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Epimutation of repeated genes in *Ascobolus immersus*

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Ascobolus immersus **artificial gene repeats were shown previously to be subject premeiotically to both cytosine methylation and inactivation. We studied sexual progenies of strains harbouring two wild type copies of the endogenous *met2* gene lying either in tandem array or at ectopic unlinked positions, by (i) investigating the methylation status, (ii) searching for mutations and (iii) analysing the inheritance of inactivation both in mitotic and sexual offspring. 100% of the 'tandem' progeny and 64% of the 'ectopic' progeny had methylated repeats and displayed gene inactivation. Similar methylation patterns involving all or most of the cytosine residues within the repeats were observed in both arrangements. The inactivated *met2* copies were totally devoid of mutation, as deduced from: (i) extensive restriction site analysis and DNA sequencing; (ii) the finding that all the Met$^-$ derivatives tested reverted to prototrophy in selective conditions; and (iii) the finding that an inactivated copy of *met2* stripped of its methylation through amplification in *Escherichia coli* regained activity when reintroduced in *A.immersus*. In the absence of selection, gene silencing and methylation were faithfully maintained through mitotic divisions and through five successive sexual cycles. Altogether, these data show the epimutational nature of this methylation induced premeiotically (MIP) process.**

Key words: cytosine methylation/epigenetic modification/filamentous fungus/gene silencing/premeiotic inactivation

Introduction

In the filamentous ascomycete *Ascobolus immersus*, artificial tandem and ectopic DNA repeats are obtained via integrative transformation (Faugeron *et al.*, 1989; Goyon and Faugeron, 1989). Genes that are carried by these repeats are inactivated through sexual reproduction (Faugeron *et al.*, 1989, 1990; Goyon and Faugeron, 1989). Inactivation of repeated genes has been found for one endogenous gene, *met2* (encoding homoserine *O*-transacetylase) and two exogenous genes, *amdS* (encoding acetamidase) from *Aspergillus nidulans* and *hph* (encoding hygromycin B phosphotransferase) from *Escherichia coli*. The process is triggered at a premeiotic stage between fertilization and karyogamy and results in *de novo* methylation of the cytosine residues of the repeats (Goyon and Faugeron, 1989; Faugeron *et al.*, 1990).

Selker and co-workers first reported a similar process triggered premeiotically in the ascomycete *Neurospora crassa* and acting only on DNA repeats (Selker *et al.*, 1987b; Selker and Garrett, 1988). Genes carried by the duplications are inactivated (Selker and Garrett, 1988) and inactivation is associated with both cytosine methylation and point mutations corresponding to transitions to A−T of 10% to 30% of G−C bp carried by the duplicated sequences (Cambareri *et al.*, 1989). This process has been called RIP, which stands for repeat-induced point mutation. Such a heavy mutational process must be extremely efficient in inactivating permanently repeated genes. The observation in *A.immersus* that a large fraction of the Met$^-$ derivatives, in which *met2* had been inactivated, were able to revert to prototrophy (Goyon and Faugeron, 1989) strongly suggested that point mutations either never or rarely occurred. If mutations are actually induced in *A.immersus*, the only difference with *N.crassa* would lie in a lower efficiency of induction and the process would not be basically different from RIP. If, on the contrary, inactivated genes are completely devoid of mutations, then only cytosine methylation would be associated with gene inactivation and the process acting in *A.immersus* would be qualitatively different from RIP.

We report on experiments designed to choose between these two hypotheses. We performed a detailed study of the methylation status of inactivated genes lying either in tandem array or at ectopic positions and we examined the duplications for evidence of base pair changes. This study indicated that: (i) all (or almost all) cytosine residues within the repeats became methylated; (ii) gene inactivation was always reversible; (iii) no base substitution was ever detected within the repeats; and (iv) after amplification in *E.coli*, and subsequent reintroduction in *A.immersus*, a formerly inactivated gene regained normal expression without any methylation. These results clearly demonstrate that genes affected by the inactivation process in *A.immersus* are just silenced. For this reason, we have chosen the acronym MIP (methylation induced premeiotically) to refer to the process acting in *A.immersus* to distinguish it from RIP. For the sake of simplicity, we will use this acronym MIP from now on, anticipating the conclusions of the results described below.

In addition, we analysed the heritability of inactivation and showed that despite the reversibility of inactivation, silencing and cytosine methylation of genes that had undergone MIP were faithfully transmitted to the progeny through both vegetative and sexual reproduction; in the latter case, silencing and cytosine methylation were both heritable in a Mendelian way. All these properties are those expected from an epimutation process (Jeggo and Holliday, 1986).

Results

Methylation status of tandem and ectopic repeats subject to MIP

We asked three questions relative to the methylation status of DNA repeats that had been subjected to MIP. (i) What proportion of the cytosine residues are methylated? (ii) To

what extent are methylation and inactivation associated, i.e. can methylated genes be active or can inactive genes be unmethylated? (iii) What is the reason for the higher proportion of progeny having lost the parental phenotype when repeated genes are in tandem array (>90%, Goyon and Faugeron, 1989) as compared with repeated genes at unlinked ectopic positions (~50%, Faugeron *et al.*, 1989)? This difference may reflect two different situations: either the probability of MIP triggering is lower when the repeats are unlinked than when they are in tandem, or the frequency is the same but the process does not always lead to gene inactivation when acting on unlinked repeats. In the latter case, we might observe that ectopic genes that are still expressed have undergone some methylation. To answer these questions, we analysed at the DNA level Met^+ and Met^- derivatives of transformants with either tandem or ectopic duplicated *met2* copies.

