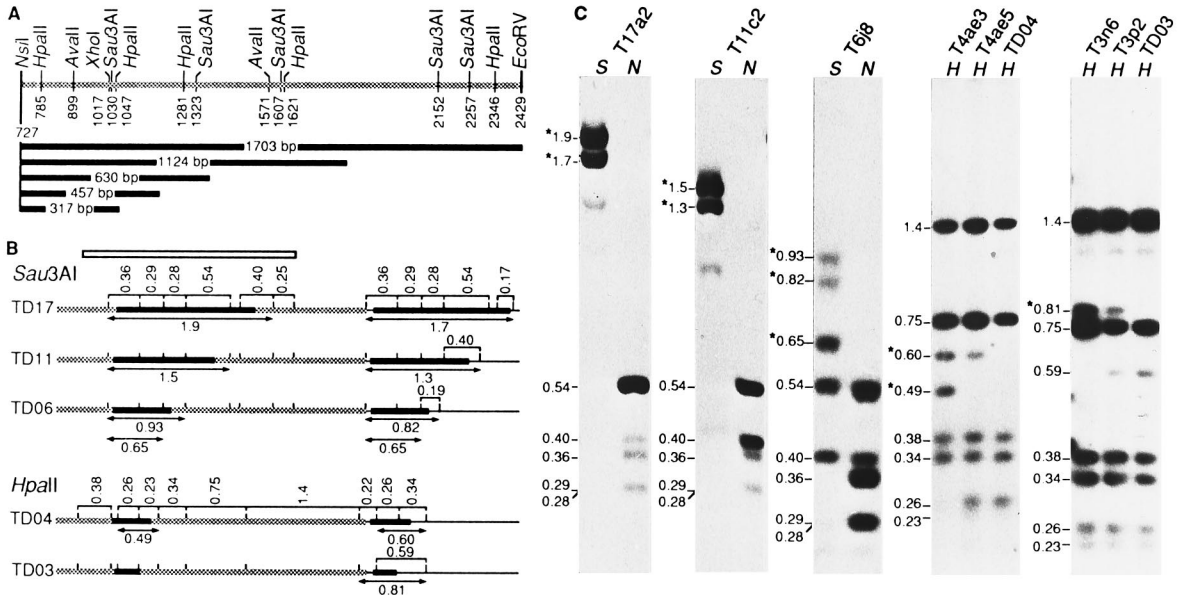

FIG. 3. Searching for methylation by Southern hybridization. (A) *Ava*II, *Hpa*II, *Sau*3AI, and *Xho*I restriction map of the *Nsi*I-*Eco*RV fragment corresponding to the *met2* ORF and of the different subfragments that have been duplicated (from the published nucleotide sequence [12]). (B) *Sau*3AI and *Hpa*II maps of the *met2* region from TD17, TD11, and TD06 strains and from TD04 and TD03$^{(2)}$ strains, respectively. Restriction fragments sizes are shown in kilobases. Arrows below the maps correspond to the hybridization fragments indicated by asterisks in panel C, which resulted from the failure to cut the methylated restriction sites. The *Xba*I-*Bgl*II fragment (12) (open box above the map) that was used as a probe in the Southern hybridization experiments shown in panel C is indicated. (C) Southern hybridization-methylation analysis of three Met$^-$ derivatives, T17a2, T11c2, and T6j8, of strains TD17, TD11, and TD06, respectively, by using the C-methylation-sensitive *Sau*3AI enzyme (*S*) and its insensitive isoschizomer *Nde*II (*N*), and of the Met$^+$ derivatives T4ae3 and T4ae5 (issued from two sister meiotic products) of strain TD04 and T3n6 and T3p2 of strain TD03$^{(2)}$ (indicated as TD03), by using *Hpa*II (*H*). Since no *Hpa*II isoschizomer insensitive to C methylation was available, we compared the *Hpa*II patterns of the TD04 and TD03 derivative DNA digests with those of the parental TD04 and TD03$^{(2)}$ strains, in which *met2* was unmethylated. In T6j8, the 0.93- and 0.82-kb *Sau*3AI fragments resulted from the failure to cut the two sites in each copy; however, methylation at the more downstream site was partial, as deduced from the presence of the 0.65-kb fragment. In the TD04 derivatives, only the more downstream *Hpa*II site was methylated, as deduced from the presence of the 0.60- and 0.49-kb fragments. In one of the derivatives (T4ae5), this site was methylated in the truncated copy only. This was also the case for both TD03 derivatives, in which the only methylated site was that of the truncated copy; in one of them (T3p2), methylation of this site was partial, as deduced from the presence in a substoichiometric amount of the 0.81-kb fragment and the persistence of the 0.59-kb fragment. Fragment sizes (in kilobases) are indicated to the left of the gels.

the progenies of strains TE11 and TE06, respectively. All of the asci issued from the TE06 strains showed the segregation 2 Met$^+$:2 Met$^-$. Such a segregation was also observed for 78 of the 87 asci analyzed issued from the TE11 strains. The other nine [4 of 43 and 5 of 44 for TE11$^{(1)}$ and TE11$^{(2)}$, respectively] showed the 0 Met$^+$:4 Met$^-$ segregation expected from the inactivation of *met2*. Methylation was searched for with *Ava*II in Met$^+$ Amd$^+$ TE11 derivatives and with both *Ava*II and *Xho*I in Met$^+$ Amd$^+$ TE06 derivatives, all issued from different asci. Of the 62 TE11 Met$^+$ derivatives tested, 2 showed methylation. Of the 84 TE06 Met$^+$ derivatives tested, 3 [1 from TE06$^{(1)}$ and 2 from TE06$^{(2)}$] showed methylation. Remarkably, in all three derivatives, only the ectopic copy showed methylation, which was detected with both enzymes. We thus analyzed methylation in all of the meiotic products from the three asci (Fig. 4). The ectopic truncated copy was always methylated, while the resident copy showed methylation only in the ascus derived from TE06$^{(1)}$ and only in one of the two meiotic products that had inherited this copy (E6r2).

