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Targeted Transformation of *Ascobolus immersus* and De Novo Methylation of the Resulting Duplicated DNA Sequences

CHRISTOPHE GOYON† AND GODELEINE FAUGERON*

Laboratoire I.M.G., Université Paris-Sud, Bâtiment 400, 91405 Orsay Cedex, France

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To develop a method to modify genomic sequences in *Ascobolus immersus* by precisely reintroducing defined DNA segments previously manipulated in vitro, we investigated the effect of transforming DNA conformation on recombination with chromosomal sequences. Circular single-stranded DNA carrying the *met2* gene and double-stranded DNA linearized by cutting within the *met2* gene both transformed protoplasts of a *met2* mutant strain of *A. immersus* to prototrophy. In contrast to the equivalent circular double-stranded DNA, which chiefly integrated at nonhomologous chromosomal sites, single-stranded and double-stranded cut DNAs recombined primarily with the homologous chromosomal *met2* sequence. Of the single-stranded DNA transformants, 65% resulted from replacement of the resident *met2* mutation by the exogenous wild-type allele. In 70% of the double-stranded-cut DNA transformants, one or more copies of the transforming DNA had integrated at the *met2* locus, leading to tandem duplications of the *met2* target region separated by plasmid DNA. These duplicated sequences could recombine, leading to progeny containing only one copy of the *met2* region. This resulted in a precise gene replacement if the wild-type allele had been retained. In addition, we show that newly duplicated sequences were most often de novo methylated at the cytosine residues during the sexual phase. Cytosine methylation was associated with inactivation of the integrated *met2* gene(s) in segregants of crosses. However, methylation was not accurately maintained at each DNA replication cycle, so that Met$^-$ segregants recovered a wild-type phenotype through successive mitotic divisions. This finding indicated that *met2* genes were silenced by methylation alone.

A procedure has recently been developed in our laboratory for integrative transformation of the filamentous ascomycete *Ascobolus immersus* (2); this procedure is based on the complementation of an auxotrophic *met2* mutation by the wild-type cloned gene (3). Transformation of a *met2* mutant strain of *A. immersus* by circular double-stranded (ds) DNA carrying the *met2* gene (2) results either in the integration of the transforming DNA at nonhomologous chromosomal sites or in the substitution of the endogenous *met2* mutation by the transforming wild-type allele. The relative frequency of both events varies according to the vector sequences carrying the transforming *met2* gene, although nonhomologous integrations are the most often obtained. Homologous integration of the transforming plasmid by a reciprocal exchange at the chromosomal *met2* locus has never been observed.

We determined whether changing the conformation of the transforming DNA might have an effect on the transformation modalities of *A. immersus* and might eventually force homologous recombination with chromosomal sequences. We report on the ability of circular single-stranded (ss) DNA and linear ds DNA carrying the *met2* gene to transform protoplasts of a *met2* mutant strain of *A. immersus*. In contrast to circular ds DNA, they both most often participate in homologous recombination with the *met2* chromosomal locus. Using this property, we show that efficient targeting of cloned DNA sequences to the resident homologous genomic locus is possible in *A. immersus*, allowing either direct or indirect gene replacement. We also report on how ss DNA can be used in transformation to molecularly localize a mutation in *A. immersus*.

We analyzed the behavior of several Met$^+$ transformants after they had undergone meiosis. We discovered that duplicated *met2* genes were inactivated at a premeiotic stage by extensive de novo methylation of the cytosine residues, so that most segregants of crosses were Met$^-$. This process is similar to that described by Selker and colleagues (17, 18) for *Neurospora crassa* transformants except that methylation is reversible in *A. immersus*, allowing reversion of the Met$^-$ segregants to wild type.

MATERIALS AND METHODS

Strains of *A. immersus* and media. Strains used belong to stock 28 (13). The RK21 (*met2.1 mt+*) strain was used as the recipient in transformation experiments. Strains used as testers in crosses were FA15 (*met2.1 mt−*) and FA01 (*b2.138 mt−*). Media have been previously described (2).

Transformation of *A. immersus* protoplasts. Protoplasts were prepared, transformed, and regenerated by procedures previously described (2).

Genetic techniques. Standard *A. immersus* genetic techniques (12, 14) were used.

Isolation and manipulation of DNA. *A. immersus* DNA was prepared by using a small-scale isolation procedure that yielded about 6 μg of DNA with a size sufficient for performing Southern blot analyses. Mycelium was grown in a Roux flask containing 100 ml of minimal medium for 48 h at 26°C without shaking. The mycelium was harvested by filtration through sintered glass (porosity 1), washed with deionized water, pressed dry, frozen in liquid nitrogen, and lyophilized overnight. About half of the mycelium was powdered in an Eppendorf tube with a spatula, suspended in 800 μl of 50 mM Tris base [pH 9.0]–50 mM EDTA–150 mM NaCl–1% sodium dodecyl sulfate, and incubated for 15 min at 70°C. The mixture was then cooled for 5 min on ice and gently treated with 500 μl of phenol-chloroform-isoamyl

* Corresponding author.
† Present address: Laboratory of Biochemistry, National Institutes of Health, Bethesda, MD 20892.

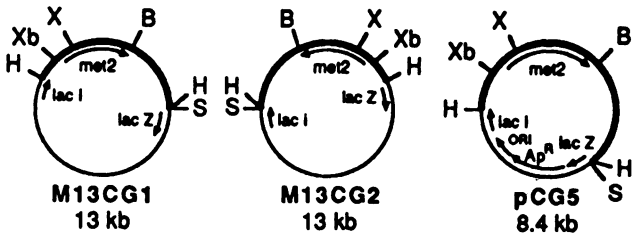

FIG. 1. Partial restriction maps of the recombinant phages DNA M13CG1 and M13CG2 and of plasmid pCG5. Thin lines correspond to phage DNA M13mp19 in M13CG1 and M13CG2 and to plasmid pUC19 in pCG5; thick lines correspond to the 5.7-kbp *Hin*dIII fragment carrying the *met2* gene from *A. immersus*. Restriction sites: B, *Bgl*II; H, *Hin*dIII; S, *Sal*I; X, *Xho*I; Xb, *Xba*I.

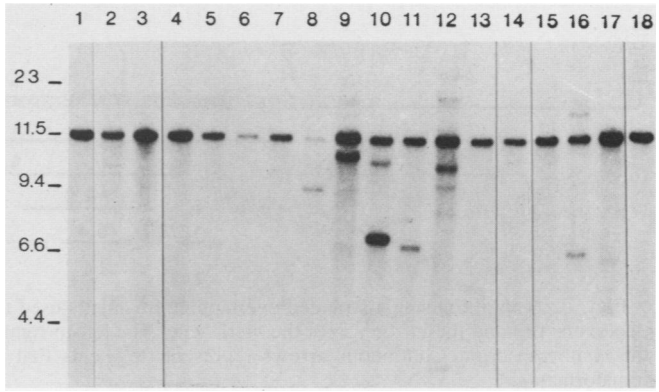

FIG. 2. Southern blot analysis of DNA from primary ssM13CG1 transformants. *Sal*I digests of DNA from the recipient strain RK21 (lane 1) and from ssM13CG1 transformants (lanes 2 to 18) were fractionated in a 0.6% agarose gel, and the gel blot was probed with ^{32}P-labeled M13CG1 DNA. Sizes of the *Sal*I fragment carrying the *met2* gene in strain RK21 and of the *Hin*dIII fragments of lambda DNA used as size markers are given in kilobase pairs.