We first crossed with a wild type strain transformant Tp1, which contains two tandemly repeated copies (one resident, the other transgenic) of the 5.7 kb *Hin*dIII fragment (Figure 1) carrying the wild type *met2* gene (Goyon *et al.*, 1988), separated by a 5.1 kb fragment carrying the *amdS* gene. We determined the Amd and Met phenotypes of the four meiotic products of asci from each cross. Since transformed protoplasts are generally multinucleate, transformants are likely to contain a mixture of both transformed and untransformed nuclei. For this reason, only asci with $2Amd^+$:$2Amd^-$ meiotic products, which had inherited the transgenic information, were considered. With this class of asci, possible excision events that would eliminate one *met2* copy and the *amdS* marker were disregarded (in another experiment, the frequency of these excision events was estimated to be close to 0.01). We analysed 91 $2Amd^+$:$2Amd^-$ asci. All of them showed a $2Met^+$:$2Met^-$ segregation. Since MIP affects repeated sequences only, the single *met2* copy from the wild type parent did not become inactivated (Faugeron *et al.*, 1990). Consequently, each Met^- segregant resulted from the simultaneous inactivation of both tandem *met2* copies from Tp1, and this occurred in 100% of premeioses. As expected, Met^- meiotic products were also Amd^+ (Figure 2). Nine such Met^- derivatives were analysed at the DNA level by Southern blot hybridizations, using restriction enzymes that allow differences in methylation patterns to be observed. We compared the hybridization patterns of *Sau*3AI and *Mbo*I DNA digests from Met^- derivatives (two examples are shown in Figure 3). *Sau*3AI and *Mbo*I each recognize the sequence GATC. Both cleave the sequence when it is unmethylated but *Sau*3AI will not cut it if the C is methylated, whereas *Mbo*I is insensitive to cytosine methylation (Nelson and McClelland, 1991). The probe used was the 2630 bp *Xba*I–*Bgl*II fragment (Figure 1), which spans the *met2* gene and contains 12 GATC sites as deduced from its nucleotide sequence (Goyon *et al.*, 1988). All Met^- derivatives showed the same *Mbo*I hybridizing fragments as those of the wild type strain (only the six larger hybridizing fragments are visible on the picture shown). This means that all *Mbo*I sites were cut. In contrast, in the nine Met^- derivatives studied, all corresponding *Sau*3AI fragments were missing and replaced by larger fragments, the largest one being ~6 kb in size. These results indicate that all the GATC sites carried by both *met2* repeats were still intact and that they all had been methylated. The presence of several large *Sau*3AI fragments of different sizes indicates that, in a fraction of the DNA molecules from the samples analysed, some GATC sites were cut and therefore that some cytosine residues were not methylated, suggesting that methylation was not accurately maintained at each DNA replication cycle. In previously published experiments (Goyon and Faugeron, 1989), both *Sau*3AI and *Mbo*I enzymes failed to cut within the duplicated *met2* sequences. Subsequently, we showed that the so-called *Mbo*I enzyme supplied by Pharmacia (France, SA) and used in those experiments, was sensitive to cytosine methylation and was unable to cut methylated GATC. Other samples of *Mbo*I supplied by various manufacturers were tested and all found to be insensitive to cytosine methylation without exception, as expected from the specificity of this enzyme.

Transformant Ep1 contains two unlinked copies of the wild type *met2* gene (one resident, the other transgenic). The transgenic copy is carried by the 5.7 kb *Hin*dIII fragment (Figure 1), integrated together with the *amdS* gene at an ectopic chromosomal site. Ep1 was crossed with wild type. Resulting asci were analysed for the Amd and Met phenotypes of their spores and only $2Amd^+$:$2Amd^-$ asci were considered. Among the 107 $2Amd^+$:$2Amd^-$ asci tested, 69 showed a $2Met^+$:$2Met^-$ segregation, attesting that both *met2* copies from Ep1 had been inactivated. The other 38 asci showed the $4Met^+$:$0Met^-$ segregation, expected if no inactivation had occurred. Southern hybridizations were performed on DNA from 13 Met^- derivatives from $2Met^+$:$2Met^-$ asci, probing *Sau*3AI and *Mbo*I digests with *met2*, as described above. Three Met^- derivatives were chosen as being also Amd^+ (Figure 4), which indicated that they all had inherited both the resident and the ectopic copies of *met2*. The other 10 Met^- derivatives were chosen as being Amd^- (Figure 4), which indicated that they all had inherited only the resident copy of *met2* and not the ectopic one. The hybridization patterns obtained with both types of Met^- derivatives were similar (two examples of each are shown in Figure 3). They were also similar to those obtained with Met^- derivatives of Tp1,

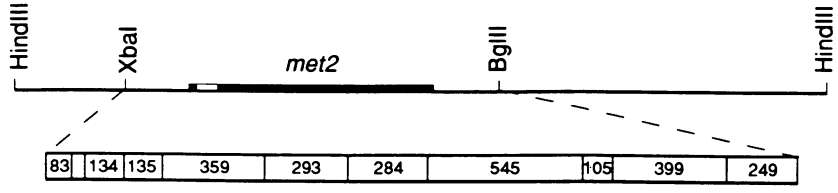

Fig. 1. Partial restriction map of the *Hin*dIII fragment carrying the *met2* gene (deduced from Goyon *et al.*, 1988). The thick line represents the two exons of the *met2* gene (black boxes) separated by a 165 bp intron (open box). The *met2* probe used in Southern hybridization experiments was the *Xba*I–*Bgl*II fragment, whose *Sau*3AI/*Mbo*I restriction map is shown (below open box; vertical bars correspond to *Sau*3AI/*Mbo*I sites, fragment sizes are in bp). Only fragments >200 bp were detected by Southern hybridization.