We did not analyze shorter ectopic duplications, assuming that they would trigger MIP with efficiencies still lower than that of the 630-bp ectopic duplication.

Genomic sequencing. The results of our Southern hybridization experiments suggested that the 317-bp tandem duplication was most often not methylated by MIP. Furthermore, the methylation of short duplications (630 down to 317 bp) that underwent MIP often appeared less intense than that of larger duplications (references 1, 9, 11, 13, and 25 and this work) and could show differential methylation of the truncated and the full-length copies. Since these observations were based on the analysis of the methylation status of a limited number of C's, we used the bisulfite genomic sequencing method (10, 13) to determine for a small number of chosen derivatives whether taking into account the methylation status of every C within the duplications would point to the same conclusions.

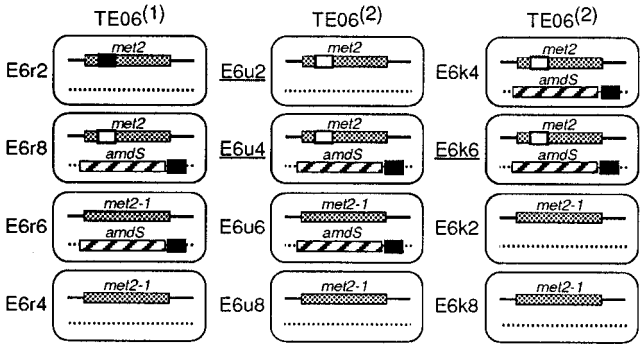

FIG. 4. Methylation of the 630-bp ectopic duplication in asci resulting from the crosses between the Met$^-$ tester strain FA24 and strains TE06$^{(1)}$ (one ascus) and TE06$^{(2)}$ (two asci). The segregation into the four meiotic products of each element of the duplication (the resident *met2* copy and the truncated copy linked to *amdS*, indicated by boxes as in Fig. 2B, except that these are either black or open; see below) and of the *met2.1* mutated allele of strain FA24 is shown for each ascus. Methylation was checked at the *Ava*II site present in each copy for all three asci and at the *Xho*I site also present in each copy for the two asci from TE06$^{(2)}$. Methylation (black box) and the absence of methylation (open box) are indicated. Genomic sequencing of the 630-bp ectopic duplication was performed for strains derived from the underlined meiotic products (the full-length copy in E6u2 and both the full-length copy and the truncated copies in E6u4 and E6k6).

Sequencing was first performed for the 317- and 457-bp tandem duplications from TD03 and TD04 derivatives of the same 4 Met^+:0 Met^- ascus issued from the cross between $TD03^{(1)}$ and TD04. Southern hybridization had shown no methylation in the TD03 derivatives T3d2 and T3d3 and had shown methylation at some of the sites tested in the TD04 derivatives T4d3 and T4d7. Sequencing of the 317-bp duplicated part of the full-length *met2* copy from T3d2 was performed with individual DNA molecules, all derived by amplification from the transcribed strand. None of the C residues was methylated (Fig. 5), confirming the Southern hybridization results. Sequencing of the 457-bp duplicated part of the full-length *met2* copies from T4d3 and T4d7 was performed with individual DNA molecules from both strands (Fig. 5 and 6). The main feature, common to both derivatives, was the scarcity of methylation. The proportions of C's that stayed unconverted by the bisulfite treatment (thus potentially methylated) were on average 6 and 7% in the nontranscribed strand and 9.5 and 6% in the transcribed strand for T4d3 and T4d7, respectively. These values may give an overestimation of the methylation levels, since we showed in control experiments (Materials and Methods) that the bisulfite treatment was not 100% efficient, leaving 0 to 3% of the unmethylated C's unconverted, which indicated that they were resistant to the treatment. Of the 26 CpGs present on both strands, 5 in T4d3 and 6 in T4d7 had the C unconverted in all or almost all of the molecules and could thus be considered methylated. Three of these methylated CpG sequences occurred at the same positions, and two and three of them were at different positions in T4d3 and T4d7, respectively. These were indeed the only C's that showed significant methylation. It is noteworthy that methylation at CpGs always occurred on both strands, i.e., at the two diagonally opposed C's of this short symmetrical sequence (Fig. 6).