alcohol (50:48:2). After 15 min of contact, the mixture was centrifuged for 15 min, and the aqueous phase was extracted with 500 μl of chloroform-isoamyl alcohol (48:2). The mixture was centrifuged for 3 min, and about 600 μl of the aqueous phase was transferred to a 2.2-ml tube. The following DNA purification steps are based on a method described by Vogelstein and Gillespie (22). A 1.2-ml sample of saturated NaI and 0.1 ml of glass milk (prepared from pulverized GF/C glass fiber filters [Whatman, Inc.]) were added, and DNA was allowed to bind to the glass for 15 min at room temperature, with occasional mixing. DNA-glass was pelleted by centrifugation (1 min at 3,000 rpm) and washed with 2 ml of 70% NaI and then with 2 ml of 50% 20 mM Tris base [pH 7.5]–200 mM NaCl–2 mM EDTA and 50% ethanol. After centrifugation, the DNA-glass was dried. DNA elution was performed by addition of 0.3 ml of TE (10 mM Tris base, [pH 7.5], 1 mM EDTA) and incubation for 15 min at 45°C, with occasional gentle mixing. Glass was removed by centrifugation at 12,000 rpm for 5 min. DNA was ethanol precipitated in 0.3 M ammonium acetate and dissolved in 100 μl of TE.

Bacterial plasmid, M13 replicative form and single-stranded M13 DNA preparations, restriction enzyme digestions, electrophoresis, and Southern blotting analyses were carried out by standard techniques (4, 6, 9). Approximately 1 or 2 μg of DNA was electrophoresed in each track of the agarose gels used in blotting experiments. Blots were hybridized with ^{32}P-labeled DNA and washed at 64°C in a buffer containing 0.04 M Na^+.

RESULTS

Direct allele replacement in transformation with ss DNA. Two major models of homologous recombination in fungi involve a strand-exchange reaction between ss and ds DNA substrates (8, 21). We tested the ability of ss DNA to transform *A. immersus* protoplasts, assuming that it might preferentially recombine with the DNA of the recipient strain by a conversion event at the homologous chromosomal locus. This would result in a direct allele replacement.

The 5.7-kilobase pair (kbp) *Hin*dIII genomic DNA fragment carrying the *A. immersus met2* gene had been previously cloned in bacteriophage M13mp19 (3). Protoplasts from the *A. immersus met2* mutant strain RK21 were successfully transformed by the ds DNA of one of the resulting recombinant phages, M13CG1 (Fig. 1). Only 5% of the transformants resulted from allele replacement, whereas the other 95% resulted from integration of the *met2* transforming DNA at nonhomologous chromosomal sites (2). In the previous cloning experiments, another recombinant phage, M13CG2, in which the 5.7-kbp *Hin*dIII fragment was inserted in the opposite direction (Fig. 1), had been obtained. ss DNA of both phages M13CG1 and M13CG2 (ssM13CG1 and ssM13CG2 DNA) was used to transform protoplasts from strain RK21. In the same set of experiments, we also used dsM13CG1 DNA for comparison. Control experiments were done either without DNA or with ssM13mp19 DNA alone. Transformants were obtained with both ss DNAs at equal relative frequencies, which varied from 10 to 20 transformants per μg of input DNA, depending on the protoplast preparation used. Transformation efficiency of dsM13CG1 DNA was 50 transformants per μg of DNA, as was obtained in previous experiments (2). No Met^+ clones appeared in the control experiments.

The DNA of 17 ssM13CG1 transformants was analyzed by Southern blotting experiments. It was previously shown that the restriction map of the *met2* region of the RK21 recipient strain was identical to that of the cloned wild-type *met2* gene used in our transformation experiments (3) and that this gene was carried by a 11.5-kbp *Sal*I fragment (2). Therefore, the DNA from the Met^+ transformants was digested by *Sal*I, which cuts M13CG1 DNA at the unique *Sal*I site present in the multiple cloning site of vector M13mp19 (Fig. 1). *Sal*I digests were probed with M13CG1 DNA (Fig. 2). Eleven transformants (65%) gave the same hybridization patterns (lanes 2 to 7, 13 to 15, and 17 and 18) as did the recipient strain (lane 1), indicating that they all resulted from replacement of the resident *met2* mutation by the wild-type allele carried by the transforming DNA. The other six (35%) all showed, besides the unmodified 11.5-kbp *Sal*I resident fragment, one or more additional *Sal*I fragments hybridizing with the probe (lanes 8 to 12 and 16), indicative of nonhomologous integrations of ssM13CG1 DNA into the *A. immersus* genome (see Fig. 4 for comparison with an integration at the homologous *met2* locus). Similar results were obtained with ssM13CG2 DNA (data not shown). This analysis shows that transforming with ss DNA increased considerably the frequency of allele replacement events (65%, compared with the 5% obtained with ds DNA).

Localization of the *met2* mutation by transformation with internal fragments of the wild-type gene. The 5.7-kbp *Hin*dIII fragment of phage M13CG1 had been previously subcloned in M13 DNA (3). This allowed us to use single-stranded internal fragments of the *met2* gene to transform the *met2*

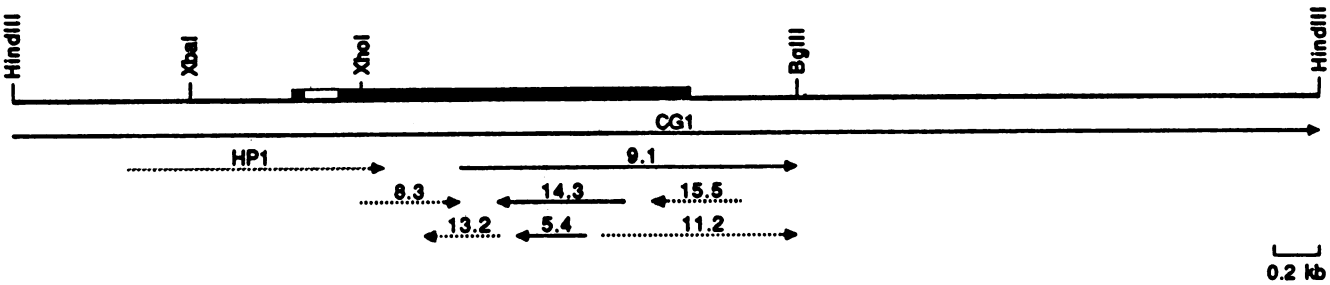

FIG. 3. Map of the single-stranded overlapping fragments used to molecularly localize the *met2* mutation of strain RK21. Shown are the two exons (■) and the intron (□) of the *met2* gene. The left-to-right direction on the restriction map corresponds to the 5′-to-3′ direction of the *met2* transcript. Continuous arrows represent fragments that gave Met$^+$ transformants; dotted arrows represent those that gave no transformants.

mutant strain RK21. The transformation ability of the eight internal fragments shown in Fig. 3 was tested. Transformants were obtained with three of them (ssM13-9.1, ssM13-14.3, and ssM13-5.4), which allowed us to localize the *met2* mutation within a definite 306-bp segment (corresponding to segment 5.4). Transformants appeared with a frequency of about 1/μg of DNA with ssM13-9.1, which is 10 to 20 times lower than the frequency obtained with ssM13CG1 DNA containing the entire *Hin*dIII fragment. Frequencies were still lower with ssM13-14.3 (4 transformants per 5 μg of DNA) and ssM13-5.4 (2 transformants per 5 μg of DNA). This result indicates that transformation efficiency decreased with the size of the ss DNA fragment used and therefore with the size of the sequence homology shared by the DNA strands that interacted during transformation.