which had two tandemly repeated *met2* copies that had undergone MIP. Here again, the size of the largest *Sau*3AI fragment is ~6 kb, indicating that all cytosine residues of the repeated 5.7 kb *Hin*dIII fragment were methylated. Interestingly, methylation of one single copy appears to be efficiently maintained through mitotic divisions. This indicates that methylation maintenance does not require two copies in the same nucleus contrary to the *de novo* methylation occurring in the MIP process (Faugeron *et al.*, 1990). We also performed Southern hybridizations on *Sau*3AI and *Mbo*I DNA digests of nine Met^+ Amd^+ segregants (which thus harboured the transgenic *met2* copy, Figure 4) found in nine different $4Met^+$:$0Met^-$ asci. All of them were completely unmethylated (not shown). This indicates that contrary to tandemly repeated copies, unlinked *met2* copies can escape MIP. This finding is consistent with the hypothesis of pairing between the repeats being required for MIP triggering (Faugeron *et al.*, 1990). Indeed, tandem repeats should find each other more efficiently than repeats located far from each other.

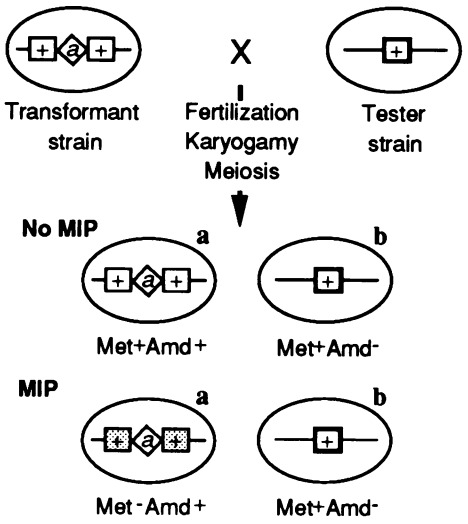

Fig. 2. Met and Amd phenotypes of the progeny of strains having two tandemly repeated *met2* copies separated by *amdS*. Each wild type *met2* copy is represented by +, *amdS* is represented by a. The only two possible a and b meiotic products are shown, together with the expected phenotypes of the resulting segregant strains depending on whether MIP did occur or not. The *amdS* gene, present as a single copy, never became silenced.

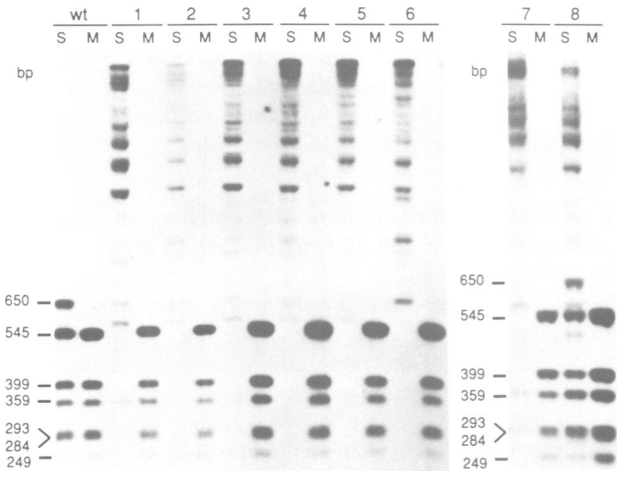

Fig. 3. Southern hybridization analysis of the methylation pattern of the *met2* region from Met^- derivatives and their prototrophic revertants. *Sau*3AI (S) and *Mbo*I (M) DNA digests were hybridized with the *met2* probe (Figure 1). wt indicates the wild type strain used as a recipient in the initial transformation experiments. 1–4 indicate Met^- derivatives from transformant Ep1, which contains two unlinked copies of *met2*, one resident the other transgenic. 1 and 2 have inherited only the resident copy. 3 and 4 have inherited both copies. 5 and 6 indicate Met^- derivatives from transformant Tp1, which contains two tandemly repeated copies of *met2*. 8 indicates a Met^+ revertant from a Met^- derivative (indicated by 7) from Tp1 having inherited the resident copy only. Fragment sizes are in bp. An extra fragment (650 bp long) appears in *Sau*3AI digests from the wild type strain and Met^+ revertants. It results from the partial digest of a GATC site which might contain a naturally methylated cytosine residue.

In addition to *Sau*3AI and *Mbo*I, other restriction enzymes sensitive to cytosine methylation (Nelson and McClelland, 1991), namely *Bgl*II, *Cla*I, *Hpa*II, *Msp*I, *Scr*FI, *Sal*I, *Sca*I, *Sma*I, *Xba*I and *Xho*I, were used in Southern hybridization experiments performed on Met^- derivatives containing two inactivated *met2* copies either tandemly repeated or at ectopic positions. In all cases, none of them was able to cut its recognition sites when these were carried by either type of *met2* repeats. The *Hph*I enzyme, which recognizes the asymmetrical sequence TCACC, was also unable to cut. This indicates that most or all cytosine residues were methylated in both tandem and ectopic repeats. It appears therefore that contrary to the frequency of MIP triggering, the intensity of the methylation resulting from MIP is not dependent on the relative position of the repeats.