We thus performed sequencing of both the ectopic and the resident copies of TE06 derivatives from two different asci in which the 630-bp ectopic duplication exhibited clear differences in the patterns of methylation between the truncated and the full-length *met2* copies (Fig. 4). For each copy, sequencing was performed for individual DNA molecules derived from the transcribed strand (Fig. 5). The methylation pattern in the resident *met2* copy from derivative E6k6 was similar to that described above for the 457-bp duplicated part of the full-length *met2* copy from derivatives T4d3 and T4d7. The proportion of unconverted C's was on average 5%, and the only significant methylation was at CpGs, although only 6 of the 32 CpG sites could be considered methylated (i.e., the C stayed unconverted in at least four of the six DNA molecules sequenced). The methylation level appeared even lower in the resident *met2* copy from derivatives E6u2 and E6u4. In E6u2, four of the six molecules analyzed did not show any methylation. In E6u4, only one C was significantly methylated, again belonging to a CpG. In contrast with the low level of methylation of the resident full-length *met2* copy, the ectopic truncated copy showed a significant level of methylation in both E6k6 and E6u4. In E6u4, the six molecules analyzed had on average 47% of the C's unconverted. In E6k6, four molecules had on average 52% of the C's unconverted and two molecules showed low levels of methylation (12 and 3%). It is remarkable again that in these two last molecules, the methylated C's mostly belonged to CpG sequences (13 of 20 and 4 of 4, respectively).

We also sequenced the 630-bp tandemly duplicated part of the full-length *met2* copy from the TD06 Met^- derivative T6j8, shown in Fig. 3C, on individual DNA molecules derived from both strands (Fig. 5 and 6). Most of the methylation features exhibited by this duplication were similar to those previously described for the large ectopic duplication of the entire *met2* ORF (13). Methylation occurred at almost every C position in the overall population of the DNA molecules, with a very efficient maintenance of the methylation of C's belonging to CpG sequences. In contrast with methylation in the ORF duplication (which left unmethylated approximately 100 internal bp next to each end), methylation in the 630-bp duplication extended up to the ends of the repeat. The presence in a minority of the molecules of unconverted C's outside the repeat suggested that some methylation might extend beyond the ends.

Altogether, the results of genomic sequencing strengthen the conclusions suggested by the Southern hybridization results. In addition, they indicate that shortening the repeats may lead to a decrease in methylation density so that the rare C's that are still methylated belong only to CpG sequences.

CpG versus non-CpG methylation. In order to check if methylation restricted to CpGs does constitute a general feature of short duplications, a comparative analysis of methylation at CpG and at non-CpG sites was performed by using a new set of restriction enzymes sensitive to C methylation (Fig. 7) in addition to those previously used in Southern hybridization experiments. This analysis was carried out with six pairs of sister TD04 derivatives (among which three pairs were previously found to be methylated and three pairs were found to be unmethylated for the 457-bp tandem duplication), the two TD03 derivatives which were previously found to be methylated for the 317-bp tandem duplication, and three pairs of sister TD06 derivatives (Fig. 7B). In addition to the previously used enzymes that are sensitive to the methylation in their target sites of C's belonging to CpG sequences (*Ava*II, *Hpa*II, and *Xho*I; see Materials and Methods), we used *Alu*I, *Bsm*AI, *Eco*RII, and *Hph*I, which, like *Sau*3AI (also previously used), have target sites containing C's not embedded in CpG sequences. We also used *Msp*I, an *Hpa*II isoschizomer, which allowed us to distinguish methylation of the external C (non-CpG) of the target site CCGG from methylation of the internal CpG.

Altogether, for each element of the duplications, methylation was checked at 15 sites (5 CpG and 10 non-CpG sites) of the 630-bp duplication, 12 sites (4 CpG and 8 non-CpG sites) of the 457-bp duplication, and 8 sites (3 CpG and 5 non-CpG sites) of the 317-bp duplication. Figure 7B shows the methylation displayed by each of these sites in the different derivatives analyzed. Methylation was completely absent in the six TD04 derivatives previously found to be unmethylated. As for the six TD04 and the two TD03 derivatives previously shown to be methylated, altogether, none of the 112 non-CpG sites tested showed methylation, whereas, among 58 CpG-containing sites tested, 17 were methylated. A straightforward illustration of the absence of methylation at non-CpG sites lies in the observation that each time a CCGG site was not cut by *Hpa*II, it was cut by *Msp*I (Fig. 7B and 8), therefore indicating that when the C belonging to a CpG was methylated, the C immediately 5′ to it was not methylated. This was observed with the nine *Hpa*II sites that were methylated in the TD04 derivatives used in this study and with three more methylated *Hpa*II sites tested in two additional TD04 derivatives (i.e., T4s8 and T4ae1; Fig. 7B). This situation contrasts with that observed for the six TD06 derivatives tested, in which most of the non-CpG sites in the 630-bp duplication did show methylation, although they tended to be less densely methylated than the CpGs, as observed with kilobase-size duplications (8, 13).