Indirect gene replacement by transformation with ds cut DNA. Indirect gene replacement can be accomplished in *Saccharomyces cerevisiae* in two steps (16) involving (i) chromosomal integration of the transforming plasmid containing a gene for selection of transformants by homologous recombination at the target region, resulting in a tandem duplication of the target region separated by vector DNA and the selectable marker, and (ii) resolution of the duplication by intrachromosomal recombination, resulting in removal of vector DNA, the selectable marker, and one or the other copies of the target region. A prerequisite for adapting this method to *A. immersus* was the feasibility of targeting integration of a transforming plasmid to the resident homologous genomic locus. Cutting a transforming plasmid within the region homologous to chromosomal DNA greatly enhances the efficiency of homologous integration in *S. cerevisiae* (11). For that reason, we examined the effect of a double-strand break within the *met2* coding region of the transforming DNA. The plasmid used in this experiment was pCG5 (Fig. 1), which contains the 5.7-kbp *Hin*dIII fragment of phage M13CG1, carried by vector pUC19. The circular plasmid pCG5 behaves in transformation of *A. immersus* similarly to dsM13CG1 DNA: (i) transformation efficiency of plasmid pCG5 is 50 Met$^+$ transformants per μg of DNA, and (ii) 90% of the pCG5 transformants result from integration of the transforming DNA at nonhomologous chromosomal sites; the other 10% result from allele replacement (2). Plasmid pCG5 contains a unique *Xho*I restriction site located within the *met2* coding sequence, 655 bp upstream of the left end of the 306-bp fragment 5.4 (Fig. 3), which encompasses the *met2* mutation of strain RK21. This strain was transformed with plasmid pCG5 first made linear by cutting at the *Xho*I site. Met$^+$ transformants were obtained at a frequency of about 20/μg of linear pCG5 DNA, which is 2.5 times less than the efficiency of the corresponding circular DNA. The DNAs of 13 transformants were analyzed by Southern blotting experiments. *Sal*I digests were probed with the *Xba*I-*Bgl*II fragment of pCG5 DNA, which encompasses only the *met2* gene and its very closely flanking sequences (Fig. 1), and with pUC19 DNA. *Sal*I cuts pCG5 at the unique *Sal*I site present in the multiple cloning site of pUC19 DNA (Fig. 1). If plasmid pCG5 has integrated at the *met2* chromosomal locus by a single crossover between the plasmidic and the chromosomal *met2* sequences, then the physical map of the *met2* region of the resulting transformant is expected to be as described in Fig. 4B, where two duplicated copies of the target *met2* region are separated by pUC19 vector sequences. The 11.5-kbp *Sal*I resident fragment (Fig. 4A) will be replaced by two 11- and 8.9-kbp fragments expected to hybridize with the *met2* probe. If more than one copy of plasmid pCG5 has integrated in tandem (Fig. 4C), then, in addition to the 11- and 8.9-kbp fragments, one will found a third 8.4-kbp hybridizing fragment corresponding to the size of plasmid pCG5. Only the 8.9- and 8.4-kbp fragments will hybridize with the vector probe (Fig. 4B and C). Among the 13 transformants analyzed, only two showed, besides the 11.5-kbp *Sal*I resident fragment, *Sal*I hybridizing fragments (not shown) resulting from integrations of the transforming DNA at nonhomologous chromosomal sites. The 11 other transformants (85%) all appeared to be the result of homologous recombination events. Among them, two showed a hybridization pattern (Fig. 5A and B, lanes 3 and 9) identical to that of the recipient strain (Fig. 5A and B, lane 1), implying that they were the result of direct allele substitution. Seven (Fig. 5A and B, lanes 2, 4, 5, and 8; Fig. 6A, lanes 2, 4, and 6) showed the pattern expected from the homologous integration of one copy of plasmid pCG5 (Fig. 4B). The two others (Fig. 5A and B, lanes 6 and 7) showed an additional 8.4-kbp *Sal*I fragment that gave a hybridization signal about twice as strong as that of the 8.9-kbp fragment with both probes. It is likely that these transformants had integrated in tandem three copies of plasmid pCG5 by homologous recombination at the *met2* locus (Fig. 4C); this hypothesis was further confirmed (see below).

The second step in the procedure leading to gene replacement is the resolution of the tandem duplication structure by recombination between the duplicated sequences, either by unequal crossing over or by intrachromosomal recombination. Gene replacement was sought by analyzing the physical map of the *met2* chromosomal region of the Met$^+$ segregants produced from crosses between transformants having tandem copies of the *met2* sequences and the *met2* mutant tester strain FA15. For this purpose, six transformants were crossed. Four of them (3II, 9II, 10II, and 11II) had two tandem copies of the *met2* region (Fig. 5A, lane 4; Fig. 6A,