Inactivation induced by MIP is always reversible

Goyon and Faugeron (1989) showed that Met^- derivatives were able to revert spontaneously to prototrophy after growth on non-selective medium (supplemented with methionine) and transfer onto selective medium (without methionine). 90% of the Met^- progeny tested reverted. One hypothesis proposed by the authors to explain the 10% stable Met^- progeny (seven out of 72) was that they might result from

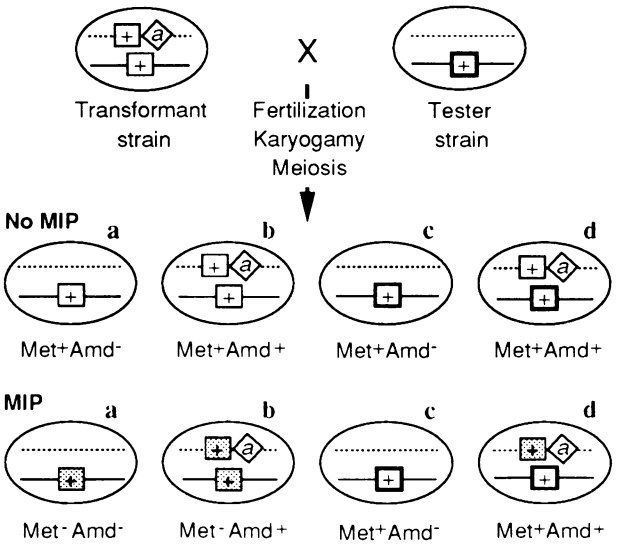

Fig. 4. Met and Amd phenotypes of the progeny of strains having two ectopic *met2* copies. Both *met2* (+) and *amdS* (a) transgenic genes are cointegrated at a chromosomal location unlinked to the resident *met2* copy. Four different meiotic products (a, b, c and d) can be formed. All other indications as in Figure 2.

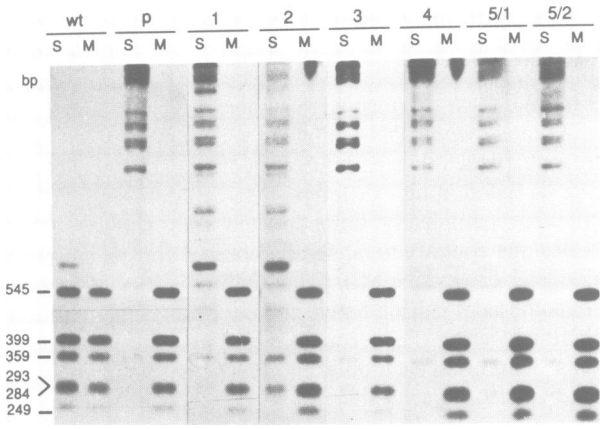

Fig. 5. Southern hybridization analysis of the methylation maintenance of a *met2* gene that has undergone MIP through five successive cycles of sexual reproduction. p indicates the Met$^-$ parent (in which *met2* has undergone MIP) of the first generation. 1, 2, 3 and 4 indicate the Met$^-$ derivatives issued from the first, second, third and fourth generations, respectively, and used as parents in the respective subsequent generations. 5/1 and 5/2 indicate two Met$^-$ derivatives issued from the fifth generation. All other indications as in Figure 3.

the heterokaryotic state of their parental primary transformants. Indeed, no selectable marker other than *met2* had been used, so that Met$^-$ progeny issued from the untransformed nuclei of the *met2* mutant recipient strain were phenotypically indistinguishable from the Met$^-$ progeny created by MIP. An alternative hypothesis was that mutations may have occurred within the genes that had undergone MIP, preventing them from recovering expression. To decide between these two hypotheses, we tested for reversion 466 Met$^-$ derivatives in which *met2* had undergone MIP. Among them, 71 contained a single copy, 361 contained two tandemly repeated copies and 34 contained both the resident and the ectopic copies. All 466 Met$^-$ derivatives reverted spontaneously to prototrophy after a period of time following their transfer onto selective medium ranging from a few days to more than a month. Revertants appeared as sectors of growing filaments which covered the plates within <3 days from the time of appearance of visible growth (similar to the wild type). In addition, reversion to the Amd$^+$ phenotype was obtained for all 37 tested Amd$^-$ derivatives having inherited either one, two or three copies of the *amdS* gene that had undergone MIP. These derivatives were obtained through a cross involving a parental strain with three unlinked *amdS* copies (Faugeron *et al.*, 1990). A fraction of these (14) reverted spontaneously to the Amd$^+$ phenotype. All the other Amd$^-$ derivatives (23) reverted after addition in the growth medium of 5-azacytidine, an analogue of cytidine, which prevents cytosine methylation (Jones and Taylor, 1980). Thus, all of the Met$^-$ and Amd$^-$ derivatives tested were able to revert to the parental transformant phenotype. This strongly suggests that inactivation is not associated with any mutation within the *met2* or *amdS* genes that have undergone MIP, and therefore that MIP results in gene silencing only.

We showed by Southern hybridization experiments that reversion was accompanied by a decrease in methylation intensity. This was done by probing with *met2* *Sau*3AI DNA digests from the Met$^+$ revertants. These revertants were obtained from several Met$^-$ derivatives containing a single copy of *met2* or two tandemly repeated copies, or both the resident and the ectopic copies. All these revertants showed similar hybridization patterns. One example is shown in Figure 3. Although large *Sau*3AI fragments resulting from cytosine methylation were still present, fragments identical to those from the wild type strain appeared in all revertants. This overall decrease in methylation might reflect either two populations of nuclei, one in which *met2* was still fully methylated and the other in which it was unmethylated, or nuclei all having methylated *met2* but less intensively. Whichever is correct, our results indicate that inactivation and methylation are intimately connected.