Differential methylation of the full-length and the truncated copies. This analysis also provided us with additional information concerning the comparison of the methylation patterns of the two elements of the same duplication (i.e., the truncated

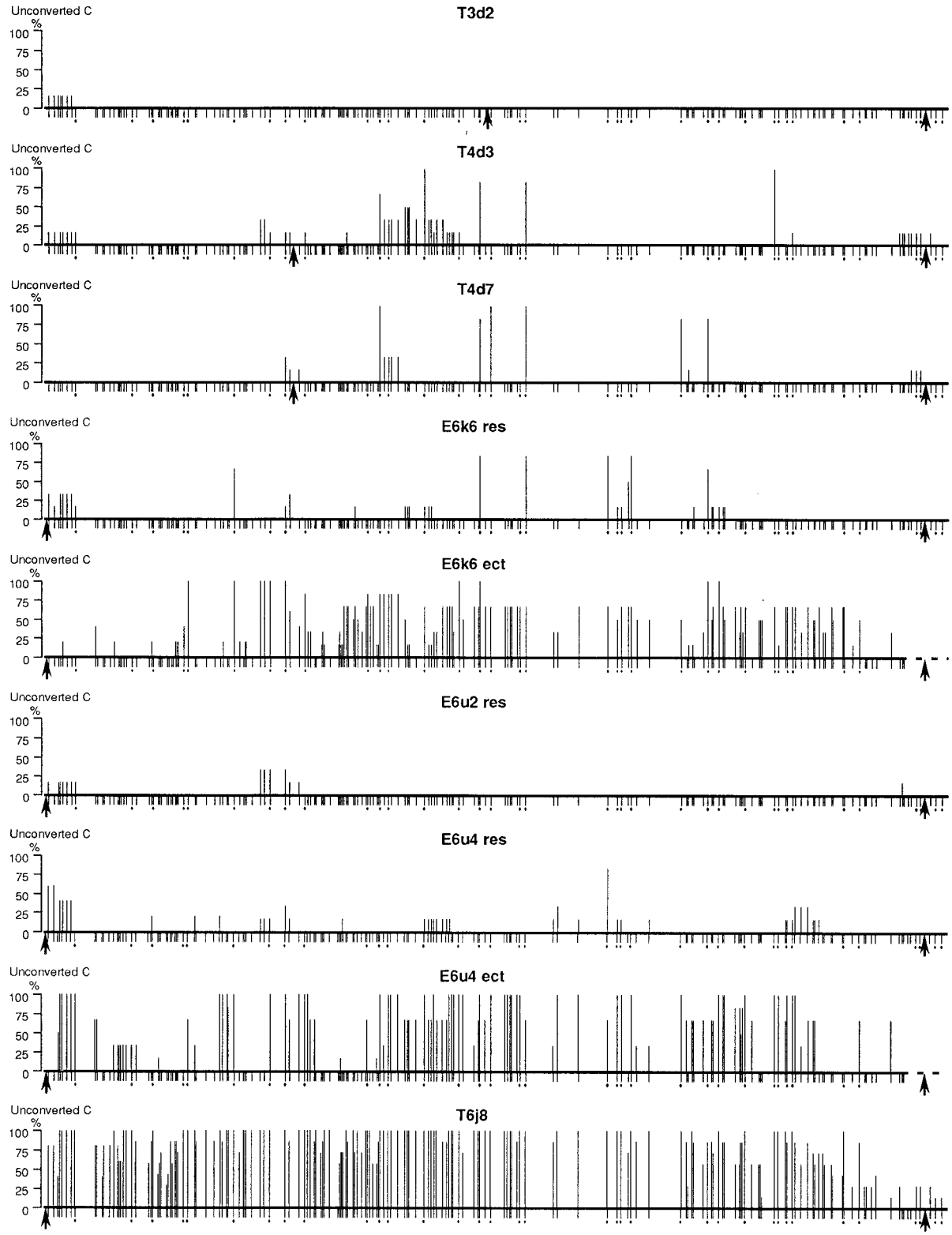

FIG. 5. Diagram of the genomic sequencing data. For each duplication, only results obtained for the transcribed strand (oriented 5′ to 3′ from left to right) are shown. The PCR-amplified sequenced fragments (abscissa thick lines), the extent of the duplicated parts (arrows), C positions (short vertical lines below the abscissa lines), and C residues belonging to CpG dinucleotides (dots) are indicated. For each C position, the length of the upper vertical line indicates the percentage of DNA molecules sequenced in which the C was found unconverted by the bisulfite treatment. In the tandem duplications of the TD03 (T3d2), TD04 (T4d3 and T4d7), and TD06 (T6j8) derivatives, sequencing was performed with the full-length *met2* copy only. In the ectopic duplications of the TE06 derivatives (E6k6, E6u2, and E6u4), sequencing was performed for both the full-length resident (E6k6 res, E6u2 res, and E6u4 res) and the truncated ectopic (E6k6 ect and E6u4 ect) *met2* copies. Each sequencing set was performed for six DNA molecules, except in the case of derivatives T4d7 and T6j8, for which four and seven DNA molecules, respectively, were sequenced.