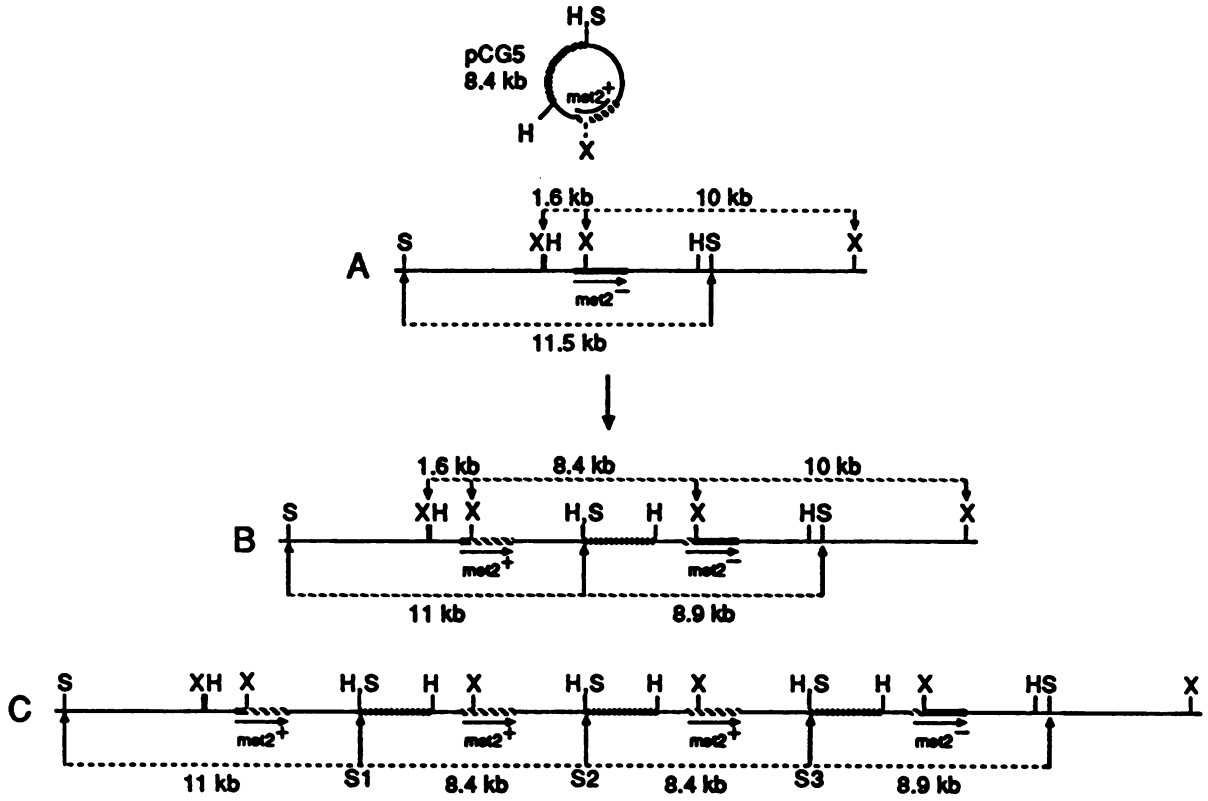

FIG. 4. One possible model of integration of *Xho*I-cut pCG5 DNA at the *met2* chromosomal locus by homologous recombination. (A) Partial restriction map of the chromosomal *met2* region; (B) integration of a single copy of pCG5 DNA by reciprocal recombination within the *Xho*I site region; (C) integration of three copies of pCG5 DNA. Restriction sites: H, *Hin*dIII; S, *Sal*I; X, *Xho*I. Sizes of the *Sal*I and *Xho*I fragments hybridizing with the *met2* probe are indicated.

lanes 2, 4, and 6); the other two (5IV and 6IV) had four tandem copies of the same region (Fig. 5A, lanes 6 and 7). In each case, 10 asci were dissected and tested for the Met phenotypes of their spores. Surprisingly, the expected $4Met^+:4Met^-$ segregation was obtained in only 3 of the 60 asci analyzed (one ascus each from transformants 9II, 10II, and 11II). All other asci showed a $0Met^+:8Met^-$ segregation. The reason for the appearance of such a high proportion of

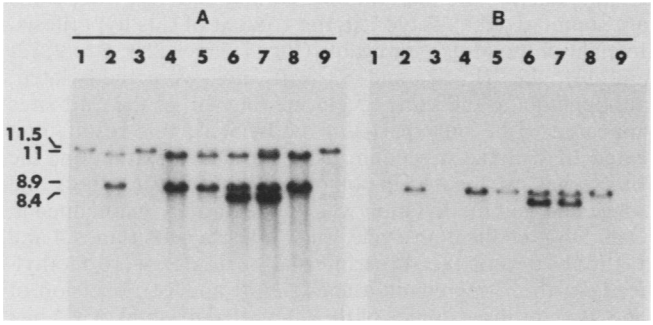

FIG. 5. Southern blot analysis of DNA from primary transformants obtained with *Xho*I-cut pCG5 DNA. *Sal*I digests of DNA from the recipient strain RK21 (lane 1) and transformants 1II, 2I, 3II, 4II, 5IV, 6IV, 7II, and 8I (lanes 2 to 9; the Roman numeral in each transformant name indicates the number of duplicated tandem copies of the *met2* region) were fractionated in 0.6% agarose gels, and gel blots were probed with the ^{32}P-labeled *Xba*I-*Bgl*II fragment encompassing the *met2* gene (A) and pUC19 DNA (B). Hybridizing fragment sizes are given in kilobase pairs.

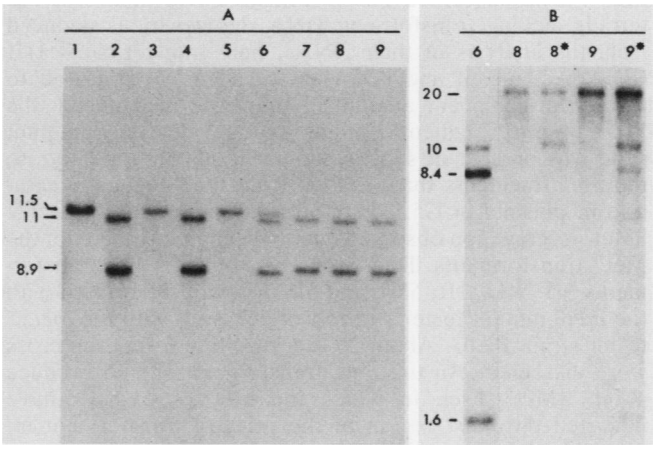

FIG. 6. Southern blot analysis of DNA from primary transformants obtained with *Xho*I-cut pCG5 DNA and from some of their meiotic segregants. (A) *Sal*I digests of DNA from the recipient strain RK21 (lane 1), the Met^+ segregants 9IIaI, 10IIaI, and 11IIaII (lanes 3, 5, and 7; the first Roman numeral in each transformant name indicates the number of duplicated tandem copies of the *met2* region) produced, respectively, from primary transformants 9II, 10II, and 11II (lanes 2, 4, and 6), and the Met^- segregants 11IIbII and 11IIcII (lanes 8 and 9), produced from transformant 11II. (B) *Xho*I digests of DNA from transformant 11II (lane 6), its two Met^- segregants 11IIbII and 11IIcII (lanes 8 and 9), and the same 11IIbII and 11IIcII segregants after they had recovered a wild-type phenotype (lanes 8* and 9*). DNA digests were fractionated in 0.5% agarose gels, and gel blots were probed with ^{32}P-labeled pCG5 DNA. Hybridizing fragment sizes are given in kilobase pairs.

Met^- progeny from primary Met^+ transformants is analyzed in the next section. The *met2* regions of three Met^+ segregants, 9IIaI, 10IIaI, and 11IIaII, produced from transformants 9II, 10II, and 11II, respectively, were analyzed by Southern blotting experiments. *Sal*I digests were probed with pCG5 DNA (Fig. 6A). One of them 11IIaII (Fig. 6A, lane 7), showed a hybridization pattern identical to the parental pattern. The two others, 9IIaI and 10IIaI (Fig. 6A, lanes 3 and 5), showed only one hybridizing *Sal*I fragment, identical in size to the 11.5-kbp *Sal*I fragment containing the *met2* gene in the recipient strain RK21 (Fig. 6A, lane 1). This finding indicated that in these Met^+ segregants, the parental tandem duplication of the *met2* region had been resolved by recombination between the duplicated sequences, leading to a single copy of the *met2* gene carrying the wild-type allele. This result shows that gene replacement can be easily obtained in transformants of *A. immersus* by a two-step procedure similar to that already described for *S. cerevisiae* (16) and *Aspergillus nidulans* (10).

The instability of Met^+ transformants is associated with methylation of their duplicated *met2* sequences. As mentioned above, most of the asci from crosses between primary Met^+ transformants resulting from a homologous integration of one or three copies of plasmid pCG5 into the recipient *met2* gene and the *met2* mutant tester strain showed a $0Met^+$:$8Met^-$ segregation. This result would be expected if all six primary transformants analyzed were heterocaryotic. In fact, since *A. immersus* is a coenocytic organism, protoplasts used for transformation are heterogeneous with respect to the number of nuclei they contain. About 80% of nucleate protoplasts have a single nucleus, the remaining 20% being multinucleate (2). Furthermore, fusion of protoplasts is most likely triggered by the polyethylene glycol used during transformation. Consequently, primary transformants may have only one of several nuclei transformed to Met^+. Untransformed nuclei will give rise in a cross to tetrads lacking transforming DNA. However, as deduced from the analysis of their DNAs, only transformants 11II (Fig. 6A, lane 6) and 6IV (Fig. 5A, lane 7) appeared to contain a significant amount of untransformed nuclei: the 11.5-kbp *Sal*I fragment containing the resident *met2* mutant gene was present in substoichiometric amounts relative to the *Sal*I fragments that resulted from homologous integration of plasmid pCG5. Therefore, the predominant $0Met^+$:$8Met^-$ segregation observed must reflect an instability of the Met^+ transformants. To address this point, the five transformants 3II, 9II, 11II, 5IV, and 6IV (previously crossed with the *met2* mutant tester strain) were crossed with the $met2^+$ tester strain FA01. About 20 asci resulting from each cross were dissected. An unstable transformant should produce $4Met^+$:$4Met^-$ asci, in which the Met^- segregants have inherited the *met2* region of the primary transformant involved in the cross. All of the asci analyzed from each cross showed a $4Met^+$:$4Met^-$ segregation except four from the cross with transformant 6IV, which showed an $8Met^+$:$0Met^-$ segregation.