Inactivation triggered by MIP does not result from mutation

In experiments described above, together with others not described, we tested a total of 720 GATC sites carried by a large number of *met2* copies that have undergone MIP. They were all structurally intact since they were all cut by *Mbo*I, even though they were not cut by *Sau*3AI due to C methylation. Thus, none of the 2880 bp (4 $\times$ 720 bp) tested was mutated. This result, together with the finding that gene inactivation is always reversible, argues for a lack of mutation within the genes that have undergone MIP. The lack of mutation was also proved by DNA sequencing. The 545 bp *Mbo*I fragment present in the coding region of *met2* (Figure 1) was sequenced in four Met$^-$ strains in which *met2* had undergone MIP. Three strains had one single copy of *met2* and one strain had a tandem repeat. The *Mbo*I fragment to be sequenced was first PCR amplified from total DNA and the resulting population of DNA fragments was subcloned into *E.coli*. The same was done with the wild type strain, as a control. The results (not shown) indicated that all four sequences and the control were identical to the wild type sequence previously published by Goyon *et al.* (1988), ruling out the presence of any mutation within 2180 bp (4 $\times$ 545 bp) from sequences that had undergone MIP.

A gene that has undergone MIP behaves as native after cloning in E.coli and reintroduction in A.immersus

In order to make sure that only methylation is responsible for gene silencing and to exclude the possibility that methylation might be triggered by some silent mutations which would have escaped the analysis, we demethylated a gene that had undergone MIP via cloning it in *E.coli* [with the exception of the Dcm methylase targets, namely the internal C of each one of the four CC(A/T)GG *Bst*NI sites present in the *met2* gene; Goyon *et al.*, 1988] and reintroduced it in *A.immersus* by genetic transformation. We started from one of the nine Met$^-$ derivatives from Tp1 having two tandemly repeated methylated *met2* copies (Figure 3), each carried by the 5.7 kb *Hin*dIII fragment (Figure 1). We first showed that the repeated *Hin*dIII sites delimiting the ends of the duplication were cut, although *Hin*dIII is sensitive to cytosine methylation (Nelson and McClelland, 1991). The observation that the borders of sequences that have undergone MIP remain unmethylated has also been made by C.Barry, G.Faugeron and J.-L. Rossignol (unpublished). The 5.7 kb *Hin*dIII fragment was inserted into vector pUC19 and then used to transform the *E.coli* strain ER1647 (*mcr*A *mcr*B; Woodcock *et al.*, 1989).

Plasmids from three independent recombinant clones were isolated and used to transform a mutant strain of *A.immersus* having a deletion removing the entire *met2* gene (this strain results from the replacement of *met2* by *amdS*; C.Barry, unpublished). As a control, transformation of *A.immersus* was also done with a plasmid identical in structure, but carrying the 5.7 kb *Hin*dIII fragment from a wild type strain. Met^+ transformants were obtained with all three recombinant plasmids at the same frequency as that of the control experiment. This indicates that once released from methylation, a gene that has undergone MIP recovers full expression. We checked by Southern hybridization experiments that *met2* remained unmethylated in the Met^+ transformants. This was done by probing with *met2 Sau*3AI and *Mbo*I DNA digests from two of them (not shown). This showed that the *met2* copy which had previously undergone MIP was devoid of signals for triggering methylation.

These results provide further compelling evidence that only methylation is responsible for gene silencing and contrast with those found with a DNA sequence having undergone RIP which, after cloning in *E.coli* and subsequent reintroduction in *N.crassa*, showed spontaneous vegetative remethylation (Selker *et al.*, 1987a; Cambareri *et al.*, 1991).

Silencing and methylation of genes that have undergone MIP are heritable

All the properties of MIP described above are those expected from an epimutation process. Indeed, the term 'epimutation' has been proposed by Jeggo and Holliday (1986) to describe 'epigenetic changes in gene activity based on DNA methylation' and 'to distinguish them from classical mutations which are due to changes in DNA base sequences'. A further property of epimutations as defined by Holliday (1991) is that they are heritable through mitotic divisions. If so, gene silencing resulting from MIP should be faithfully maintained through vegetative reproduction, in the absence of any selective pressure. For this purpose, four Met^- strains, which contained one *met2* copy that had undergone MIP (its ability to revert to prototrophy on selective medium was checked), were grown on minimal medium supplemented with methionine for a large number of generations. On such a medium, all nuclei, no matter whether they are Met^- or Met^+, can efficiently divide. Mitotic subclones were obtained by making protoplasts; this is the only way by which mitotic subclones can be obtained, since *A.immersus* does not produce conidia. Protoplasts were regenerated on the appropriate medium supplemented with methionine. Subclones thus obtained were then transferred onto selective medium to check their Met phenotype. In this way, a roughly equal number of subclones of each strain, totalling 967 subclones, were tested. Only one was Met^+, the others were Met^-. We checked that all Met^- subclones were able to revert to prototrophy, which indicated that in all of them *met2* was still silenced and had not undergone *de novo* mutation. It should be noted that (i) protoplasts were not all uninucleate (Faugeron *et al.*, 1989), so that the number of nuclei analysed for reversion was certainly higher than 967; and (ii) the Met^+ subclone might have arisen during protoplast regeneration from reversion of a still Met^- nucleus. Consequently, reversion to prototrophy occurred in probably $< 1 \times 10^{-3}$ nuclei. This shows that *met2* silencing is efficiently maintained and thus heritable through vegetative reproduction. This conclusion does not contradict the finding that 100% of Met^- derivatives revert to prototrophy. Indeed, reactivation of *met2* in only one nucleus out of the thousands of nuclei present in the piece of mycelium transferred onto selective medium is certainly enough for producing a strain exhibiting a Met^+ phenotype.