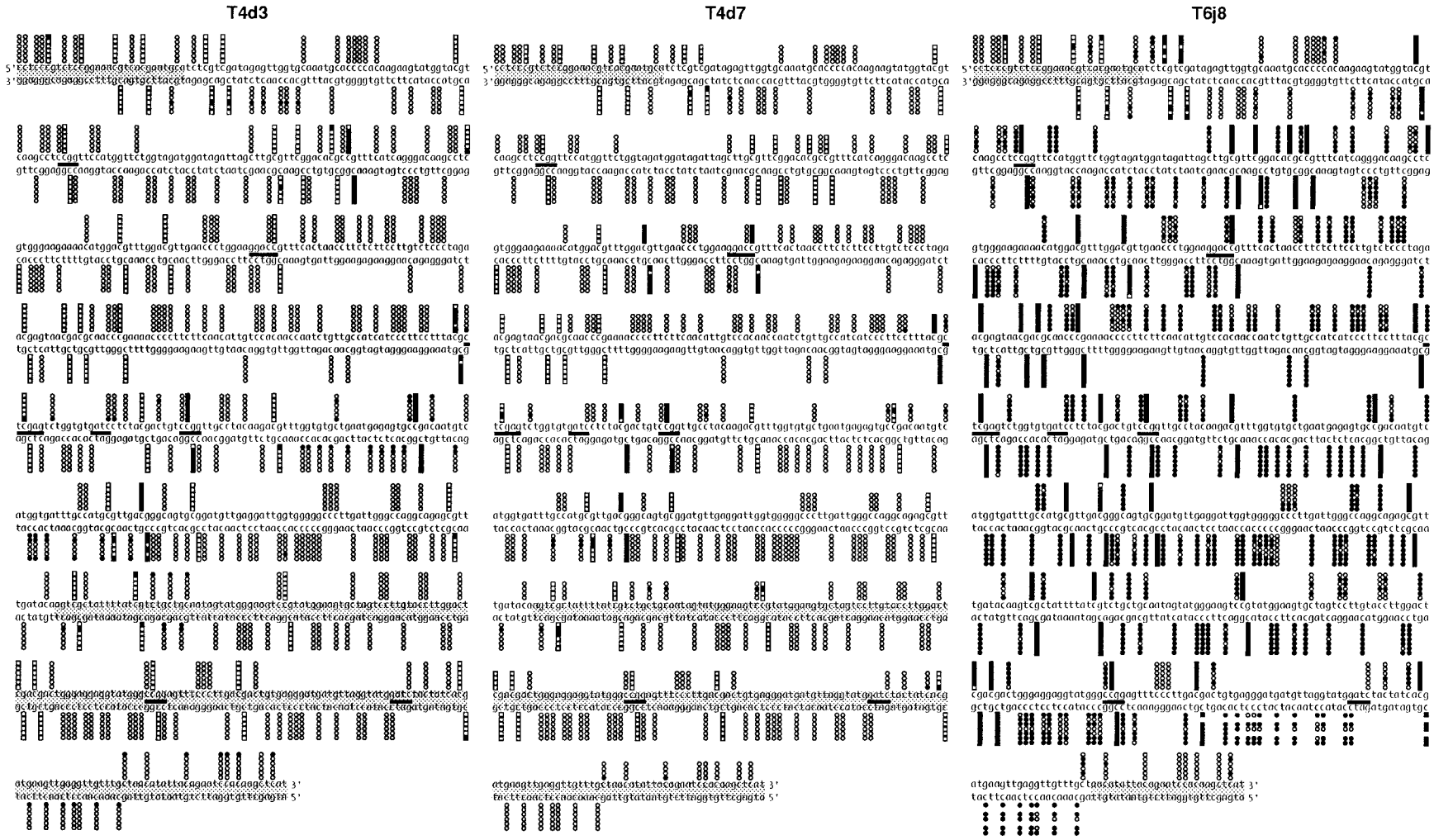

FIG. 6. Distribution of methylation on individual transcribed (bottom) and nontranscribed (top) DNA strands in the sequenced *met2* segments of derivatives T4d3, T4d7, and T6j8, shown in Fig. 5. For each derivative, sequencing was performed for six DNA molecules derived from the nontranscribed strand and six, four, and seven DNA molecules derived from the transcribed strand for T4d3, T4d7, and T6j8, respectively. The duplicated parts (nonshaded regions of the nucleotide sequences), the flanking nonduplicated parts (shaded regions), C residues belonging to CpG dinucleotides (squares), other C's (circles), unmethylated C residues (open symbols), and C's that remained unconverted by the bisulfite treatment (filled symbols) (they correspond mostly to methylated C's; see Materials and Methods) are indicated. Each DNA molecule is represented by one horizontal symbol alignment. For strain T6j8, sequencing data for the 5′ part of the transcribed strand were not accurate enough for two of the DNA molecules analyzed to determine the methylation status of the C's (indicated by blank spaces in the symbol alignments). Restriction sites whose methylation was tested by Southern hybridization are underlined (*Ava*II, GGACC; *Hpa*II, CCGG; *Sau*3AI, GATC; and *Xho*I, CTCGAG).