The *met2* regions of several Met^- segregants were analyzed by probing with pCG5 DNA their *Sal*I and *Xho*I DNA digests. We first analyzed *Sal*I digests of the DNAs from eight Met^- segregants obtained in eight different $4Met^+$:$4Met^-$ asci found in the progeny of transformant 11II, which contains two tandem copies of the *met2* region separated by a unique copy of vector DNA (Fig. 4B; Fig. 6A, lane 6). All showed a *Sal*I hybridization pattern identical to that of transformant 11II (Fig. 6A; only two of them are shown [lanes 8 and 9]). This finding indicated that all of these segregants had inherited the *met2* region from the primary transformants and that their Met^- phenotype was not associated with any detectable sequence rearrangement or with any change in the overall size of the duplication. We next analyzed *Xho*I digests of DNAs from the same eight Met^- segregants. *Xho*I cuts within the *met2* gene (Fig. 4B), and three *Xho*I fragments (10, 8.4, and 1.6 kbp) of transformant 11II hybridized, as expected, with the pCG5 probe (Fig. 6B, lane 6). All eight Met^- segregants analyzed showed a *Xho*I hybridization pattern consisting of one major 20-kbp hybridizing fragment (Fig. 6B; only two of them are shown [lanes 8 and 9]). The size of this fragment corresponds exactly to the sum of the sizes of the three *Xho*I fragments hybridizing in the parental 11II transformant (Fig. 6B, lane 6). This result implied that none of the duplicated *Xho*I sites were cut. This might have been the result of local sequence rearrangements that created mutations in the *met2* coding sequence without affecting the size of the whole *met2* region. The possibility that identical rearrangements could have occurred simultaneously in eight independent meiotic events is hardly conceivable.

A more attractive hypothesis is that both *met2* copies became methylated at their cytosine residues during crosses by a process acting on duplicated sequences only and resulting in inactivation of the integrated wild-type *met2* gene (see reference 1 for a review of the effect of DNA methylation on gene activity). Indeed, both *Xho*I and *Sal*I are sensitive to methylation and do not cut their respective recognition sites, CTCGAG and GTCGAC, when the internal C is methylated (7). In our hypothesis, the *Sal*I site is cut because it is located in the unique unmethylated copy of pUC19 DNA separating the two elements of the duplication. However, in transformants where three copies of plasmid pCG5 had integrated in tandem, the internal C of the three *Sal*I sites, each present on each copy of pUC19 DNA (Fig. 4C; *Sal*I sites are indicated as S1, S2, and S3), are expected to be methylated, inhibiting the enzyme cutting activity. We therefore analyzed *Sal*I digests of 13 Met^- segregants obtained from 13 different asci found in the progeny of such a transformant (5IV). All 13 Met^- segregants (Fig. 7, lanes 2 to 14) exhibited a *Sal*I hybridization pattern different from that of the parental 5IV transformant (lane 1) and indicative for most of them of partial *Sal*I digestion, in agreement with the hypothesis. Since the results described below give still stronger support to the idea that cytosine methylation is responsible for the inhibition of cutting activity, the results are summarized in Table 1 in the context of this hypothesis. In eight of the Met^- segregants (Fig. 7, lanes 2, 3, 6 to 9, 12, and 13), the S1, S2, and S3 *Sal*I sites were methylated, although not at the same level: one (or two) of the *Sal*I sites appeared to be only partially methylated; this result indicated that in the mycelium used for DNA extraction, the site(s) was methylated in some nuclei but not in others and suggested that methylation was not accurately maintained at each DNA replication cycle. In two segregants (lanes 4 and 11), only two of the three internal *Sal*I sites were methylated. In three segregants (lanes 5, 10, and 14), excision of one, two, or three copies of the integrated plasmid pCG5 had occurred. One segregant (lane 10) had lost one copy; the remaining two internal *Sal*I sites were totally methylated. The second segregant (lane 5) had lost two tandem copies; its single internal *Sal*I site was not methylated. Three tandem copies had been excised from the third segregant (lane 14), leading to a single copy of a nonfunctional *met2* gene.

To show definitely that methylation of the cytosine residues was responsible for the loss of cutting activity of

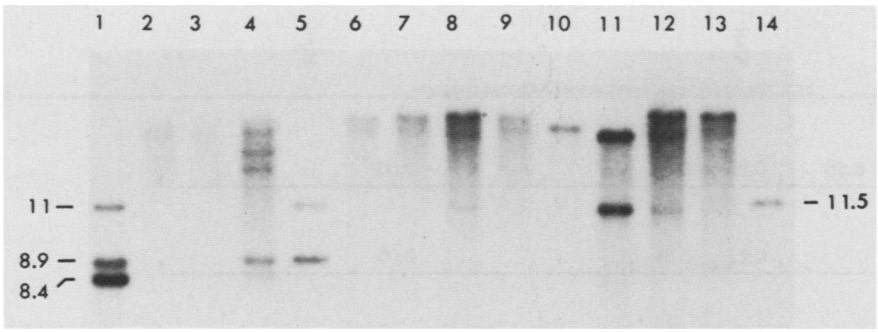

FIG. 7. Southern blot analysis of DNA from Met$^-$ segregants produced from primary transformant 5IV. *Sal*I digests of DNA from the parental transformant 5IV (lane 1) and from 13 Met$^-$ segregants (lanes 2 to 14) were fractionated in a 0.6% agarose gel, and the gel blot was probed with ^{32}P-labeled pCG5 DNA. Sizes of the hybridizing fragments from the parental transformant 5IV (lane 1) and from the segregant retaining one copy of the *met2* gene (lane 14) are indicated in kilobase pairs.