We then asked whether *met2* silencing was also heritable through sexual reproduction. Four Met^- derivatives, each containing one *met2* copy that had undergone MIP (the resident one), were crossed with wild type. Fifty asci harvested in the progeny of each cross were dissected and tested for the Met phenotype of their spores. They all showed the $2Met^+$:$2Met^-$ segregation expected for a Mendelian transmission of the Met^- phenotype. Reversibility of *met2* silencing was checked on the two Met^- meiotic products of 10 different asci from each cross. In the progeny of two out of the four crosses, we chose one of the Met^- strains checked for reversion and backcrossed it to wild type. Here again, 50 asci issued from each of the two crosses were analysed. They all showed a $2Met^+$:$2Met^-$ segregation. The same was repeated for three more generations. We analysed 50 asci in the third and fourth generation progenies and 100 asci in the fifth generation progenies. Thus, a total of 700 asci were analysed. We always observed a $2Met^+$:$2Met^-$ segregation, indicating that no Met^+ progeny derived from the Met^- parent. The perfect maintenance of *met2* silencing through five successive cycles of sexual reproduction proves that silencing is heritable. We then showed that cytosine methylation also was perfectly maintained and was heritable by probing with *met2 Sau*3AI and *Mbo*I DNA digests from Met^+ progenies of each generation (Figure 5).

Discussion

All our data indicate that gene inactivation resulting from MIP is associated with cytosine methylation only and is not due to mutation. (i) All of the Met^- derivatives tested were able to revert to prototrophy under selective pressure, recovering a phenotype identical to that of the Met^+ parental transformant. The same was observed with all Amd^- derivatives tested. The possibility that reversion to the parental phenotype resulted from reversion of some missense mutations in the genes that had undergone MIP is ruled out by two observations. First, very early reversion is the rule if spore germination and subsequent growth are performed on medium with 5-azacytidine, since none of the transformant derivatives has the Met^- phenotype (Goyon and Faugeron, 1989; unpublished results). Insofar as 5-azacytidine is a powerful demethylating agent (Jones and Taylor, 1980) and is not known to induce base substitutions, reversion triggered by 5-azacytidine is very probable to result from a decrease in cytosine methylation rather than from back-mutation. The second argument comes from the finding that the time necessary for observing reversion of Met^- derivatives, in which *met2* had been silenced following the duplication of different parts of the gene, is dependent on which part has been duplicated. When the region 5′ to the ORF is duplicated, reversion occurs much earlier than when either the entire ORF or the region 3′ to the ORF are duplicated (C.Barry, G.Faugeron and J.-L.Rossignol, unpublished). This would not be the case if reversion resulted from base substitutions. (ii) No mutations were found among 5060 bp tested in *met2* sequences which had undergone MIP,

analysed by restriction site cutting and DNA sequencing. (iii) After cloning in *E.coli* and reintroduction in *A.immersus*, an initially silenced *met2* gene immediately recovered expression. We can therefore conclude that MIP is an epigenetic silencing process.

Another important observation is that epimutations in silenced genes that have undergone MIP are heritable not only through mitotic divisions, as was previously found in mammals (see Holliday, 1991) and maize (see Fedoroff, 1989), but also through meiosis. Being an ascomycete and thus having all four meiotic products (four pairs of sister spores) grouped together in an ascus, *A.immersus* allows the determination of the meiotic segregation of mutations into the four products. Using this property, we could show that gene silencing and cytosine methylation segregate according to Mendelian laws and that in this respect epimutations behave as classical mutations.

If one considers the prerequisites for triggering MIP and RIP (Faugeron *et al.*, 1990; Selker, 1990b), both processes appear identical. The difference consists in the occurrence of G−C to A−T transition mutations in sequences subject to RIP, whereas sequences that undergo MIP are totally devoid of mutations. Transition mutations in *N.crassa* are triggered together with the overall RIP process. They are usually numerous (Cambareri *et al.*, 1989), although sometimes their number appears to be quite small (Fincham, 1990). Three hypotheses have been proposed to account for the connection between RIP and cytosine methylation. The first one (Selker, 1990a) is that the point mutations resulting from RIP might themselves be responsible for rendering the sequence a good substrate for DNA methylation. This is mainly based on the observation that sequences altered by RIP become faithfully *de novo* methylated when reintroduced through transformation into *N.crassa* (Selker *et al.*, 1987a; Cambareri *et al.*, 1991). This hypothesis is also consistent with the finding by Fincham (1990) of RIP-induced mutations in the *am* gene of *N.crassa*, in which methylation was undetectable. This hypothesis cannot account for MIP, since mutations are not the cause of methylation in this process. Insofar as RIP is closely related to MIP, it is likely to use the same methylation mechanism, which would not fit this hypothesis. The second hypothesis (Selker, 1990b) rests on the spontaneous deamination of 5,6-dihydrocytosine, which is thought to be an intermediate in the enzymatic mechanism of cytosine methylation and is 10 000-fold more prone to deamination than cytidine. Thus, C to T changes could occur directly without involving a 5-methylcytosine intermediate. In this hypothesis, the absence of C to T changes in sequences that have undergone MIP can only be explained if the chemical environment leads to a much lower rate of spontaneous deamination of 5,6-dihydrocytosine in *A.immersus* as compared with *N. crassa*, or if the enzymatic complex responsible for MIP is able to stabilize the 5,6-dihydrocytosine intermediate by preventing its deamination. The third hypothesis (Cambareri *et al.*, 1989) simply rests on an enzymatically driven deamination of 5-methylcytosine producing thymine. In this hypothesis, the only difference between RIP and MIP would be the expression or non-expression of 5-methylcytosine deaminase at the precise premeiotic stage when both processes act.