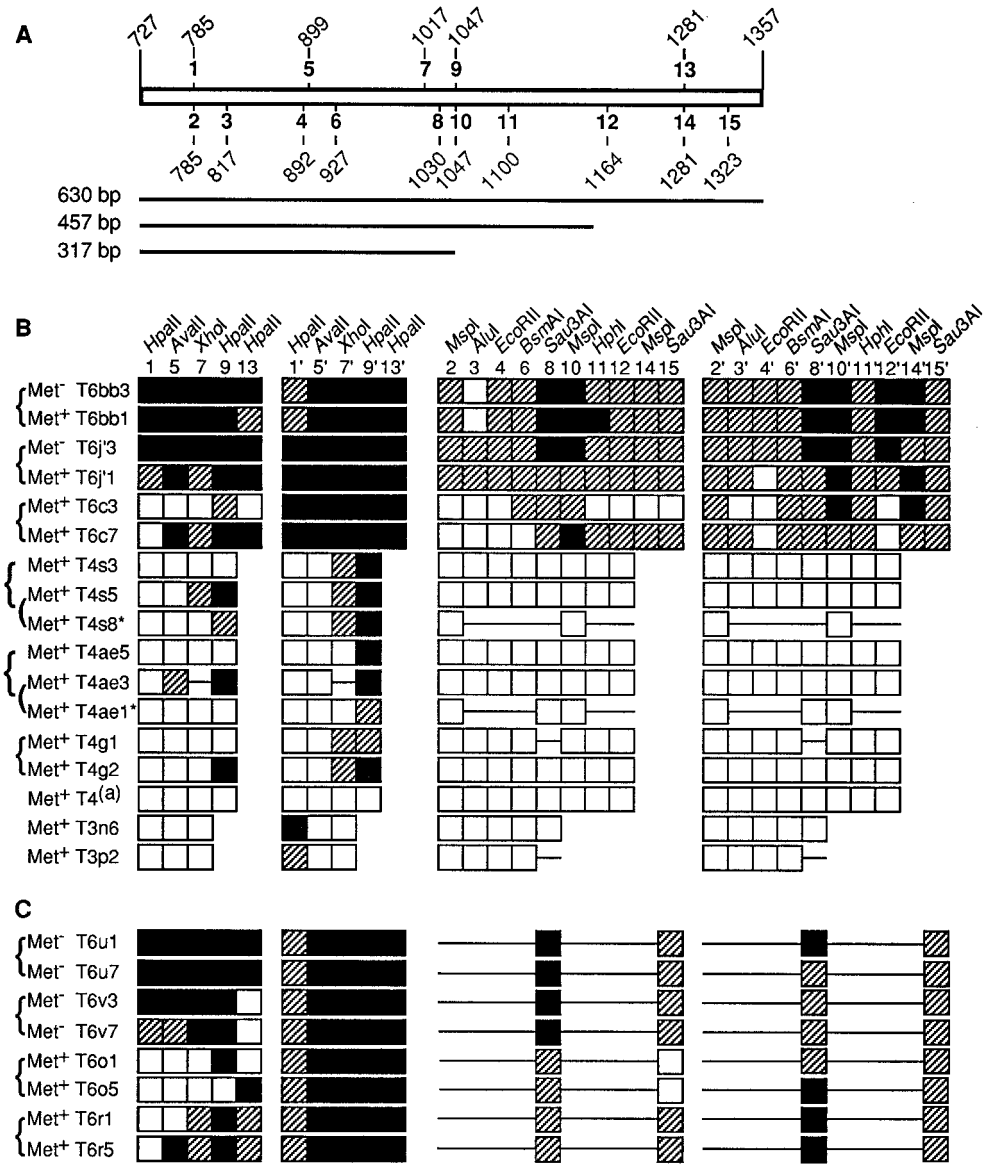

FIG. 7. Analysis of methylation at CpG dinucleotides and at non-CpG sites. (A) Map of the restriction sites tested for methylation in the 630-, 457-, and 317-bp tandem duplications. The extent of each of the duplicated fragments is shown below the map. Restriction sites are numbered from 1 to 15 as in panel B, in which the names of the matching enzymes are also indicated; the position numbers are as in the published sequence of *met2* (12). The restriction sites whose cutting is blocked by methylation of a CpG (above the map) and non-CpG sites (below the map) are shown. (B and C) Methylation of each restriction site tested as deduced from Southern hybridization experiments performed with TD06, TD04, and TD03 derivatives. The names of the derivatives are indicated together with their Met phenotype. Sister derivatives (from the same ascus but from two different meiotic products) (braces) and twin derivatives (issued, after postmeiotic mitosis, from the same meiotic product) (parentheses) and the two additional TD04 derivatives that were tested for methylation of twin derivatives (asterisks) are shown. Met^+ $T4^{(a)}$ represents the set of the three pairs of sister TD04 derivatives that were totally unmethylated at any of the sites tested. The TD03 derivatives, T3n6 and T3p2, came from different asci. Completely methylated sites (black boxes), partial methylation (striped boxes), totally unmethylated sites (open boxes), and untested sites (horizontal lines) are indicated. The left two panels show the methylation levels of CpG-containing sites; the right two panels show the methylation levels of non-CpG sites. Sites 1 to 15 are those present in the full-length copy; sites 1′ to 15′ are those present in the truncated copy.

and the full-length copies). Indeed, the full-length copy appeared less methylated than the truncated copy in most derivatives. This held true for the two TD03 derivatives and six of the eight TD04 derivatives (Fig. 7B). As for the TD06 derivatives, this held true for the two sister derivatives (i.e., issued from the same ascus) T6c3 and T6c7. In contrast, derivative pair T6bb3 and T6bb1 and pair T6j′3 and T6j′1 displayed similarly dense methylation of each element of the 630-bp duplication. These two latter pairs consisted of one Met^+ and one Met^- sister derivatives, whereas T6c3 and T6c7 were both Met^+, like all TD03 and TD04 derivatives, which exhibited lighter methylation of the full-length copy. To test whether the decrease in methylation intensity could be related to the expression of the full-length copy, we compared the methylation of the two elements of the 630-bp duplication in two pairs of Met^- and in two pairs of Met^+ derivatives (Fig. 7C). The decrease of the methylation of the full-length copy compared with that of the truncated copy was indeed the most pronounced in the four Met^+ derivatives.