restriction enzymes, DNA from the Met$^-$ segregants 11IIbII and 11IIcII (Fig. 6A, lanes 8 and 9) resulting from transformant 11II, carrying two tandem copies of the *met2* region, was analyzed further by Southern hybridizations, using enzymes that allow differences in methylation patterns to be observed. Each DNA digest was probed with the *Xba*I-*Bgl*II fragment from plasmid pCG5, carrying the *met2* gene (Fig. 1). We first compared *Bst*NI and *Scr*FI digests. *Bst*NI recognizes the sequence CCXGG (X = A or T) and is insensitive to methylation of either or both C residues (7). *Scr*FI recognizes the sequence CCNGG (N = A, C, G, or T) and will not cut it if the internal C is methylated (7). A map of the *Bst*NI and *Scr*FI fragments overlapping the *Xba*I-*Bgl*II fragment from plasmid pCG5 is shown in Fig. 8. Four of the five *Scr*FI sites present in this region were also recognized by *Bst*NI, so that three of the four *Scr*FI or *Bst*NI fragments expected to hybridize with the *met2* probe were identical. Bands expected from the four *Bst*NI fragments of the *met2* region as found in the nontransformed strain RK21 (Fig. 9A, lane 1) and in both copies of the primary transformant 11II (Fig. 9A, lane 3) were also present in both Met$^-$ segregants (Fig. 9A, lanes 5 and 7). On the other hand, the four expected *Scr*FI fragments (Fig. 9A, lanes 2 and 4) were missing in both Met$^-$ segregants (Fig. 9A, lanes 6 and 8), and a number of novel fragments appeared. This finding demonstrated that *Scr*FI restriction sites were still intact in both Met$^-$ segregants and that methylation of the internal C was indeed the reason for the failure of enzyme *Scr*FI to cut. We then probed *Msp*I and *Hpa*II digests (Fig. 9B). *Msp*I and *Hpa*II recognize the sequence CCGG. Both cleave this sequence when it is unmethylated; however, *Msp*I will not cut it if the external C is methylated, and *Hpa*II will not cut it if either C is methylated (7). As in the case of *Scr*FI digests, all eight *Msp*I and *Hpa*II fragments of the *met2* region as found in the nontransformed strain RK21 (Fig. 9B, lanes 1 and 2) and in both copies of the primary transformant 11II (Fig. 9B, lanes 3 and 4) were missing in both Met$^-$ segregants and replaced by a number of novel fragments resulting from partial digestions (Fig. 9B, lanes 5 to 8). This was obviously the result of cytosine methylation, which appeared to be extensive. Finally, we compared in the same way *Mbo*I and *Sau*3AI digests. *Mbo*I and *Sau*3AI 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 (7). *Mbo*I is insensitive to cytosine methylation but will not cleave if the adenine residue is methylated (7). As expected from cytosine methylation, all 11 hybridizing *Sau*3AI fragments of the nontransformed strain RK21 (Fig. 9C, lane 2) and the primary transformant 11II (Fig. 9C, lane 5) were missing in both Met$^-$ segregants (Fig. 9C, lanes 8 and 11). Surprisingly, similar results were obtained with *Mbo*I (Fig. 9C, lanes 9 and 12), since expected *Mbo*I fragments (Fig. 9C, lanes 3 and 6) were replaced by novel ones. Digestions with *Dpn*I (Fig. 9C, lanes 1, 4, 7, and 10), which cuts the sequence GATC only if the adenine is methylated (7), demonstrated that the inability of *Mbo*I to cut the sequence GATC was not due to adenine methylation. This finding suggested that *Mbo*I may be sensitive to cytosine methylation when its recognition site is embedded in an heavily methylated sequence. Finally, the sizes of the largest fragments obtained from digestions of DNAs from the Met$^-$ segregants with all five restriction enzymes exhibiting sensitivity to cytosine methylation (*Scr*FI, Fig. 9A, lanes 6 and 8; *Msp*I and *Hpa*II, Fig. 9B, lanes 5 to 8; *Sau*3AI and *Mbo*I, Fig. 9C, lanes 8, 9, 11, and 12) were about 6 kbp. This is exactly the size expected if all of the restriction sites present in the duplicated 5.7-kbp *Hin*dIII fragment carrying the *met2* gene were uncut, whereas those present in the unique sequences (the chromosomal and the vector sequences) next to the borders of the *Hin*dIII fragment were all cut. This result is a further indication that unique sequences were left unmethylated.

TABLE 1. Methylation of the internal C residues of the duplicated *Sal*I sites

Lane[a]	Methylation at given site[b]		
	S1	S2	S3
2	+++	+++	+
3	+++	+++	+
4	+++	+	−
5[c]	One internal *Sal*I site, unmethylated		
6	+++	+++	+
7	+++	+++	+
8	+	+++	+++
9	+++	+++	+
10[c]	Two internal *Sal*I sites, methylated		
11	−	+++	+++
12	+	+++	+++
13	+	+++	+
14[c]	No internal *Sal*I site		

[a] Numbers correspond to lanes shown in Fig. 7.
[b] +++, High methylation; +, partial methylation; −, no methylation.
[c] See text for comments.

Mitotic stability of the Met$^+$ primary transformants. Southern blotting experiments performed with the DNAs of pri-

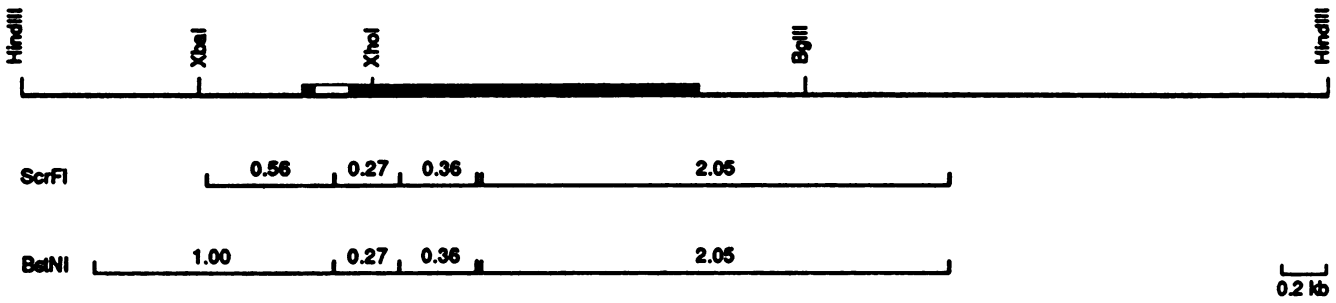

FIG. 8. Partial restriction map of the *Hin*dIII fragment carrying the *met2* gene. Sizes of the *Scr*FI and *Bst*NI fragments hybridizing with the *met2* probe are indicated in kilobase pairs. Two tightly clustered sites cut by both *Scr*FI and *Bst*NI lie between the 0.36- and 2.05-kbp fragments; they were discussed as one site in the text. All other notations are as in the legend to Fig. 3.

mary transformants 11II (Fig. 6B, lane 6; Fig. 9A and B, lanes 3 and 4; Fig. 9C, lanes 5 and 6) and 5IV and 6IV (Fig. 5A, lanes 6 and 7) indicated that the tandemly repeated *met2* sequences were unmethylated. This observation suggested that the methylation process that inactivated the integrated wild-type copies of the *met2* gene was not acting during vegetative growth. However, methylation could have inactivated the *met2* gene in some nuclei. These could have escaped analysis while being counterselected during growth (without methionine) of the mycelium used for DNA extraction. This possibility was checked by assessing the mitotic stability of primary transformant 3II, which carries two tandemly duplicated copies of the *met2* region. As deduced from the analysis of its DNA, this transformant did not seem to contain untransformed nuclei (Fig. 5A, lane 4). Transformant 3II was subcloned by making protoplasts. These were subsequently filtered through sintered glass (porosity 4) so that more than 90% of them were uninucleate, as shown by 4,6-diamidine-2-phenylindole dihydrochloride staining of the nuclei (2). The mycelium used for preparing protoplasts was grown on minimal medium (M1M) supplemented with methionine. Protoplasts were plated on minimal regeneration medium supplemented with methionine. A total of 100 regenerating protoplasts were picked and tested for ability to grow on M1M (without methionine). They all appeared to be Met^+, which indicated that each of the nuclei of transformant 3II carried a functional *met2* gene and that this transformant was therefore mitotically stable. Consequently, the