The finding that most, if not all, cytosine residues are methylated in genes that have undergone MIP and that methylation is transmitted to the progeny at each generation raises the question of the mechanism by which methylation is maintained. Methylation at cytosine residues belonging to short symmetrical sequences might be maintained according to the model proposed by Holliday and Pugh (1975) and Riggs (1975), who have postulated a eukaryotic methylase capable only of completing methylation of hemimethylated sites generated by DNA replication. Because the CpG dinucleotide, which bears most of the vertebrate methylation, is a simple symmetrical sequence, methylation on one strand could direct the methylation on a newly replicated strand through the action of the postulated maintenance methylase. Compelling evidence has been obtained in favour of this model (Bird, 1978; Gruenbaum *et al.*, 1982; Smith *et al.*, 1991). In this model, the maintenance methylase in *A.immersus* may resemble some phage-encoded methyltransferases that methylate more than one sequence and contain several independent target-recognizing domains, each responsible for recognizing a different site (Wilke *et al.*, 1988; Lange *et al.*, 1991). However, we have observed methylation at cytosine residues that do not belong to symmetrical sequences. Maintenance of such methylation requires a different mechanism. In this case, the methylase might recognize a hemimethylated region rather than a hemimethylated sequence, the recognition signal being a change in the chromatin structure. A model based on a change in chromatin structure has been proposed (Selker, 1990b).

The fact that methylation of a single copy of a gene that has initially undergone MIP is efficiently maintained through mitotic divisions, could provide interesting applications in the study of the effects of cytosine methylation upon the main aspects of DNA metabolism. By duplicating chosen chromosomal regions via genetic transformation, it is possible to construct strains identical to wild type, but in which a gene, a cluster of genes or part of a gene have been methylated by the MIP process. This device has been used to study the effect of methylation on gene expression (C. Barry, G.Faugeron and J.-L.Rossignol, unpublished). It could also be used to perform *in vivo* studies of the effects of methylation upon homologous recombination, mutation, transposition, chromatin configuration and, more generally, susceptibility of DNA to enzymes involved in DNA metabolism.

We previously proposed that MIP could act on natural DNA repeats in *A.immersus* and contribute to genome stability by preventing chromosome rearrangements and invasion by mobile elements (Faugeron *et al.*, 1990). MIP, just as RIP, even though their immediate consequences appear to be different, could also have important evolutionary effects in organisms where they act. First, duplications of essential genes should have a tendency for being eliminated insofar as they entail the lethality of the carrying strains. Secondly, these processes should contribute to a fast accumulation of mutations at the level of the duplicated regions (if the genes are non-essential), either instantaneously (RIP) or possibly during further mitotic divisions (MIP), through secondary deamination of the 5-methylcytosine residues, causing G−C to A−T transitions and hence participating in an accelerated evolution of some DNA sequences (Kricker *et al.*, 1992). In plants, several examples of gene inactivation caused by the presence of several copies of a gene and sometimes shown to be correlated with

methylation, have been described (Jorgensen, 1990). Unlike MIP and RIP in such cases, gene inactivation occurs vegetatively. This suggests that a family of processes related to MIP and RIP, but not identical among themselves, might involve a large spectrum of organisms and play a major evolutionary role.

Materials and methods

Strains of A.immersus, genetic procedures and media

Strains containing either tandem or ectopic *met2* repeats both resulted from transformation of the wild type strain RL95 with plasmid pJF2 (J.-J.Godon, F.Pâques and G.Faugeron, unpublished). Plasmid pJF2 resulted from the integration of the 5.1 kb *Sal*I–*Eco*RI fragment carrying the *amdS* gene (encoding acetamidase) from *Aspergillus nidulans* (Hynes *et al.*, 1983) into plasmid pCG5, which consists of the 5.7 kb *Hin*dIII fragment carrying the *met2* gene (encoding homoserine *O*-transacetylase) from *A.immersus* inserted into vector pUC19 (Goyon *et al.*, 1989). *AmdS* was used as a selectable marker. Indeed, non-transformed strains of *A.immersus* are not able to grow on a medium containing acetamide as sole nitrogen source as they lack the *amdS* gene. The ectopic duplicated copy of *met2* resulted from the non-homologous integration of plasmid pJF2, used as circular in the transformation experiment. The tandem *met2* repeats resulted from the integration of linearized plasmid pJF2 (cut at the *Not*I site of the *met2* coding sequence) by homologous recombination between the resident and the plasmidic *met2* copies.

Strains belong to stock 28 (Rizet *et al.*, 1969). The recipient strain RL95 and the tester strain FA35 are of opposite mating types. They both have Met^+ and Amd^- phenotypes as both are wild type except that FA35 harbours 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 by Faugeron *et al.* (1990), each pair corresponding to one meiotic product. Most media were as in Bennett and Lasure (1991). The Met phenotype was checked on minimal medium. The Amd phenotype was checked on medium containing acetamide as nitrogen source instead of urea (Faugeron *et al.*, 1990) and supplemented with methionine (20 μM/ml). 5-azacytidine was used at a concentration of 10 μM.