Maintenance of methylation in short DNA segments. It is noteworthy that differences in methylation patterns were almost always observed between the duplications harbored by

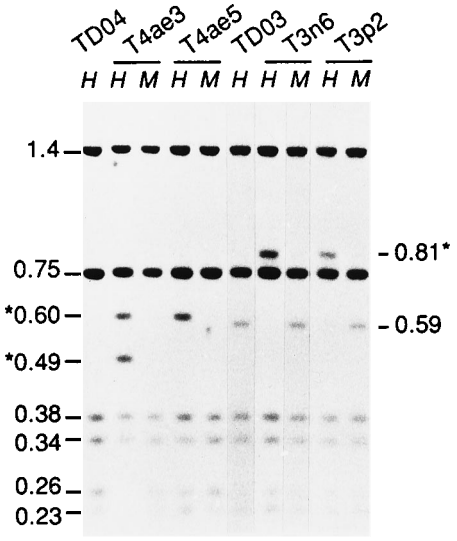

FIG. 8. Methylation at CCGG sites. Some examples of the results obtained from the Southern hybridization analysis of the TD04 and TD03 derivatives, using *Hpa*II (*H*) and *Msp*I (*M*), are shown. See Fig. 3 for the derivatives analyzed, the unmethylated controls, the probe used, and other details.

the sister derivatives (Fig. 7). In order to test whether such differences would also exist between duplications harbored by twin derivatives (i.e., derivatives obtained from one pair of ascospores issued after the postmeiotic mitosis from the same meiotic product), we also studied the derivatives T4s8 and T4ae1, which are twin derivatives of T4s5 and T4ae3, respectively, with *Ava*II, *Hpa*II, *Msp*I, *Sau*3AI, and *Xho*I (Fig. 7B). Again, differences in the methylation patterns were found. In twin derivatives, the differentially methylated duplications are derived through at least two cycles of DNA replication (premeiotic and postmeiotic) from the same individual MIP event. This indicates that the differences observed in the methylation patterns of short duplications subjected to MIP can result from differences in the maintenance of methylation.

In order to test the stability of methylation over a large number of DNA replication cycles, the six TD06 derivatives shown in Fig. 7B and which showed different levels of methylation were subjected to continuous growth on nonselective agar plates for a large number of nuclear divisions (about 100). DNA was then extracted and analyzed by Southern hybridization using *Hpa*II, *Msp*I, and *Hph*I. Comparison with controls consisting of DNA extracted after standard growth (which allowed about 30 nuclear divisions after ascospore germination) failed to show any difference in the methylation patterns of five of the six derivatives tested, regardless of the enzyme used. The only difference was found with T6c3, which lost methylation at the external C of CCGG *Msp*I site 2′ (Fig. 7B), in the truncated copy (Fig. 9). Interestingly, this site was still fully methylated at the internal CpG, as shown by its failure to be cut by *Hpa*II. With this exception, this experiment indicates that regardless of its initial level, the methylation of the 630-bp duplications was remarkably stable through replication cycles.

DISCUSSION

The minimal size of the region of homology required for MIP is close to 300 bp. We detected methylation in all derivatives analyzed that had inherited a 630-bp or longer tandem duplication and in a majority of derivatives (20 of 26) that had inherited the 457-bp tandem duplication. In contrast, only a minority of the derivatives (2 of 26) that had inherited the 317-bp tandem duplication showed methylation. Thus, shortening the tandem repeats from 630 to 317 bp caused a drastic drop in the frequency of MIP triggering and/or in the methylation maintenance efficiency. In contrast to the situation with tandem duplications, reduction of the size of ectopic repeats was correlated with a progressive decrease in the frequency of MIP. This decrease (64, 35, 26, and 3% for the 5.7-kb and 1,703-, 1,124-, and 630-bp duplications, respectively) is likely to reflect a decrease in the probability that ectopic repeats will find each other and pair to form a target for MIP.

When monitoring the effect of the size of the region of homology on the efficiency of recombination between tandem repeats in mammalian cells, Liskay et al. (20) observed that for repeats longer than 300 bp (this is the minimal length for a region of homology required for efficient homologous recombination in mammals), the frequency of recombination decreased progressively with the repeat size. Such a progressive decrease in recombination frequency is expected if the recombination process requires a previous break in the region of homology: this break will be rarer as the region becomes shorter. The finding that the frequency of MIP stays close to 100% when the size of the tandem repeats decreases to 630 bp suggests that MIP proceeds via a DNA pairing mechanism which does not require such a preliminary break; this pairing process could be related to the premeiotic pairing observed in *S. cerevisiae* (33).

MIP was still detected, although with low frequency, with the 317-bp duplication, indicating that a repeat size of ~300 bp is sufficient to trigger MIP. However, this size did not allow efficient methylation, because of defects either in de novo or in maintenance methylation. This precludes checking whether a smaller repeat is efficient in triggering MIP. The only conclusion we can arrive at concerning the minimal size required for pairing in MIP is that it is ≤317 bp.