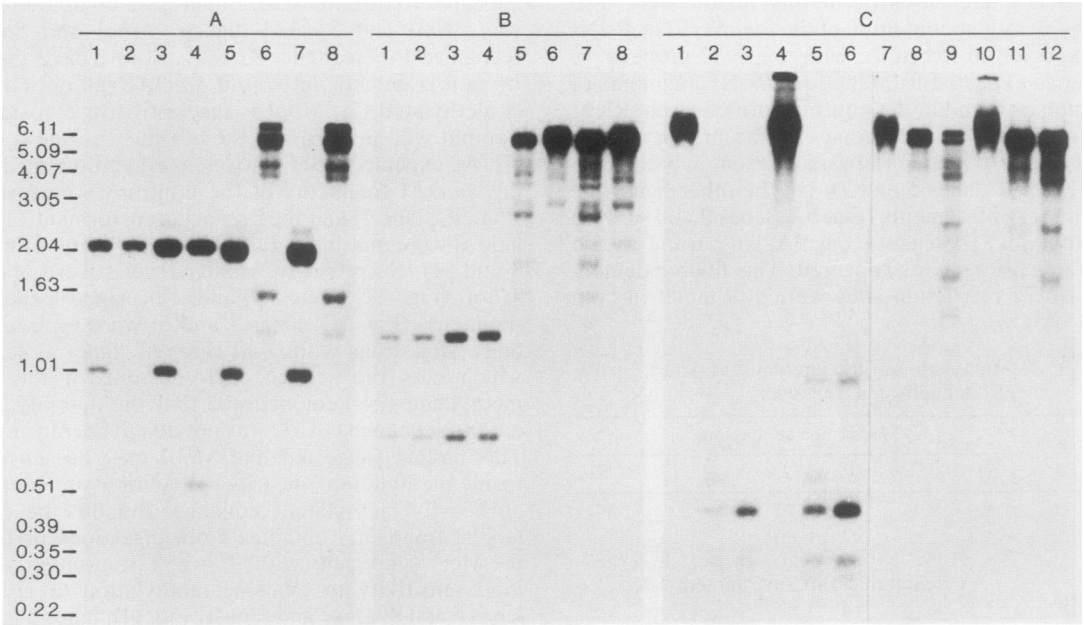

FIG. 9. Southern blot analysis of the methylation pattern of the *met2* region carried by Met^- segregants produced from transformant 11II. (A) *Bst*NI DNA digests from the recipient strain RK21 (lane 1), the Met^+ primary transformant 11II (lane 3), and the Met^- segregants 11IIbII (lane 5) and 11IIcII (lane 7); *Scr*FI DNA digests from strain RK21 (lane 2), transformant 11II (lane 4), and segregants 11IIbII (lane 6) and 11IIcII (lane 8). (B) *Msp*I DNA digests from strain RK21 (lane 1), transformant 11II (lane 3), and segregants 11IIbII (lane 5) and 11IIcII (lane 7); *Hpa*II DNA digests from strain RK21 (lane 2), transformant 11II (lane 4), and segregants 11IIbII (lane 6) and 11IIcII (lane 8). (C) *Dpn*I DNA digests from strain RK21 (lane 1), transformant 11II (lane 4), and segregants 11IIbII (lane 7) and 11IIcII (lane 10); *Sau*3AI DNA digests from strain RK21 (lane 2), transformant 11II (lane 5), and segregants 11IIbII (lane 8) and 11IIcII (lane 11); *Mbo*I DNA digests from strain RK21 (lane 3), transformant 11II (lane 6), and segregants 11IIbII (lane 9) and 11IIcII (lane 12). DNA digests were fractionated in 1.2% agarose gels, and the gel blots were probed with the ^{32}P-labeled *Xba*I-*Bgl*II fragment encompassing the *met2* gene. Sizes of the 1-kilobase ladder (Bethesda Research Laboratories) used as size markers are indicated in kilobase pairs. Several small hybridizing fragments are not visible on this short-exposure autoradiograph but were visible after longer exposure.

methylation process responsible for the Met$^-$ phenotype of most transformed segregants resulting from crosses involving transformants carrying duplicated *met2* genes was induced during the sexual phase alone.

Reversion to wild type of the Met$^-$ segregants through vegetative growth. We tested the ability of Met$^-$ segregants to recover a wild-type phenotype through vegetative growth. This procedure was based on our previous observation that methylation might randomly fail to occur at hemimethylated sites generated by DNA replication; we made the assumption that, after a number of mitotic divisions, this failure might lead to a reduction of the level of methylation, allowing expression of the *met2* gene. For this purpose, we picked 72 Met$^-$ segregants in 72 asci produced by primary transformants that had integrated one or three copies of plasmid pCG5 and transferred them onto plates containing M1M supplemented with methionine. After 3 days of growth, the mycelium obtained from each strain was transferred onto M1M without methionine and, as a control, onto methionine-supplemented M1M. Three days later, all control plates were invaded by mycelia, whereas no filaments or only very short ones appeared on the M1M plates. After 5 to 10 days, some filaments started to grow in sectors of most of the M1M plates; these plates were invaded 3 days later by vigorously growing mycelium. The latter was transferred again onto an M1M plate, where it displayed perfect wild-type growth. In this way, 17 of the 17 Met$^-$ segregants from 3II, 12 of the 14 from 9II, 12 of the 13 from 11II, 16 of the 16 from 5IV, and 8 of the 12 from 6IV reverted to wild type. Interestingly, the Met$^-$ segregants in which methylation of the duplicated *met2* regions was obvious from analysis of their DNAs (Fig. 6, 7, and 9) had all recovered a functional wild-type *met2* gene. Even the Met$^-$ segregant that had lost three tandem copies of the *met2* region and therefore contained a single copy of a nonfunctional *met2* gene (Fig. 7, lane 14) reverted to wild type. This result indicates that this segregant had indeed retained one wild-type copy of the *met2* gene but that the gene was silenced by methylation. Since our results suggest that unique sequences are not altered by the methylation process, one may assume that, in this transformant, the parental tandem copies were methylated before the excision event. Finally, the stable Met$^-$ segregants (7 of the 72 tested) might have arisen because of the heterocaryotic state of their parental primary transformants (this is probable for transformants 6IV and 11II; Fig. 5 and 6), because excision of the tandem repeat units had led to restoration of the *met2* mutant allele in these segregants, or because methylation had triggered G · C to A · T mutations via deamination of the 5-methylcytosine residues (19).

To confirm that restoration of the wild-type phenotype of the Met$^-$ segregants was definitely associated with imperfect maintenance of methylation in the *met2* gene, we probed, using pCG5 DNA, the *Xho*I DNA digests of six revertants from transformant 11II and the *Sal*I DNA digests of four revertants from transformant 5IV. All 10 DNA samples analyzed showed a decrease in methylation level. *Xho*I digests of the DNA samples from segregants 11IIbII and 11IIcII were examined just after the segregants had reverted to wild type (Fig. 6B, lanes 8* and 9*). Although the 20-kbp fragment resulting from methylation of the duplicated *Xho*I sites (lanes 8 and 9) was still present, fragments identical to those of the parental transformant 11II (lane 6) became visible. The 10-kbp hybridizing band in lane 8* must correspond to two comigrating fragments (10 and 8.4 + 1.6 kbp), indicating that in some nuclei the *Xho*I site of one of the duplicated copy of the *met2* gene was not methylated. In lane 9*, the presence of the three hybridizing 10-, 8.4-, and 1.6-kbp fragments indicated that again in some nuclei, the *Xho*I sites of both copies of the *met2* gene were not methylated. Although the lack of methylation was far from complete at this stage, there obviously were enough nuclei containing at least one unmethylated functional *met2* gene to allow wild-type growth of the mycelium.