Isolation and manipulation of DNA

Most experimental procedures were performed as described by Goyon and Faugeron (1989). For Southern hybridization experiments, DNA (3 μg) was digested with 5-fold excess of enzymes to ensure complete digestion. Digests were fractionated in 1.8% agarose gels and gel blots were hybridized to a ^{32}P-labelled *met2* probe (Figure 1). The 1 kb ladder from Bethesda Research Laboratories was used as size marker.

PCR amplification and DNA sequencing

The 545 bp fragment (Figure 1) to be sequenced was amplified by PCR using 20mer primers respectively located upstream and downstream from the bordering *Sau*3AI sites (positions 1582–1601 and 2160–2179 in the sequence published by Goyon *et al.*, 1988). Amplifications were performed in 100 μl reaction volumes containing 0.5 μg of genomic DNA, 30 pmol of each primers, 200 μM (each) dNTPs, 0.5 units of Taq polymerase (Bioprobe) and the buffer supplied by the manufacturer. Reactions were carried out in a Perkin–Elmer Cetus DNA Thermal Cycler as follows: 30 s at 94°C, 30 s at 57°C and 1 min at 72°C for 25 cycles. Following the synthesis, each mixture was phenol extracted, ethanol precipitated, digested with *Sau*3AI and ligated into the *Bam*HI site of pBluescript II KS+ (Stratagene) using standard techniques. The ligation products were introduced into the *E.coli* strain DH5αF′ (Woodcock *et al.*, 1989) by electroporation. Recombinant plasmids were characterized by restriction analysis and the *Sau*3AI insert was sequenced on both strands using the T7 and KS primers.

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References

Bennett,J.W. and Lasure,L.L. (1991) In Bennett,J.W. and Lasure,L.L. (eds), *More Gene Manipulation in Fungi.* Academic Press, pp. 441–458.
Bird,A.P. (1978) *J. Mol. Biol.*, **118**, 49–60.
Cambareri,E.B., Jensen,B.C., Schabtach,E. and Selker,E.U. (1989) *Science*, **244**, 1571–1575.
Cambareri,E.B., Singer,M.J. and Selker,E.U. (1991) *Genetics*, **127**, 699–710.
Faugeron,G., Goyon,C. and Grégoire,A. (1989) *Gene*, **76**, 109–119.
Faugeron,G., Rhounim,L. and Rossignol,J.-L. (1990) *Genetics*, **124**, 585–591.
Fedoroff,N.V. (1989) *Cell*, **56**, 181–191.
Fincham,J.R.S. (1990) *Curr. Genet.*, **18**, 441–445.
Goyon,C. and Faugeron,G. (1989) *Mol. Cell. Biol.*, **9**, 2818–2827.
Goyon,G., Faugeron,G. and Rossignol,J.-L. (1988) *Gene*, **63**, 297–308.
Gruenbaum,Y., Cedar,H. and Razin,A. (1982) *Nature*, **295**, 620–622.
Holliday,R. (1991) *Mutat. Res.*, **250**, 351–363.
Holliday,R. and Pugh,J.E. (1975) *Science*, **187**, 226–232.
Hynes,M.J., Corrick,C.M. and King,J.A. (1983) *Mol. Cell. Biol.*, **3**, 1430–1439.
Jeggo,P.A. and Holliday,R. (1986) *Mol. Cell. Biol.*, **6**, 2944–2949.
Jones,P.A. and Taylor,S.M. (1980) *Cell*, **20**, 85–93.
Jorgensen,R. (1990) *Trends Biotechnol.*, **8**, 340–344.
Kricker,M.C., Drake,J.W. and Radman,M. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 1075–1079.
Lange,C., Jugel,A., Walter,J., Noyer-Weidner,M. and Trautner,T.A. (1991) *Nature*, **352**, 645–648.
Nelson,M. and McClelland,M. (1991) *Nucleic Acids Res.*, **19**, 2045–2071.
Riggs,A.D. (1975) *Cytogenet. Cell Genet.*, **14**, 9–25.
Rizet,G., Rossignol,J.-L. and Lefort,C. (1969) *C. R. Acad. Sci. Paris*, **269**, 1427–1430.
Selker,E.U. (1990a) *Annu. Rev. Genet.*, **24**, 579–613.
Selker,E.U. (1990b) *Trends Biochem. Sci.*, **15**, 103–107.
Selker,E.U. and Garrett,P.W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6870–6874.
Selker,E.U., Jensen,B.C. and Richardson,G.A. (1987a) *Science*, **238**, 48–53.
Selker,E.U., Cambareri,E.B., Jensen,B.C. and Haack,K.R. (1987b) *Cell*, **51**, 741–752.
Smith,S.S., Kan,J.L.C., Baker,D.J., Kaplan,B.E. and Dembek,P. (1991) *J. Mol. Biol.*, **217**, 39–51.
Woodcock,D.M. *et al.* (1989) *Nucleic Acids Res.*, **17**, 3469–3478.
Wilke,K., Rauhut,E., Noyer-Weidner,M., Lauster,R., Pawlek,B., Behrens,B. and Trautner,T.A. (1988) *EMBO J.*, **7**, 2601–2609.

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Note added in proof

We have determined the complete sequence (2910 bp) of an entire *met2* copy that had undergone MIP. The sequenced fragment, which corresponds to the *Hin*cII–*Bgl*II fragment described in Goyon *et al.* (1988), contains all the signals necessary for the full expression of the gene. We found a sequence strictly identical to the wild type sequence. This constitutes a straightforward demonstration that MIP is definitely not associated with any mutation within the target gene.