DNA methylation associated with the extensive amplification of a variable CGG repeat within the CpG island of the

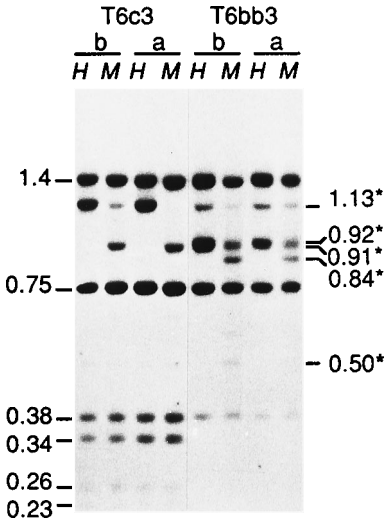

FIG. 9. Test of the stability of methylation after about 100 DNA replication cycles in TD06 derivatives. Two examples of the results obtained from the Southern hybridization analysis of derivatives T6c3 and T6bb3 before (b) and after (a) they had grown continuously on nonselective medium, using *Hpa*II (*H*) and *Msp*I (*M*), are shown. All other indications are as in Fig. 3 and 8. The loss, after growth, of methylation of the external C of the CCGG site 2′ (Fig. 7B) in the truncated copy of T6c3 is deduced from the disappearance of the 1.13-kb *Msp*I fragment.

FMR-1 gene has been shown to be involved in the fragile-X syndrome, which is the most common form of inherited mental retardation in humans (for a review, see reference 2). The disease is observed only when the trinucleotide expansion exceeds 200 repeats or so, i.e., around 600 bp, which triggers methylation of C residues of all CpG dinucleotides within the trinucleotide repeats and in the surrounding CpG island. Shorter expansions do not trigger methylation and constitute a silent mutation. A minimal size of the expanded region is thus required for the triggering of methylation. This could reflect the existence of a MIP-like process taking place when the trinucleotide expansion has reached a certain threshold in such a way that the expanded region is composed of two halves that can recognize each other as two elements of a tandem duplication.

Two mechanisms for C methylation. In contrast with previous work, which showed that methylation of kilobase-size parts of the *met2* gene prevented gene expression at the transcription level (1), we observed that methylation of part of this gene could be compatible with its expression. Methylation of the duplicated part of the full-length copy in the 630-bp ectopic and in the 457-bp tandem duplications had no detectable effect on *met2* expression. Also with the 630-bp tandem duplication, methylation did not always repress *met2*; when it led to *met2* silencing, this silencing was particularly unstable, since reversion occurred spontaneously within an unusually short period. This indicates that the extent and/or the density of methylation in short duplications was not sufficient to systematically impair transcription.

Methylation of short duplications differed by two other features from that of kilobase-size duplications.

First, the two elements of a duplication often displayed differences in their methylation patterns. Typically, the full-length copy was less methylated than the truncated one. This was documented with the 630-bp ectopic duplications, the 457- and 317-bp tandem duplications, and a significant fraction of the 630-bp tandem duplications in which the full-length copy was expressed. This suggests that *met2* expression may have an inhibitory effect on either the setting up or the maintenance of the methylation in short segments. That methylation maintenance is involved in the divergence of methylation patterns is indicated by reproducible differences in these patterns between sister derivatives and even between twin derivatives, which must have inherited, after a small number of DNA replication cycles, duplications methylated through the same individual MIP event. However, such divergence in the methylation patterns must take place mainly during early divisions, since we have shown that once established, the pattern of methylation can be faithfully maintained for a large number of replication cycles.

Second, in both the full-length copies and the truncated copies of all the 457- and 317-bp tandem duplications and in the full-length copies of the 630-bp ectopic duplications tested by either genomic sequencing or Southern hybridization, methylation was restricted to C's belonging to CpG sequences. Genomic sequencing further indicated that methylation at CpGs always occurred on both strands, at the two diagonally opposed C's of this short symmetrical sequence. Thus, MIP, which usually results in the methylation of every C when acting on kilobase-size duplications (1, 9, 11, 13, 25), typically leads to the methylation of CpGs only when targeted at short duplications, thus mimicking the situation typically found in mammals (4, 6). This observation indicates that two distinct mechanisms underlie methylation maintenance in *A. immersus*. The first mechanism, which acts at CpGs, likely involves a methyltransferase similar to that found to be acting in mammals (19), which recognizes individual hemimethylated CpGs. The finding that heavy methylation was limited to discrete CpGs which were not all located at the same positions in sister and twin derivatives suggests that maintenance and loss of methylation at these sites are clonally propagated, in keeping with the classical methylation maintenance model (15, 26). The efficiency of the second mechanism, which would ensure methylation maintenance at non-CpG sites, is dependent on the size of the repeat region. The methylase would recognize the overall methylated region via a signal that can be lost in short DNA segments. The nature of this signal still has to be determined. It may correspond either to the presence of particular DNA stretches or to a minimal number of methylated C's or to a minimal DNA segment size required for allowing a local change in chromatin conformation. The observation that in short repeats only part of the CpG sequences are methylated indicates that the methylation of C's at CpG sites is also size dependent. This suggests that the two postulated mechanisms are interconnected. The second mechanism could ensure methylation at all C's, including those in CpG sites, thus contributing to the efficient methylation maintenance at these dinucleotides in larger repeats. The existence of two mechanisms of methylation maintenance may or may not imply the existence of two different enzymatic complexes.

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