Finally, since DNA methylation requires *S*-adenosylmethionine as a methyl group donor, one may ask to what extent the methionine starvation applied to the Met$^-$ segregants, which were forced to rescue their wild-type phenotype on minimal medium, contributed to preventing methylation from proceeding at the newly duplicated DNA sequences. We therefore tested whether recovery of a functional wild-type *met2* gene occurred at a lower rate when methionine was present in the growth medium. We checked the phenotype of 20 Met$^-$ segregants (10 from transformant 11II and 10 from transformant 5IV) after serial passages on M1M supplemented with methionine. After two to three passages, most of them (six from 11II and eight from 5IV) started to grow on M1M (without methionine) as quickly as did the wild type. Four to five passages were necessary for the others. This result indicates that even if exogenous methionine helps to maintain a certain level of methylation of the duplicated *met2* regions at each DNA replication cycle, this process is not accurate enough that unmethylated and therefore functional copies of the *met2* gene are finally produced.

Additional results concern the meiotic instability of transformants resulting from nonhomologous integration of a *met2*$^+$ transforming plasmid. Such transformants carry two unlinked copies of the *met2* region. They were shown to lose their Met$^+$ transformed phenotype through 50% of meiosis on average. The reason for this genetic instability was unknown (2). We have shown, by using the method described above, that Met$^-$ segregants resulting from such transformants all revert to wild type through vegetative growth. Moreover, when spores issued from a cross between a transformant carrying two unlinked copies of the *met2* gene and a *met2* mutant tester strain were germinated and transferred onto minimal medium containing 5-azacytidine, all asci showed the 4Met$^+$:4Met$^-$ segregation expected if the integrated *met2* gene were fully functional. 5-Azacytidine is an analog of cytidine which, once incorporated into DNA, prevents cytosine methylation (5). These results strongly suggest that inactivation of unlinked duplicated *met2* genes through crosses also is due to cytosine methylation and results from the same process as that operating on tandemly duplicated *met2* sequences.

DISCUSSION

We have developed molecular genetic methods that enable the targeting of a cloned gene of *A. immersus* to its homologous chromosomal locus. The *met2* mutation carried by the recipient strain RK21 can be directly replaced by the wild-type allele of the *met2* gene in transformation experiments performed with the single-stranded form of a recombinant M13 DNA in which the *met2* gene is inserted. This leads to an apparent gene replacement and occurs in a majority of the Met$^+$ transformants obtained in this way. The likeliest hypothesis is that gene replacement occurs by a gene conversion event resulting from a recombination process between the transforming ss DNA and the resident *met2* mutant gene. ss DNA has also been shown to transform *S. cerevisiae* cells, leading, as in *A. immersus*, to an apparent

gene replacement (20). Using M13 constructs in opposite orientations, we have found that the two DNA strands behave equally in transformation. Consequently, the ability of ss DNA to transform *A. immersus* protoplasts is not dependent on its ability to be transcribed. Transformation with in vitro-modified ss DNA could be used to directly mutagenize targeted chromosomal sites in *A. immersus*, provided the mutated transformed strains have a selectable phenotype.

We used the behavior of ss DNA in transformation to localize the *met2* mutation within a rather short DNA fragment (306 bp) of the *met2* gene. Transformation with ss DNA promises to be an effective way to physically map any point mutation in *A. immersus* and perhaps in other eucaryotes, provided the corresponding cloned wild-type gene is available and the phenotype of the transformants is directly selectable. Sequencing of the mutated allele could then be achieved by using the polymerase chain reaction amplification technique (15).

Gene replacement in *A. immersus* can also be accomplished by a two-step procedure involving integration followed by excision. Linearization of a transforming plasmid by a double-strand cut made within the *met2* coding region highly targeted integration of the entire plasmid at the homologous *met2* locus of the recipient strain RK21. Indeed, 70% (9 of 13) of the transformants obtained in this way had integrated one or more copies of the exogenous plasmid and therefore contained tandem duplications of the *met2* target region separated by plasmid DNA. Such a genome structure appears to be rather unstable physically and may yield progeny in which only one copy of the *met2* region carrying the wild-type allele is still present as a result of a recombination event between the duplicated sequences. This leads to a perfect gene replacement. This method opens the possibility of modifying genomic sequences in *A. immersus* by precisely reintroducing defined DNA segments that have been manipulated in vitro.

A striking feature of the Met^+ transformants that have integrated one or more copies of the exogenous plasmid is that they frequently lose their transformed phenotype after undergoing meiosis. Tandemly duplicated *met2* regions become modified by an extensive de novo methylation of the cytosine residues during the sexual phase of the life cycle, leading to inactivation of expression of the integrated *met2* gene in the segregants of crosses in which such transformants are involved. Cytosine methylation is likely to be induced at a stage of the sexual phase before the premeiotic DNA replication, since (i) the Met^+ phenotype of a primary transformant is totally stable through mitotic divisions and (ii) after meiosis, both pairs of spores issued from a transformed $met2^+$ nucleus always exhibit simultaneously an inactivated phenotype. Inactivation of duplicated genes may cause difficulties in experiments in which gene replacement is achieved in two steps. However, some nuclei escape this specific methylation process, since a small number of Met^+ segregants are found in the progeny of crosses. In addition, methylation appears not to be accurately maintained at each DNA replication cycle. This allows, after a number of mitotic divisions, reactivation of the expression of one (or more) of the integrated copies of the *met2* gene and consequently a reversion of the Met^- segregants to wild type.

The methylation process operating in *A. immersus* is reminiscent of that described by Selker et al. (17), which specifically acts on duplicated sequences of *N. crassa* transformants during the sexual phase, at a premeiotic stage between fertilization and karyogamy. This process, termed RIP (rearrangements induced premeiotically), leads, according to the authors, to local sequence alterations, as seen by changes in the positions of restriction sties. Alterations are associated with a de novo methylation of the cytosine residues of both elements of the duplication, whereas, as in *A. immersus*, unique sequences are left unaltered. RIPing is responsible for gene inactivation in *N. crassa* transformants (18), but it is not yet known whether this inactivation is the result of sequence alterations, of DNA methylation, or of both. Unlike the case with *N. crassa*, no detectable sequence alterations appeared to be associated with cytosine methylation in *A. immersus*, and the fact that methylation is reversible in *A. immersus* clearly demonstrates that the *met2* gene of Met^- segregants from crosses is silenced by methylation alone. Therefore, whether the mechanism triggering methylation of duplicated sequences in *A. immersus* is equivalent to that operating in *N. crassa* remains an open question. Whatever the answer, there obviously is in both organisms a mechanism by which duplicated sequences are detected or find each other. Whether this mechanism is related to that operating in homologous recombination is so far unknown. This is an important question, since recognition between two homologous DNA segments might be a prerequisite common to both DNA de novo methylation and recombination processes. Another question concerns the biological significance of the de novo methylation of duplicated sequences, which is not yet understood. Answering these questions will probably reveal an important and unexplored aspect of the cellular machinery.

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